EFFECT OF MECHANICAL FLUID FLOW ON CALCIUM DEPOSITION OF DENTAL PULP STEM CELLS

WITSANU YORTCHAN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Master of Sciences in Dentistry (Pediatric Dentistry) - Type A 2)
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By WITSANU YORTCHAN

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Master of Sciences in Dentistry (Pediatric Dentistry) - Type A 2 of Naresuan University

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Title EFFECT OF MECHANICAL FLUID FLOW ON CALCIUM DEPOSITION OF DENTAL PULP STEM CELLS

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ABSTRACT

Cleft lip and/or palate is a congenital disorder with a high incidence in Thailand (0.11-0.24%). To fill the defect with autologous bone graft is the gold standard of treatment, however resulting in donor site morbidity, more operation time and time to stay at hospital. Bone tissue engineering may be used to restore such problems with mechanosensitive cells which enhance a bone production. Dental pulp cells (DPCs) are one of the promising cell sources which are collected from human dental pulp tissues. Oscillatory fluid flow (OFF) has been shown to enhance bone production in osteoprogenitor cells. However, the frequency of OFF for increasing bone production is still unclear. This study aimed to determine whether the frequencies of OFF enhance the DPCs' osteogenesis. DPCs from 3 donors were cultured in an osteogenic medium and rocked under an incubated rocking shaker at frequencies of 10, 20, 30, 40, and 50 rounds per minute (RPM) for 1 hour/day, 5 days/week compared to a static group (0 RPM) from day 4 of culture. Cell proliferation was measured by measuring the total protein and osteogenic activity was measured by alkaline phosphatase (ALP) activity and calcium deposition on days 7, 14, and 21 of culture. DPCs' in the 30 rpm group produced more calcium deposition, suggesting the best frequency in enhancing osteogenesis in the human DPCs. High frequency (50 RPM) showed negative effect on cell proliferation. The results were consistent with the previous study that a low shear stress (less than 1 Pa)

could promote cell differentiation. The method using the incubated rocking shaker may be a simple treatment and cost effective method for producing cells in bone tissue engineering to restore cleft defects. More donors of DPCs will be used for reducing cell variation and extended days of culture for receiving more results in future work.

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WITSANU YORTCHAN

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CHAPTER I

THESIS PROPOSAL

Statements and Significance of the Problems

A common craniofacial congenital disorder is a cleft lip and/or cleft palate (CL/P) with 1.1-2.4 per 1000 live newborns (1). The overall treatments take about 20 years of treatment and undergo many surgical steps with a high treatment cost. In alveolar bone graft surgery on 8-9 years old, the patients have to harvest bone from the iliac crest (hip bone) to fill the cleft defect. The current treatment problems involve many surgical sites, both hip and oral sites. As a result, the patients could suffer from surgical areas, gesture, extend time in operation, and limit bone graft amount. Bone tissue engineering would be a new method to treat a bone defect. Adult stem cells from patients will be harvested and cultured in a laboratory. Patient-derived stem cells could be subsequently differentiated into osteoblastic-like cells, which could produce a calcium deposition. These differentiated cells could be implanted to repair the cleft defect. Recently, mesenchymal stem cells (MSCs) serve as an essential cell source in bone-tissue engineering. They could be harvested from the bone marrow, but the procedure is invasive and can causes donor site morbidity. Many studies reported that dental pulp cells (DPCs) from human teeth served as a renewable cell source for bone repair in tissue engineering (2-4).

Moreover, the mechanical loading takes part in the bone cell regulatory process through muscle movement. It's well known that the bone densities in athletes are higher than in non-athletes. Similar to the oral bones are stimulated with mechanical loading through the movement of surrounding muscles, such as, chewing. Su KC et al., reported that the mechanical loading could induce fluid flow in dentinal tubules, which stimulates dentin in the pulp chamber and affects a tooth structure. Many oral clinical findings showed the pulp obliteration (continuous formation of dentine) in senior patients induced by fluid flow while the teeth were chewed (5). Li et al., reported that oscillatory fluid flow (OFF) could enhance the mineralization of

osteoblastic cells (6). However, knowledge about the frequency of OFF in stimulating DPCs and to enhance calcium deposition is undiscovered.

Research Question

What suitable frequency of OFF whether 10, 20, 30, 40, or 50 round per minute (RPM) could enhance the calcium deposition of human DPCs compared to static group?

Research objective

This project is aim to evaluate the effect of the frequency of oscillatory fluid flow (OFF) on osteogenesis of human dental pulp cells on a incubated rocking shaker.

Significance of the Research

This research could scale-up procedure for stimulating calcium deposition of bone tissue engineering by using human DPCs and OFF in the laboratory before implant this graft to repair cleft defect in clinic. This method could reduce morbidity on the harvesting site (hip bone), time in operation, limited bone amount to fill the cleft defect.

Scope of the Research

Dental pulp tissue will be collected from patients who attend for oral surgical treatments at Naresuan University from September 2019 to April 2020.

Hypothesis

The suitable frequency of OFF either 10, 20, 30, 40, or 50 RPM could enhance calcium deposition on human DPCs.

CHAPTER II

LITERATURE REVIEW

Orofacial cleft

The most common orofacial defect is cleft lip and/or cleft palate (CL/P). The formation of facial structure is complex, which involves many tissues and organs during pregnancy. The interference of these processes can cause an orofacial cleft. CL/P can cause significant problems with facial profile and also dysarthria, infection of middle ears, hearing, swallowing, chewing, and body development (7). It can affect the patients, their parents, and also the national economics.

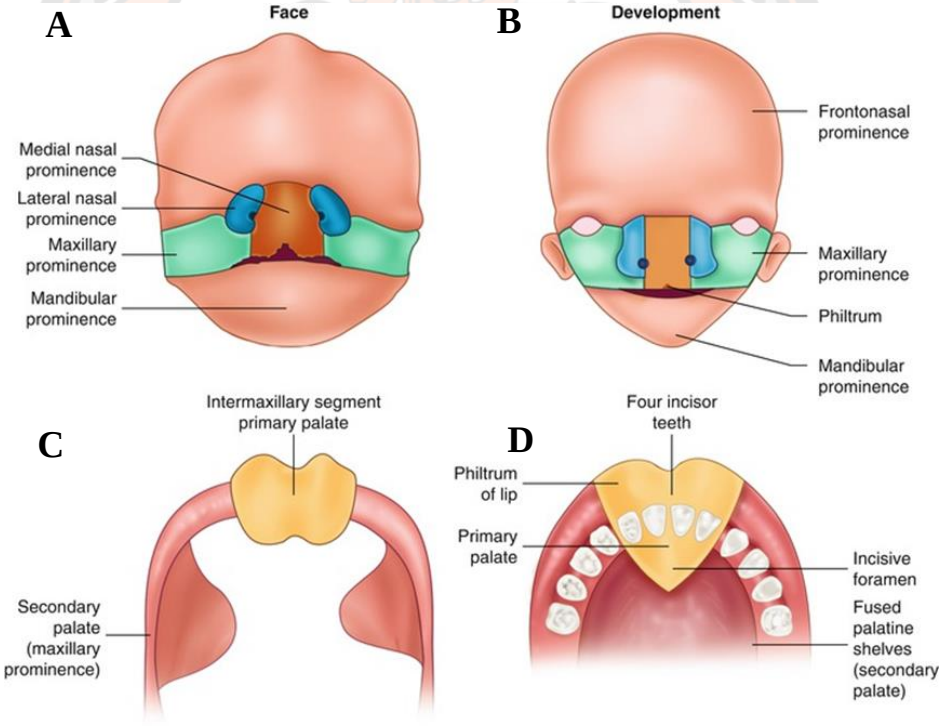

Figure 1 Schematic of normal development of face and palate (8)

The worldwide prevalence of CL/P is about 1/700 live births (8). A significant factor of CL/P was seen in a racial variation. In the Caucasian race, CL/P occurs in 1 per 1000 live births. It is more frequent in Mongoloid race or about twice

times than in the Caucasian race. On the other hand, Negroid is lower about half than the Caucasian race (9, 10).

In Thailand, the prevalence of oral cleft is around 1.1-2.4 per 1000 live births (11). The highest incidence of CL/P is in the Northeast region or about 2.5 per 1000 live births associated with using vitamin A or retinoic acid. Chowchuen B et al., (2015) reported that in Phitsanulok province, the incidences of CL/P is about 2.01 per 1000 live births (12).

Human face development starts in the 4th week of pregnancy. Lip and palate formation can occur around the 7th to 10th weeks of fetus development (8). A cause of cleft lip was a failure in fusion of the maxillary prominences with the medial nasal prominences (figure 2A). As a result, a separation of a lateral lip to a philtrum (figure 2B). In contrast, a cleft palate cause was a failure in the fusion between two maxillary prominences and intermaxillary segment primary palate (figure 2C).

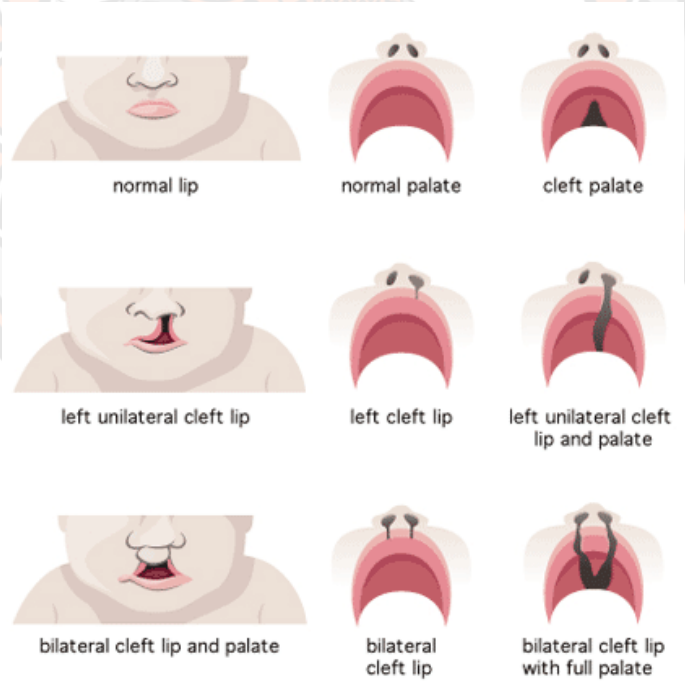

Figure 2 Schematic of variation of cleft lip and/or palate (CL/P) compare to normal development (13)

The protocol for CL/P treatment depends on the type of cleft defect (Figure 2). Managements of these problems could be solved with an interdisciplinary team. Naresuan University (NU) founded Naresuan University Cleft and Craniofacial Center (NUCCC), which consisted of professional staffs who volunteer to improve the quality of life in CL/P patients (table 1) (13). Moreover, they generated the protocol for cleft patient management from the patient is born until a complete treatment. From the newborn - 3 months, the patient could be applied nasoalveolar molding (NAM) to help swallow milk or breastfeeding and adjust a nasal shape. At the next three months of age, the patient could attend in cheiloplasty (lip repair). The patient could be attended in palatoplasty from 9 to 12 months of age (palate repair). During 8 to 11 years of age, the patient could be attended in alveoloplasty (bone graft on a tooth-bearing area). From 8-20 years of age, the patient could be aligned with the teeth and facial shape to be normal.

Table 1 show the NUCCC protocol for cleft patient management (14)

Age	Pediatrician	Orthodontist	Plastic surgeon and maxillo-facial surgeon	Pedodontist	Otolaryngologist	Speech-language pathologist
Newborn – 3 months	-Neonatal evaluation -History taking of pregnancy -Genetic counseling -Counseling of breastfeeding -Neonatal care	Impression taking for presurgical nasoalveolar molding and adjustment of NAM	Patient evaluation	Oral health care, patient instruction	Hearing test	-
3-6 months	-Medical and developmental examination -Healthcare guidance	-	Cheiloplasty + primary rhinoplasty	Dental check-up	Hearing test	Language and speech development counseling
6-9 months	Medical and developmental examination -Healthcare guidance	Post-operative evaluation	Post-operative check-up	Dental check-up	Hearing test	Semi-annual check-up

Age	Pediatrician	Orthodontist	Plastic surgeon and maxillo-facial surgeon	Pedodontist	Otolaryngologist	Speech-language pathologist
9-12 months	Medical and developmental examination -Healthcare guidance		Palatoplasty	Dental check-up, dental development evaluation	Ear examination, myringotomy if necessary	Language & speech counseling and instruction
1-3 years	Medical and developmental examination -Healthcare guidance	Examination of occlusion	Post-operative evaluation	Dental check-up, dental development evaluation	Ear examination, check for otitis media	Semi-annual check-up
3-5 years	Medical and developmental examination -Healthcare guidance	Examination of occlusion	Cheiloplasty + rhinoplasty (If necessary)	Dental check-up, dental development evaluation	Annual ear examination	Language & speech correction
5-8 years	Medical and developmental examination -Healthcare guidance	Examination of occlusion, orthodontic treatment	Palatoplasty (If necessary)	Dental check-up, dental development evaluation	Annual ear examination, nasoendoscopy	Language & speech correction
8-11 years	Medical and developmental examination	Examination of occlusion, orthodontic treatment preparing for	Alveolar bone grafting	Dental check-up before alveolar bone grafting	Annual ear examination	Follow up until normal language &

Age	Pediatrician	Orthodontist	Plastic surgeon and maxillo-facial surgeon	Pedodontist	Otolaryngologist	Speech-language pathologist
	-Healthcare guidance	alveolar bone grafting				speech
11-12 years	Medical and developmental examination -Healthcare guidance	Orthodontic treatment (growth modification/orthognathic surgery)	Post-operative check-up	Dental check-up	Annual ear examination	Follow up until normal language & speech
12-18 years		Orthodontic treatment, retainer application			Annual ear examination	Follow up until normal language & speech
>19 years		Orthodontic treatment evaluation, preparing for orthognathic surgery, post-operative orthodontic treatment, retainer application	orthognathic surgery (If necessary), rhinoplasty (corrective surgery)		Follow up, treatment evaluation	Follow up, treatment evaluation

In general, alveolar bone graft usually uses an autologous bone graft by harvesting from the iliac crest (hip bone) region. It can cause a donor site morbidity, discomfort from gesture and increased time to operation and stay in the hospital. Therefore, tissue engineering's utility by using stem cells could be a material of choice to reduce those disadvantages as above. The stem cells could be collected from their patients' tissue. Then, the tissues were explanted become the stem cells which were expanded in the laboratory. The stem cells could be induced to osteoblastic-like cells and implanted back into the patient.

Adult stem cells

Adult stem cells could be found throughout the body, such as dental pulp tissue or bone marrow (15). Adult stem cells' abilities are to self-renew and a variant of differentiation (with limited cell life span). Their undifferentiation stage could be differentiated into many cell types for tissue regeneration.

1. Bone marrow mesenchymal stem cells (BMSCs)

BMSCs are usually used as cell sources in regenerative medicine for many years (14). Their abilities could be self-renewal and differentiated into adipocytes, chondroblasts, and osteoblasts using specific culture media. They could be harvested from the patient and used as autologous cells and implant their cell productions back to the same patient. This process would be a patient-specific treatment and avoid immune rejection. Behnia et al., used the autologous BMSCs from 10 to 14 years old patients, which were seeded on biodegradable scaffolds to repair their alveolar clefts. They showed new bone formations on the defect regions after the operation and sufficient to move on to the next treatment (orthodontic treatment) (15). However, the amount of MSCs in bone marrow is about 0.01 percent of the total mononucleated cells, and their differentiated ability was reduced when their passage number increase (16-18). Therefore, other cell sources might show an advantage for use in bone regeneration.

2. Dental pulp cells (DPCs)

Mesenchymal stem cells (MSCs) are usually used as cell sources in tissue engineering applications. Iezzi I. et al., reported that dental pulp was a rich specialized cranial neural crest-derived mesenchymal tissue responsible for dentine production (odontoblasts) and nerve fibers (19). Dental pulp cells (DPCs) from the dental pulp tissue had been described as an alternative cell source for an autologous MSCs to substitute the maxillofacial bone defects. Many studies found that the dental pulp tissue contains some source of dental pulp derived mesenchymal stem cells (DPSCs) (4), which can be easily harvested, explanted, and cultured DPCs *in vitro*. DPSCs can be derived from the extracted teeth due to wisdom or non-functional teeth. Dominici et al., reported that DPSCs showed their expressions, which were similar to MSCs; for example, they showed CD73, CD90, CD105 expressions more than 95%, and CD45, CD34, CD11b, CD19, and HLA-DR expressions less than 5%. MSCs are capable of undergoing osteogenic, adipogenic, or chondrogenic differentiation (20, 21).

Besides, DPSCs can differentiate into the neurogenic, odontogenic, and myogenic lineages (22). Age of donor (between 20-39 and 40-59 years of age) influences the DPCs, which exhibited senescence features, such as enlarged cell shape, decreased proliferation and differentiation potentials (19).

Therefore, bone regeneration DPSCs cultures are adherent on the cell culture plate, rapidly growing, showing high proliferative capacity. Moreover, DPSCs can be stored in liquid nitrogen for long periods while retaining the MSCs' characteristics, making them applicable for tissue banking technology. DPSCs could decrease the expression of immune capacity. Therefore, these cells type could be used as a cell source in tissue engineering, which decreases the risk of cell tissue rejection compared to bone marrow MSCs (21). DPSCs, *in vitro,* can be stimulated to calcified deposit on the extracellular matrix. They also express important bone markers, including osteonectin, bone sialoprotein, osteocalcin, fibronectin, and alkaline phosphatase (ALP) (23). Paino et al., reported that DPSCs could form a new lamellar bone containing the Haversian canals with appropriate vascularization and osteocytes *in vivo* (24). Furthermore, the researchers could get the DPCs without any surgical intervention using the dental pulp tissue derived from deciduous teeth. These data

suggest that PDSCs are interesting cell sources in tissue engineering for craniofacial regenerative, the dental, and periodontal field (25).

Successful bone tissue engineering requires that a newly formed bone adapts its mass, shape, and trabecular architecture to the prevailing mechanical load. This inherent adaptive nature of bone ensures an optimal resistance against mechanical loads and prevents possible mechanical failure. The adaptation of bone to the mechanical load relies on mechanosensitive cells' ability to recognize mechanical changes and integrate this information into cellular responses to maintain bone integrity.

Effect of mechanical force on bone tissue

Bone remodeling is the balancing between bone resorption and bone formation. There is an essential process to maintain bone integrity. If the loss of these processes, it could occur skeletal pathologies that affected bone function.

Wolff's Law stated that the mechanical force could affect a bone remodeling process (26). The local mechanical directions led to the formation and adaptation of bone tissue. The cellular mechanism was changed by mechanical forces into a signal which called "the mechanotransduction pathways"(27). This pathway involved two cells that played an essential role in the bone mechanism. The first cell is an osteocyte, which performs as a primary mechano-receptor in an intact bone. The other cell is an osteoprogenitor cell that plays a role in bone formation and involves ectopic calcification of cardiovascular tissues (figure 3).

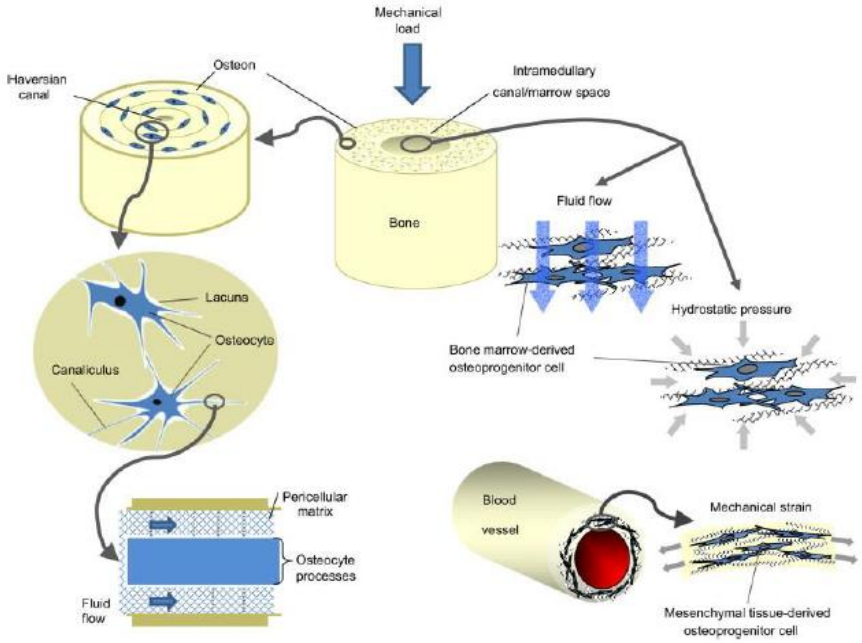

Figure 3 Schematic of fluid flow which affects osteoprogenitor cells in bone marrow space (26)

Effect of external mechanical stimuli on MSCs

Mesenchymal stem cells can be affected by mechanical stimuli such as a fluid flow, a compressive or a tensional force using the internal cell properties, such as a cell-matrix or cell shape. Type, frequency, and magnitude of them can affect MSCs in different results.

1. Fluid flow induced shear stress

Fluid flow has been used for mechanical stimulation in bone in vivo (28). It can be created mechanical stimuli when fluid transport over cell surfaces called “Fluid flow-induced shear stress.”

Several studies show that this type of mechanical stimuli can affect the behavior of MSCs (6, 29, 30). Bancroft et al., explained that a fluid flow increased calcium content and matrix distribution in MSCs derived from a rat bone marrow (31). Huang et al., explained that the fluid flowed stimuli could increase cardiomyogenic mRNA and protein marker in MSCs from rat bone marrow (32).

Arnsdolf et al., explained that fluid flow could increase Runx2, Sox9, RPARγ, RhoA, and RockII, which were the crucial markers in osteogenesis (33).

Many types of applications were used in experiments such as cone and plate system, rotating disk, plate flow chamber system, oscillating orbital shaker system, and rocking shaker see-saw system (34). These experiments could be made from a 2D culture which was impacted by the fluid flow-induced shear stress.

Moreover, 3D culture can be tested by different designs, such as spinner flasks or perfusion systems. The advantages of 2D culture over 3D culture were uncomplicated methods, which was easy to determine the quantity of fluid flow affected to MSCs. Moreover, the direction of flow magnitude was more consistent (figure 4), reduce the risk of contamination.

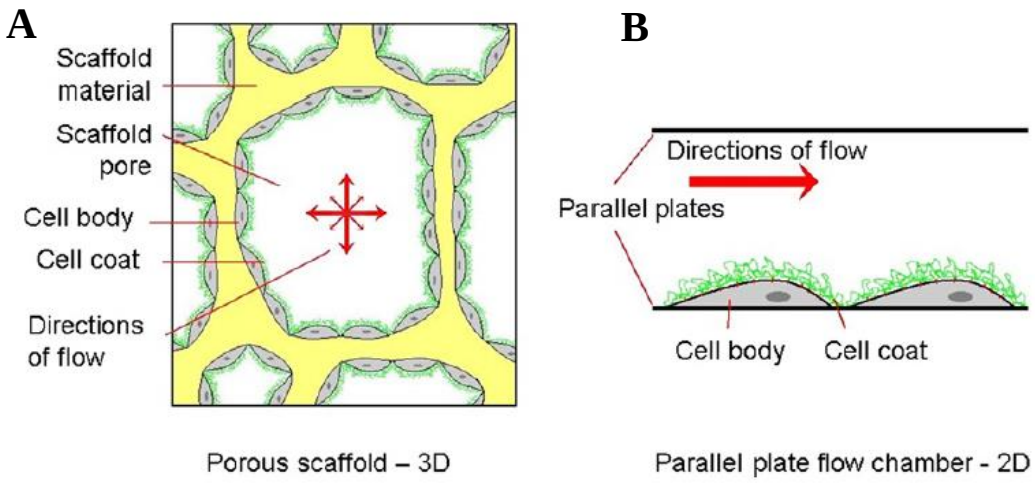

Figure 4 Schematic of fluid-flow induced shear stress (A) Monolayer 2D culture and (B) 3D culture (35)

2. Compressive force

The compressive force will express when the objects are withstanding to reduce the object size. Two principle designs were often used in many studies, such as a hydrostatic pressure and direct compression (figure 5) (35). The hydrostatic design can be created the force with no direct contact with cells. Their advantages are a homogeneous force, easy to set up and reduce possible resistance from cells.

The direct contact compression resembles the bone marrow environment (36) in which they get a compression force from muscle directly. On the other hand, it can create a fluid-induce shear stress from culture media which drawn in and out on scaffolds as a disadvantage.

Many studies showed that the compression force could stimulate MSCs differentiation. It can induce MSCs into chondrogenic and osteogenic lines (37). A study by Li et al., showed that a compression could increase sulfated-glycosaminoglycan (sGAG) synthesis and chondrogenic gene expression in hBMSCs (38). Sittichokechaiwut et al., found that compressive loading could increase matrix production in hBMSCs in 3D scaffold culture (39). Also, cell-matrix can play a role in MSCs differentiation. The dental pulp stem cells were seeded in a stiffer hydrogel scaffold. The cells express more chondrogenic lineage than those seeded in the softer scaffold (40).

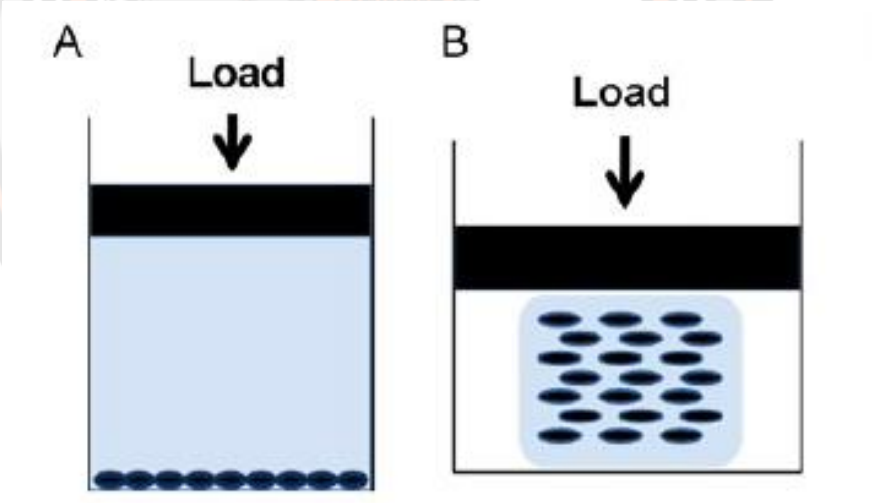

Figure 5 Schematic of compression design. (A) Hydrostatic pressure and (B) direct contact pressure (35)

3. Tension force

The tension force will express when the object is stretched in changing its length. There are two experiment designs, such as uniaxial grip tension and substrate bending (figure 6). The uniaxial grip tension design comprises seeding cells on the membrane; then, the substrate was gripped and pulled to express a uniaxial tension.

The substrate bending can be made by seeding cells on the substrate, which obtained three-or four-point bending (35). This could also create tension on the substrate surface. Many studies stated that the tension force could induce MSCs differentiation (40-42).

Hanson et al., showed that the tension force could increase the amount and rate of calcium deposition of human adipose cells (43). Rui et al., showed that the tension force could increase BMP-2 mRNA expression, ALP activity, and calcium deposition (44). Recently, the studies were made up in the 3D design. Therefore, an effect of force on the cells seeded scaffold may be different. The cells' seeded scaffold on the outer region may receive the force better than the cells located in the inner region. An advantage of the tension force could randomly create a non-uniform force to each cell on the scaffold.

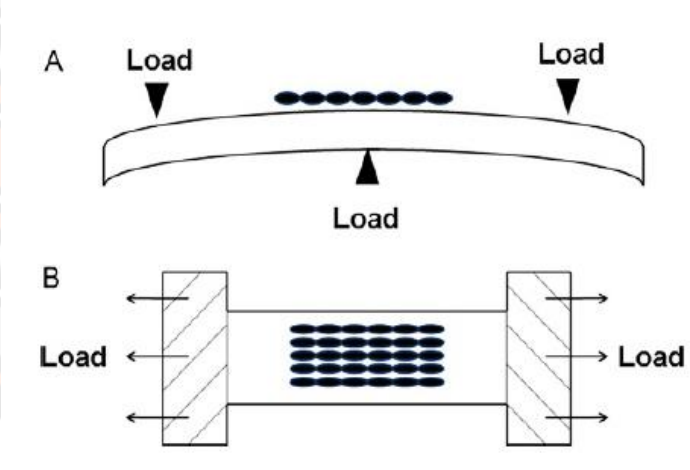

Figure 6 Schematic of tension loading design. (A) Substrate bending technique and (B) grip tension technique (35)

Effects of Mechanical Stimuli on the Behavior of DPSCs

An effect of mechanical loading in a bone could result a bone deformation, which induced the interstitial fluid surrounding the calcified matrix's osteocyte network to flow from high-pressure regions to a low pressure. The fluid flow exerted a shear stress on the osteocytes could produce signaling molecules, such as nitric oxide (NO) and prostaglandins (PG), which responses to a bone-remodeling process.

The effect of fluid shear stress, which is a physiological magnitude by pulsating fluid flow, may mimic a normal bone situation, *in vivo*. The loading via the lacuno-canalicular network on osteocytes could increase NO and prostaglandin E2 (PGE_2), and cyclooxygenase 2 (COX_2) gene expression by bone cells *in vitro* (figure 7) (45). Cyclooxygenase activity is essential and rate-limiting for prostaglandin production.

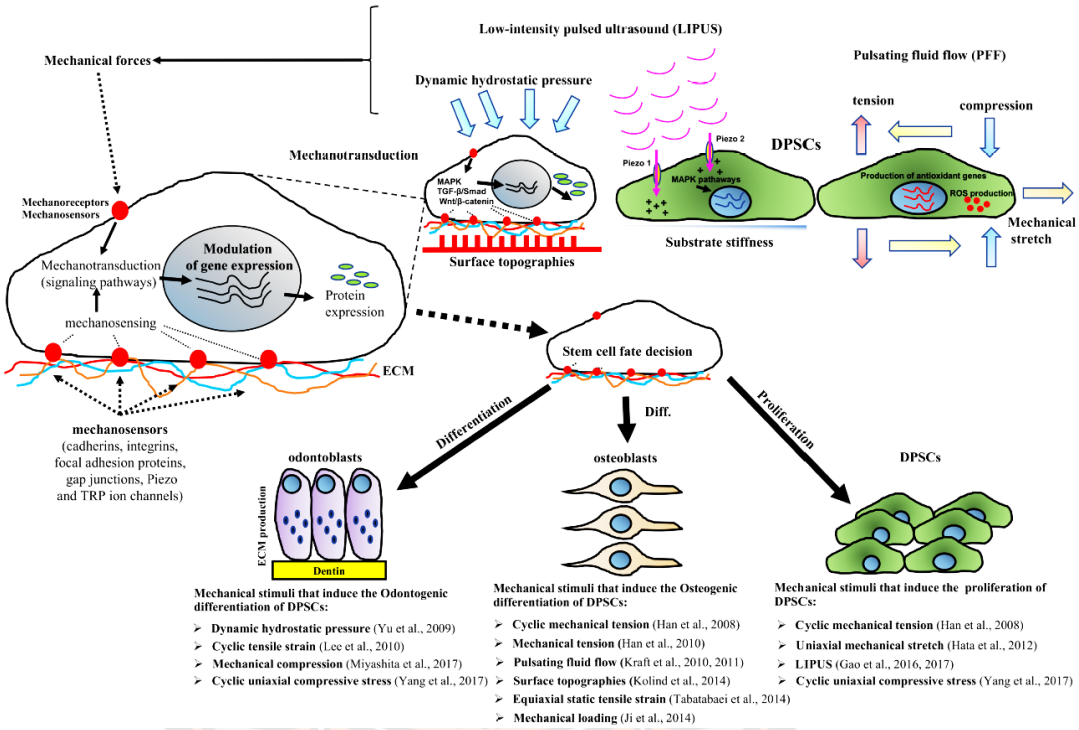

Figure 7 Schematic of mechanical factors which affect stem cells (46).

The mechanical sensitivity of dental pulp cells could be recognized as a mechanical loading of teeth. The loading could affect the fluid in dentinal tubules so it could activate the dental pulp cells in the pulp chamber. DPCs had mechanoreceptors and played an essential role in maintaining tooth integrity (45, 47). *In vitro* study applied a mechanical loading to DPCs by a cyclic strain stimulation showed increased production of inflammatory cytokines, collagen, osteopontin, antioxidant gene expression, and inhibits α-smooth muscle actin gene expression (48).

It is well known that DPCs are sensitive to a physiological environment. However, there were not investigated the effect of mechanical loading induced fluid flow to DPCs, recently. This study could present a preparation procedure of bone tissue engineering in the laboratory before implanting back to the patient.

CHAPTER III

RESEARCH METHODOLOGY

Sample

The study was applied to the Naresuan University ethics approval. The patients attended for maxillofacial surgeries at dental hospital, Naresuan University, from August 2019 to April 2020 with informed consent.

Inclusion Criteria

1. All sexes were included
2. Age between 20-25 years
3. Healthy
4. Non-smoking
5. No history of orofacial trauma or surgery
6. Dental pulp tissue from an impacted tooth with no decay and no infection

Sample Size

All patients were recruited into this project with informed consent by their voluntaries between August 2019 - August 2020.

Materials

1. Dental pulp tissues were collected from the patients who attended dental surgery in Dental Hospital, Naresuan University.
2. Basic culture media (BCM) which was consisted of α-MEM culture medium (Lonza®, Basel, Switzerland), supplement with 10% fetal bovine serum (FBS; v/v) (Capricorn, Ebsdorfergrund, Germany), 2 mM L-glutamine (Hyclone, Massachusetts, USA), 100 mg/ml penicillin, and streptomycin (P/S) (Caisson labs, Utah, USA).

3. Osteogenic induction media (OIM) contained with BCM, 50 µg/mL ascorbic acid (AA), 5mM ß-Glycerolphosphate (ß-GP), and 10nM Dexamethasone (Dex) (Sigma, Singapore)

4. 0.25% Trypsin-EDTA (Trysin-EDTA) (Sigma, Singapore)

5. Collagenase type I (Gibco, Massachusetts, USA)

6. Dispase (Gibco, Massachusetts, USA)

7. Dimethyl Sulfoxide (DMSO) (Sigma, Singapore)

8. Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific, USA)

9. Bovine Serum Albumin (BSA: known protein) for create a standard curve

10. Alizarin red (Sigma, Singapore): at 1mg/mL diH2O, pH 4.2, for calcium staining

11. Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System) (Sigma, Singapore) for quantitative determination of cellular alkaline phosphatase (ALP) activity

12. Formaldehyde

13. 6-well plate (Nunc, Massachusetts, USA)

Instruments

1. Laminar flow cabinet (BioSafety Cabinet class II, LABCONCO, USA)
2. Incubator (Thermo Fischer Scientific, USA)
3. Microcentrifugator (Beckman, USA)
4. Spectrophotometer (SpectraMax, USA)
5. Laboratory digital balance (Mettler Toledo, UK)
6. Data analysis software: SPSS (Statistic 21, IBM)
7. Inverted light microscope (IX70, Olympus, Japan)
8. Incubated rocking shaker (VWR, USA) (Figure 8)

Figure 8 Incubated rocking shaker

Experiment design

All dental pulp tissues were collected from the impacted teeth with no dental decay within 24 hours after tooth extraction. The dental pulp tissues were isolated to explant dental pulp cells, which were called DPCs. Various frequencies of oscillatory fluid flow (OFF) under the incubated rocking shaker (0, 10, 20, 30, 40, and 50 round per minute: RPM) were evaluated by using DPCs from 3 donors. The samples were rocking for 1 hour a day, five days a week. Then the samples in each group were evaluated for 3 assays such as bicinchoninic acid (BCA) assay, alkaline phosphatase (ALP) activity, and alizarin red staining (figure 9).

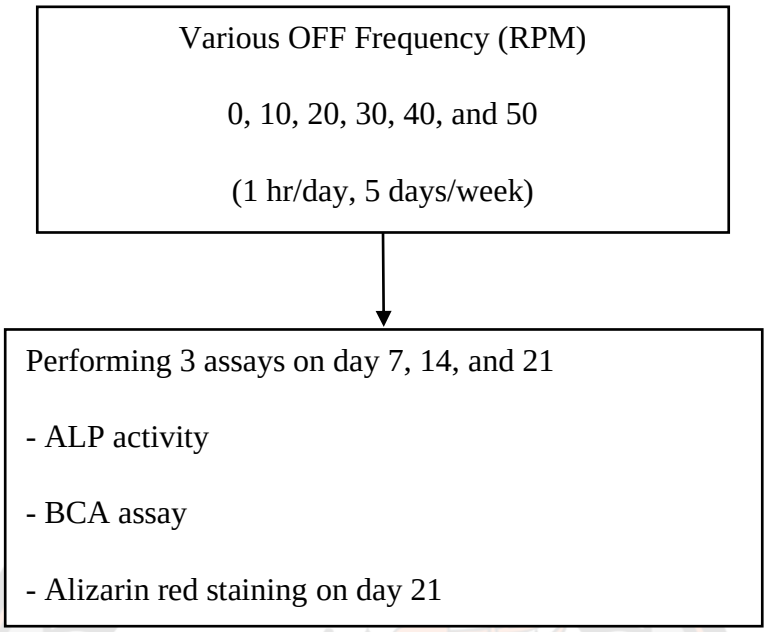

Figure 9 Flow chart of this experimental design

Cell isolation

DPCs were isolated from dental pulp tissue. Briefly, the impacted teeth were transferred from the dental hospital, Naresuan University, and collected the dental pulp tissue within 24 hours. The pulp tissue was removed using a sterile dental diamond bur and followed by a chisel and a hammer to split the tooth into two parts (49). The tissue was rinsed with phosphate-buffered saline (PBS) containing 100 mg/ml P/S, minced into smaller pieces, and added to a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 hour until the tissue digested (50). After this time, the cells were centrifuged, the supernatant were removed, and the cells were placed into a 25 cm^2 tissue-culture flask (Nunc, Massachusetts, USA) in 5 ml of fresh BCM. Fresh media were added to the flask every 2-3 days for seven days. After seven days, the culture media and non-adherent cells were removed, and the adherent population were termed “passage 0” (21, 52). DPC was sub-cultured when they reach 80% confluence. The medium was removed and washed twice with PBS. 2.5ml of the trypsin-EDTA solution was added to release the DPCs from the old tissue culture flask. The enzyme was stopped by using the fresh BCM. Cell suspension was

centrifuged at 1,000 rounds per minute (RPM) for 5 minutes. The supernatant was removed, and a known volume of the BCM was added. The cell pellet was resuspended in 1ml of the medium; 40 µl of cell working solution was evaluated several viable cells, which were counted by using a hematocytometer. Finally, a minimum of 10^5 cells was transferred to a new flask or seeded onto samples or cryopreservation. For future experiments, DMSO was used as a cryoprotectant at 10% final concentration in FBS. The cells were transferred to a cryovial and stored at -80ºC for one day and then moved to -196 ºC the next day for long term storage. The other cells were used for experiments from passage 2-5 to ensure their initial functions are maintained (51).

Dental pulp cell culture

DPCs from 3 donors were cultured in the 75 cm^2 tissue culture flasks (Nunc, Massachusetts, USA) with BCM. 10 nM Dex were added to DPCs cultures based on previous experiments in our laboratory to establish the best Dex concentration for the osteogenic induction media (52). All cells were cultured at 37ºC in 5% CO_2 in a humidified atmosphere. The media were changed every 2-3 days. DPCs were used between passages 2-5, as suggested by De Bari and colleagues (51).

Total protein quantification

Total protein quantitation was measured using the BCA assay kit (53). BCA is a substance that has the ability to react with cuprous ions (Cu^+). This method is based on the reduction activity of Cu^{2+} to Cu^{1+} under an alkaline medium (the biuret reaction). The amount of reduction refers to the amount of the present protein. After assay reaction, BCA forms a purple-colour product by chelation of two molecules of BCA with Cu^{1+}. The colour of the solution was measured with an absorbance at 526 nm, quantifying protein over a broad range (20-2000 µg/mL). Besides, there was highly sensitive and specific colorimetric detection of Cu^{1+}.

The samples were added to the cell digestion buffer which consisted of cell lysate buffer 10 ml, Triton-X-100 1 ml, and dH_2O 90 ml to extract protein; stored at -80°C for 10 minutes; incubated at 37 °C for 15 minutes, triplicated, and vortexed for 15 seconds. BCA working reagent was created by the 50:1 ratio mixture of BCA

reagent A and B. 25 µL of sample and 200 µL of BCA working reagent mixture was transferred into a 96-well plate, mixed well, covered the plate with aluminum foil, and incubated at 37°C for 60 minutes. The samples were measured at the wavelength of 562 nm using a spectrophotometer (Bio-Rad®, USA).

The value of absorbance was plotted against the known protein concentration (bovine serum albumin: BSA) to create a standard curve (n=9) (Table 2).

Table 2 Preparation of Diluted Albumin (BSA) Standards (53)

Vial	Volume of cell lysate buffer (µl)	Volume and source of BSA (µl)	Final BSA concentration (µg/mL)
A	600	400 of stock	800
B	500	500 of vial A dilution	400
C	500	500 of vial B dilution	200
D	500	500 of vial C dilution	100
E	500	500 of vial D dilution	50
F	500	500 of vial E dilution	25
G	500	0	0 = blank

The intensity of absorbance was plotted against the known protein concentration to create a standard curve. The line graph presented R^2 parameter (R^2 = 0.9915), resulting accurate equation (Figure 10) to be used. The equation from the standard curve was created to determine the unknown protein quantification (µg/mL) converted from absorbance:

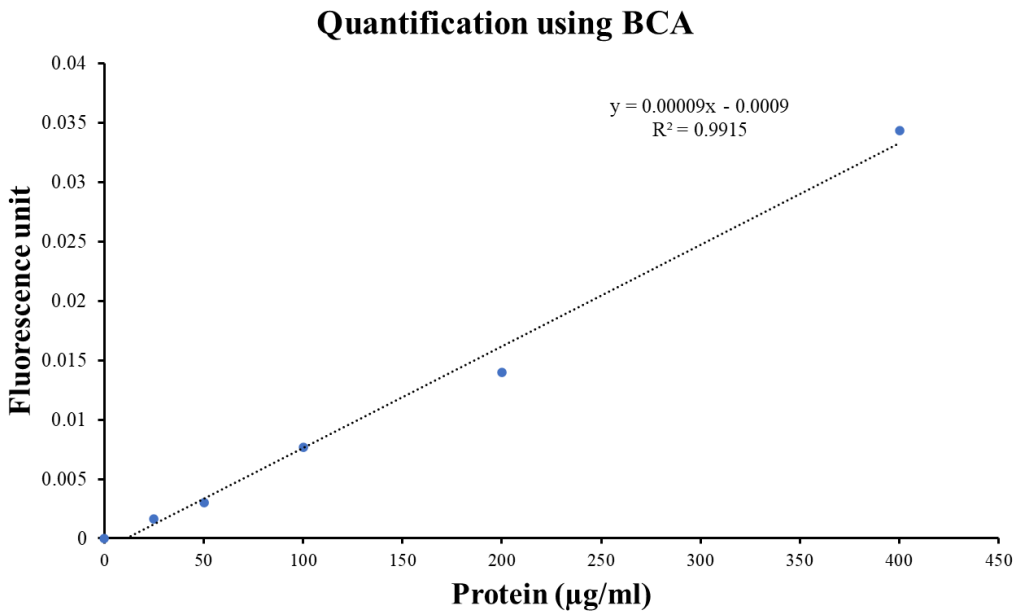

Figure 10 Linear quantification of BSA (known protein) by using BCA Protein Assay Kit, from 0 to 400 µg/ml with linear regression.

The formula from the standard curve was used to determine absorbance's value to a volume of protein concentration (µg/mL):

Protein quantification (µg/ml) = (Fluorescence emission +0.0009)/0.00009

The normalized total protein quantification presented as a result in which the total protein was divided by the total protein quantification of the control (static group) on Day 7, as described in equation:

$$\text{Normalized total protein quantification} = \frac{\text{Total protein}}{\text{Total protein (control group on Day 7)}}$$

Alkaline phosphatase (ALP) activity

ALP is an enzyme that can evaluate an early stage of osteogenic differentiation. ALP can hydrolyze an inorganic form of pyrophosphate (PPi) and produce an inorganic form of phosphate (Pi) to enhance mineralization. Samples were evaluated for ALP activity by washing twice times with PBS. 500 µL of cell lysate buffer (10% of cell assay buffer, which is composed of 1.5M Tris-HCL, 1mM $ZnCL_2$, 1mM $MgCL_2$ in double-distilled water and subsequently 1% Triton X-100) was added to the cells for 20 min at 37°C before scraping to remove cell lysate. Cell lysates were freeze-thawed three times, vortexed, and centrifuged with 1,000 RPM for 5 minutes. The cell lysates were mixed with an 'Alkaline Phosphatase Yellow Liquid Substrate' (Sigma-Aldrich, USA) based on p-nitrophenol phosphate. The absorbance was measured using a spectrophotometer (Bio-Rad, USA) at 405 nm every minute for 30 min. Then normalization of ALP activity with total protein could be derived into 3 steps, as followed:

Step1: The enzyme activity was calculated from the absorbance as nano-mol of para-nitrophenol per min (nmol pNP/min) as described in the equation:

$$\text{ALP activity} = \Delta A \times K \times \frac{V_{total}}{V_{sample}}$$

As ΔA : Maximum slope of each example

K : Constant of pNP concentration (54 mmol)

V_{total} : Volume of total cell lysate solution (500 µL/well)

V_{sample} : Volume of cell lysate solution used for calculating ALP activity

Step 2: the ALP activity (nmol of pNP/min/µg) was divided to total protein in each group (µg/0.5mL) as described in equation:

$$\text{ALP activity /protein} = \frac{\text{ALP activity}}{\text{Total Protein}}$$

Step 3: The formula for the calculation of normalization of the ALP activity was determined by mean ALP activity /protein divided by the mean of ALP activity/protein on day 7, as described in equation:

$$\text{Normalization of the ALP activity} = \frac{\text{Mean of ALP activity/protein}}{\text{Mean of ALP activity/protein on day 7}}$$

Total calcium deposition

Calcium mineralization staining was performed on day 21 for monolayer culture. Total calcium mineralization was measured by staining with 1 mg/ml alizarin red in distilled water (dH_2O), adjusted to pH 4.1 with ammonium hydroxide for 20 min at room temperature. All unstained dye were removed with dH_2O and left to air-dry. The stained samples were dissolved by adding 500 µl 5% perchloric acid in ddH_2O, leaving for 30 min at room temperature. Pipette out 150 µl of the eluted solutions were measured at 570 nm using a spectrophotometer (54). The spectrophotometric optical density was used for estimating mineralization from cell culture (55, 56).

Statistics

Data were expressed as mean values ± standard error of the mean (SEM). The numbers of replicates were stated in the figure legend. ‘N’ represented a biological repeat (separate experiment), and ‘n’ represented a technical repeat (different samples within one experiment). Statistical analysis was performed using SPSS (IBM SPSS statistics 23). Total protein quantification, ALP activity, and total calcium deposition were analyzed using an independent-samples Kruskal-Wallis test. The differences were considered to be statistically significant when the *p*-value was less than or equal to 0.05 *($p \leq 0.05$).*

Study Plan

Activities of this study included these following

1. Preliminary study

1.1 Literature review

1.2 Study proposal writing and ethical consideration applying

2. Start experiments and data collection
3. Data analysis and conclusion
4. Documentation and presentation

A duration of each activity of study was concluded in a study plan (Table 3).

Table 3 The Study Plan table

Activity	**Duration**	
	Start	**End**
1. Preliminary study 1.1 Literature review 1.2 Study proposal writing and ethical consideration applying	August 2018 April 2019	March 2019 September 2019
2. Data collection	September 2019	August 2020
3. Data analysis and conclusion	September 2020	November 2020
4. Documentation and presentation	December 2020	January 2021

CHAPTER IV

RESULTS

Cell morphology

The morphology of DPCs after day 3 (figure 10A) and 14 of culture (figure 10B) in the osteogenic induction medium. The morphology was gradually transformed from spindle cell shape similar to fibroblastic-like cells, into cuboidal cell shape similar to osteoblast-like cells.

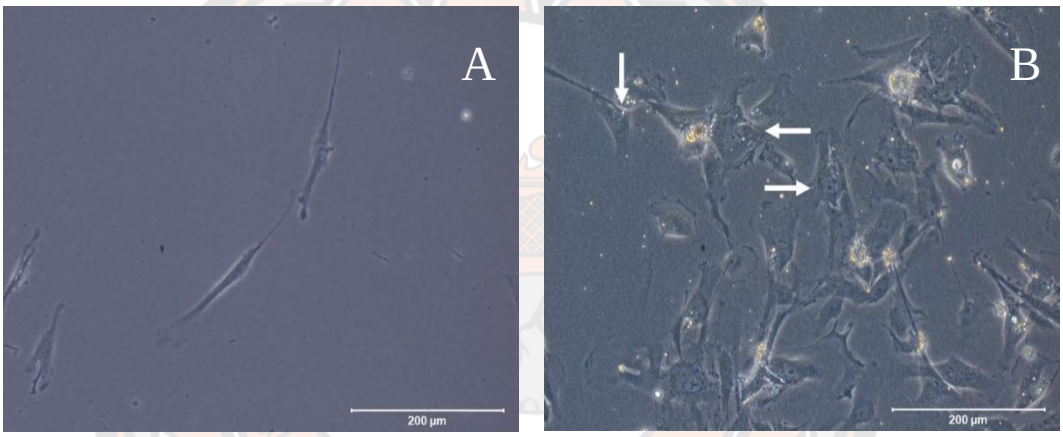

Figure 11 Phase-contrast images of the DPCs cells from (DPC-1) on day 3 (A) and day 14 (B) of cultures. (A) The cell morphology of DPCs-1 showed a spindle cell shape. (B) The cell morphology of DPCs-2 showed a distinct cuboidal cell shape (white arrows). Scale bar = 200 µm.

Total protein quantification

Pierce^{TM} BCA protein assay was used to determine the protein concentration of the samples.

The cell proliferation of DPCs was measured using the total protein quantification after day 7, 14, and 21. Total protein increased over 21 days (0, 10, 20, 30, 40, and 50 RPM) in all groups (Figure 12). The 30 RPM group was slightly high among the others, implying that the 30 RPM group had the highest cell proliferation than the other groups with no significantly difference. On the other hand, the cell population of 50 RPM groups detached from cell culture plates in all donors. The sample in these group was insufficient and inconsistent; therefore, the 50 RPM group was excluded from this project (Figure 13).

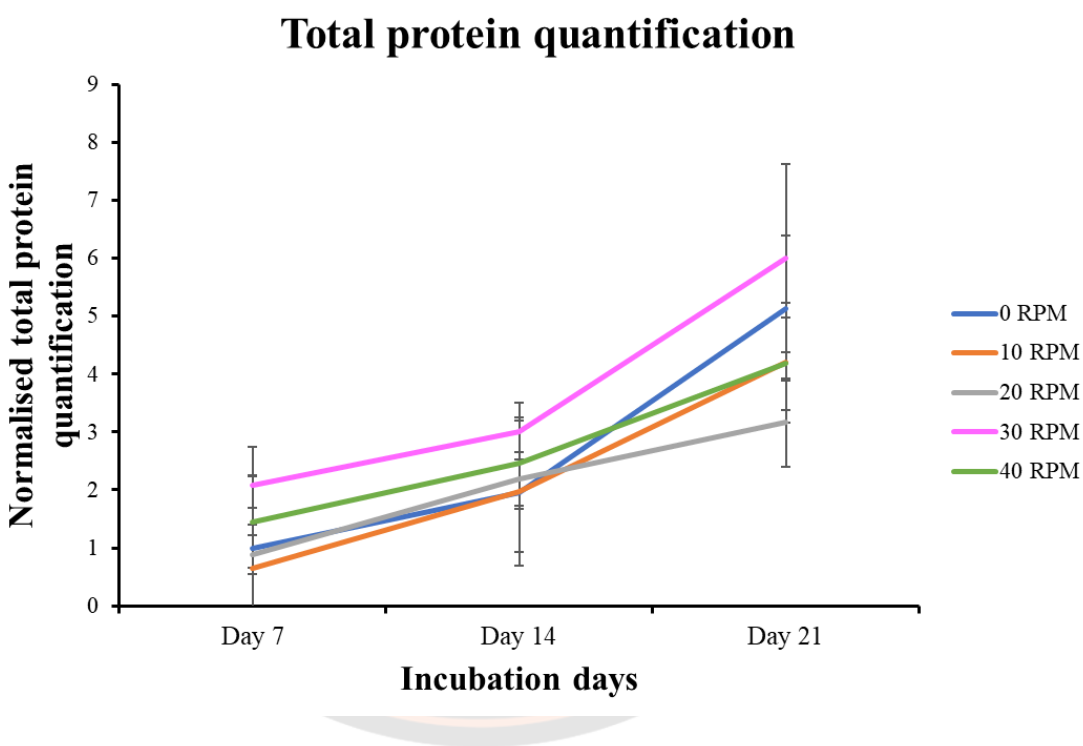

Figure 12 Total protein quantification of DPCs measured by BCA protein assay varying in different OFF frequencies on the osteogenic induction medium on 7, 14, and 21 days of culture. Data were presented as mean ± SEM (N=3, n=3).

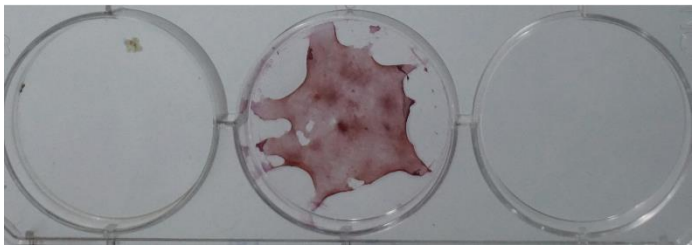

Figure 13 The photo image of 50 RPM group using alizarin red staining showed a diminished cell population.

ALP activity/protein quantification

The ALP activity is the early osteogenic marker of DPCs. The ALP activity normalized to the total protein of DPCs was increased overtime in all group for 21 days of culture (figure 14). On day 7, the highest ALP activity was on the 10 RPM group, but appeared not to be different from others. Days 14 and 21, the highest ALP activity were the static group suggesting that the ALP activity was diminished prior to cell start mineralization.

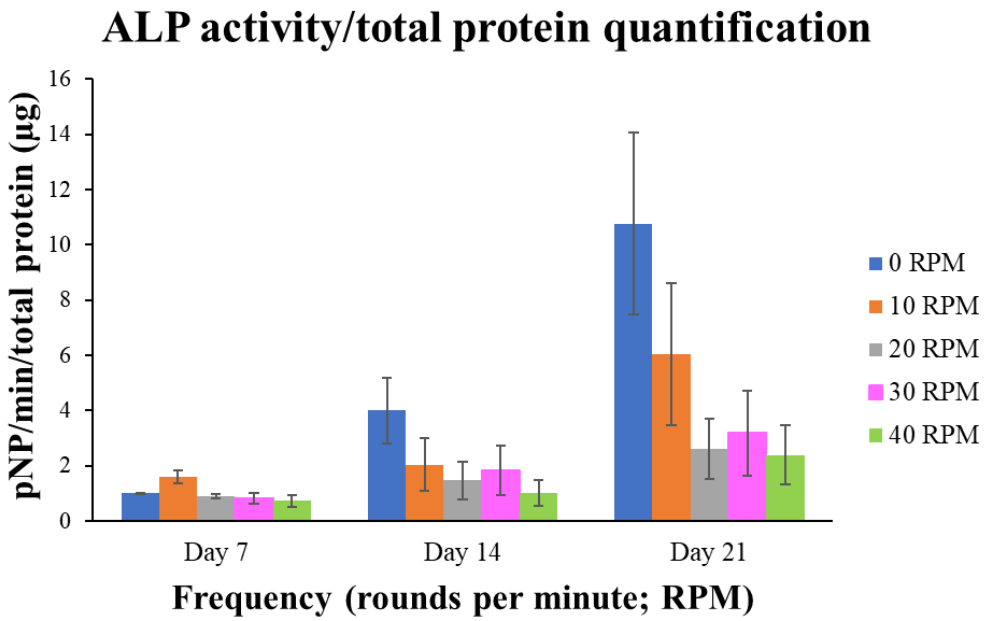

Figure 14 ALP activity normalized to total protein (µg) of the DPCs under the osteogenic induction medium on 7, 14, and 21 days of culture. Data were presented as mean ± SEM (N=3, n=3).

Total calcium deposition

Total calcium deposition was evaluated by using alizarin red staining after day 21 of culture, which represented the late osteogenic differentiation. The highest calcium deposition was in the 30 RPM group with significant differences *(p<0.05)* (Figure 15A). However, the lowest deposition was on the 20 RPM group. The photo image of alizarin red staining showed the darkest coloration after 21 days compared to the other groups (Figure 15B), indicating that there was the most calcium deposition than the other groups.

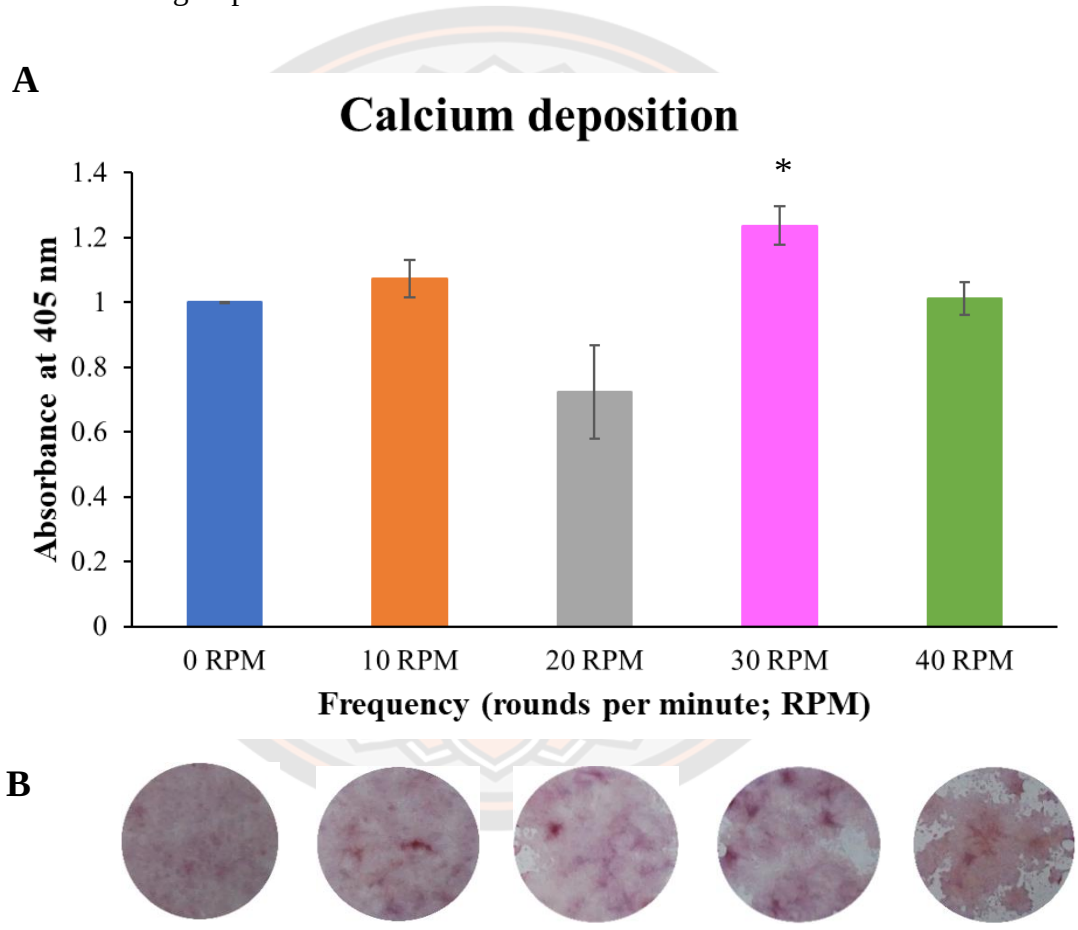

Figure 15 Total calcium deposition of DPCs using alizarin red staining (A) on 21 days of culture. The photo images showed representative sets of alizarin red staining varying by different oscillatory fluid flow (OFF) frequency (B). Data were presented as mean ± SE, *=*p<0.05* compared to the static and rocking groups, an using independent-samples Kruskal-Wallis test

CHAPTER V

DISCUSSIONS

To the best of our knowledge, this is the first study that showed that dental pulp cells (DPCs) respond to the incubated rocking shaker induced OFF in the 30 RPM group which showed the significantly high calcium deposition on monolayer cultures. OFF was no effect to cell proliferation and ALP activity (early marker of osteogenesis differentiation) in the proper shear stress.

It is known that DPCs can differentiate into osteogenic cells by adding the osteogenic induction media included Dex (2). This is a promising cell source for bone tissue engineering. OFF under the incubated rocking shaker can enhance osteogenic differentiation and may be used as a pre-treatment in the alveolar bone graft. A previous report from Delaine-Smith and colleagues showed that OFF, using a standard see-saw rocker, at room temperature for 1 hour a day, five days a week, can stimulate ALP, collagen, and calcium in the 6-well plate culture on human embryogenic mesenchymal progenitors (hESMPs). The sufficient fluid flow pressure described in the study was between 0-0.051 Pa (57). Furthermore, Li and colleagues' study reported that OFF in monolayer cultures of hBMSCs with the parallel type of plate flow chamber, the peak shear stress, is up to 2 Pa (6). hESMPs, human jaw periosteum cells (HJPs), and human bone marrow stem cells (hBMSCs) were used as typical cells in the study of OFF using a standard see-saw rocker in monolayer culture. HJPs and hBMSCs were already well-presented that they had osteogenic potentials and have been used in clinic for bone reconstruction. The incubated rocking shaker could be used in this project to avoid changing of temperature during cell cultures. Temperature is an important environment factor for cell culture. It may take some time for cell culture media to balance the media pH (58). Therefore, the incubated rocking shaker could reserve the pH balance of culture media. Bone tissue engineering may become an option for cleft palate surgical reconstruction. One crucial part is a cell source for autologous bone formation.

The oscillatory fluid flow acted like the fluid flow in the lacuno-canaliculi system (59). Applying OFF using the incubated see-saw rocker was selected in this study as the previous study, which showed osteogenic differentiation in osteoprogenitor cells (34, 57). Several studies used the parallel plate flow system in monolayer culture (60, 61). Its disadvantages was inlet, and an outlet tube port could lead to infection, and used a high amount of cells, culture medium, and reagents are needed (62). The parallel plate flow chamber often generated 0-2 Pa OFF, which could detach half of the attached cell on monolayer culture (critical fluid flow more than 1 Pa) (63). So the incubated rocking shaker were used in this study was less likely to peel off cells from a 6-well plate. OFF in this study were calculated using the equation from Zhou and colleagues' study (2010) (34). The shear stress values generated from either 10, 20, 30, 40, and 50 RPM were 0.50, 0.92, 1.24, 1.34, and 1.59 Pa, respectively.

This study aimed to evaluate suitable OFF frequencies for enhancing osteogenesis of DPCs in monolayer culture. This study were used the human dental pulp tissue from 3 donors and performed in triplicate. The culture media were used in the study was α-MEM culture medium, which was approved that facilitated cell proliferation and osteogenesis similar to the human bone marrow cells, as reported in many studies (64, 65). Nakamura and colleagues' study (2008) showed that human MSCs were cultured on both DMEM added osteogenic induction media and specific MSCs media. Their growths and osteogenic differentiated behaviors were not different (66). Danupon and colleagues' study (2018) showed that DPCs were cultured under α-MEM media. As a results, DPCs presented the osteogenic differentiation on monolayer cultures (2). Therefore, this medium may facilitate DPCs in cell proliferation and differentiate into osteoblast-like cells. Base on a previous study on hESMPs and HJPs showed the OFF could increase in ALP activity, calcium mineralization, and collagen production (67). Some studies showed that the low frequency of fluid flow (1 Hz and 1 hour/day), it could increase ALP and calcium deposition of hES-MPs.

In this study total protein quantification indicated cell proliferation was not different in all groups except the 50 RPM group. The results implied that OFF (0, 10, 20, 30, and 40 RPM groups) was neither deteriorated the DPCs on monolayer culture

nor increased cell proliferation. The results were consistent with Nauman et al., which cultured on the rat femoral and tibial osteoprogenitor cells under applied the pulsatile fluid flow between 0.1-1.1 Pa from day 14 to day 21 of culture showed an increased cell proliferation on day 21 (60). In the 50 RPM group (1.59 Pa) presented the detachment of DPCs from culture plate. Similar to study of Pre' and colleagues (2013) which showed that high frequency of shear stress reduced rate of cell proliferation in human marrow stromal cells under culture an osteoinductive media without a cell dead (68). Similar to Huber et al, (2018) reported that the high fluid shear stress more than 1 Pa could lead to alter cell adhesion properties, for example, detachment from cell culture plate (69). Similar to the report of Kreke et al., which MSCs from bone marrow were subjected to fluid flow-induced shear stress of 0.16 Pa showed no effect in cell proliferation (61).

In this study, the effect of OFF showed no differences in ALP activities (the early markers for osteogenic differentiation) in all groups. It was consistent with the study of Nauman et al., by using the rat osteoprogenitor cells were subjected to pulsatile fluid flow (unidirectional) showed no effect on ALP activity whether the static group of various magnitudes of flow (60). However, the study of Li et al., reported that fluid flow could exhibit a negative effect on ALP activity (6). Yourek and colleagues reported that hBMSCs were stimulated with unidirectional fluid flow in 100 nM of Dex (70). The ALP activity in cell lysate did not affected but the ALP in the media increased. The fluid flow may remove ALP from the cell membrane.

Calcium deposition refers to the late marker of osteogenic differentiation. The calcium depositions were the highest significantly different in the 30 RPM group compared to the others. The OFF combined with osteogenic media can enhance osteogenic differentiation in the late stage by more produce calcium deposition. Consistent with the study of Huber et al., reported that the low shear stress (<1 Pa) could affect cell differentiation such as an extravascular flow or interstitial flow in the body. James et al., reported that MSCs from bone marrow presented the donor unique markers to control functions about differentiation efficiency (71). *In vitro* study of Han et al., reported that a mechanical loading to DPCs by a cyclic strain stimulates showed the increased productions of collagen and osteopontin (OPN) (48). OPN is a secreted protein which is necessary for calcium deposition and cell proliferation (72,

73). The OFF could increase the mineralization of cell by nutrient distribution. The effect of fluid shear stress, which is a physiological magnitude by pulsating fluid flow, may mimic a normal bone situation, *in vivo* such as the lacuno-canalicular network on osteocytes. It could increase NO and prostaglandin E2 (PGE_2) and cyclooxygenase 2 (COX_2) gene expression by bone cells *in vitro* (74). COX has two isoforms, COX-1, and COX-2, as the COX-2 played a crucial role in prostaglandin (PGE) production to control bone cells' responsiveness to mechanical loads. OFF could also stimulate bone cells by increasing endothelial nitric oxide synthase enzymes (eNOS), which promotes bone formation. The eNOS enzymes regulated NO, which induced differentiation of osteoblasts and inhibited bone resorption by osteoclasts (75).

This finding will be an essential consideration for future bone-tissue engineering applications by using DPCs as the cleft repair.

CHAPTER VI

CONCLUSIONS

Conclusions

DPCs can be a promising cell source for bone tissue engineering. Applied OFF using the incubated rocking shaker, enhances calcium deposition on DPCs cultured with OIM. The incubated rocking shaker could be easily setting, cost-effective to prepare bone tissue engineer in graft in preliminary for cleft palate repair.

Future work

Future work should evaluate an effectively increased osteogenic potential by using OFF which can be inferred from this study. In addition, more benefit could be made in order to clarify the accuracy of OFF, for example;

1. More donors of DPCs should be measured in the osteogenic potential to reduce donor variations.
2. The study should be considered the extent of more culture days to receive further results.
3. Studying the response to OFF by cells in 3D culture.

REFERENCES

1. Ittiwut R, Siriwan P, Suphapeetiporn K, Shotelersuk V. Epidemiology of cleft lip with or without cleft palate in Thais. Asian Biomedicine. 2017;10(4):335-8.
2. Jantapalaboon D, Kaewsuwan K, Yortchan W, Choorujiporn P, Puwanun S. The Effect of Osteogenic Induction Medium on Mineralization between Human Jaw Periosteum Cells and Dental Pulp Cells. Naresuan University Journal: Science and Technology. 2018;26:40-8.
3. Mitsiadis TA, Barrandon O, Rochat A, Barrandon Y, De Bari C. Stem cell niches in mammals. Exp Cell Res. 2007;313(16):3377-85.
4. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97(25):13625-30.
5. Su KC, Chuang SF, Ng EY, Chang CH. An investigation of dentinal fluid flow in dental pulp during food mastication: simulation of fluid-structure interaction. Biomech Model Mechanobiol. 2014;13(3):527-35.
6. Li YJ, Batra NN, You L, Meier SC, Coe IA, Yellowley CE, et al. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. Journal of Orthopaedic Research. 2004;22:1283-9.
7. ARTS NVS. Schematic of cleft lip and cleft palate formation. 2019.
8. Peter J. Taub JMM. Embryology of the Head and Neck. 2014.
9. Wyszynski DF BT, Maestri NE Genetics of nonsyndromic oral clefts revisited. Cleft Palate Craniofac J. 1996;33:406-17.
10. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Felix TM, Rahimov F, et al. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. PLoS Genet. 2005;1(6):e64.
11. Usa Boonplian PS, Kornkiat Snidvongs. Improvement of caregivers' life quality after children with cleft lip cleft palate undergoing surgical correction: The Thai Red Cross project. . Chula Med J. 2017;61(5):577-87.
12. Chowchuen B, Thanaviratananich S, Chichareon V, Kamolnate A, Uewichitrapochana C, Godfrey K. A Multisite Study of Oral Clefts and Associated Abnormalities in Thailand: The Epidemiologic Data. Plast Reconstr Surg Glob Open. 2015;3(12):e583.
13. Sittichokechaiwut A. Cleft lip and palate patient care for general people. 2018.
14. Asatrian G, Pham D, Hardy WR, James AW, Peault B. Stem cell technology for bone regeneration: current status and potential applications. Stem Cells Cloning. 2015;8:39-48.
15. Behnia H, Khojasteh A, Soleimani M, Tehranchi A, Khoshzaban A, Keshel SH, et al. Secondary repair of alveolar clefts using human mesenchymal stem cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108(2):e1-6.
16. Jaquiery C, Schaeren S, Farhadi J, Mainil-Varlet P, Kunz C, Zeilhofer HF, et al. In vitro osteogenic differentiation and in vivo bone-forming capacity of human isogenic jaw periosteal cells and bone marrow stromal cells. Ann Surg. 2005;242(6):859-67, discussion 67-8.
17. Andrea Banfi AM, Beatrice Dozin, Maddalena Mastrogiacomo, Ranieri Cancedda, Rodolfo Quarto Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells-Implications for their use in cell therapy.

Experimental Hematology. 2000;28:707-15.
18. Caplan AI. Mesenchymal Stem Cells: Cell-Based Reconstructive Therapy in Orthopedics. Tissue Engineering. 2005;11:1198-21.
19. Iezzi I, Pagella P, Mattioli-Belmonte M, Mitsiadis TA. The effects of ageing on dental pulp stem cells, the tooth longevity elixir. European cells & materials. 2019;37:175-85.
20. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
21. Graziano A, d'Aquino R, Laino G, Papaccio G. Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev. 2008;4(1):21-6.
22. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage Differentiation Potential of Stem Cells Derived from Human Dental Pulp after Cryopreservation. Tissue Engineering. 2006;12(10):2813-23.
23. Wei X, Ling J, Wu L, Liu L, Xiao Y. Expression of mineralization markers in dental pulp cells. J Endod. 2007;33(6):703-8.
24. Paino F, La Noce M, Giuliani A, De Rosa A, Mazzoni S, Laino L, et al. Human DPSCs fabricate vascularized woven bone tissue: a new tool in bone tissue engineering. Clin Sci (Lond). 2017;131(8):699-713.
25. Jeon B-G, Jang S-J, Park J-S, Subbarao RB, Jeong G-J, Park B-W, et al. Differentiation potential of mesenchymal stem cells isolated from human dental tissues into non-mesodermal lineage. Animal Cells and Systems. 2015;19(5):321-31.
26. Wolff J. The Law of Bone Remodelling. 1986.
27. Chen JH, Liu C, You L, Simmons CA. Boning up on Wolff's Law: mechanical regulation of the cells that make and maintain bone. Journal of biomechanics. 2010;43(1):108-18.
28. S.Weinbaum SCC, Yu Zeng. A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. Journal of biomechanics. 1994;27:339-60.
29. Marlene Knippenberg MNH, Behrouz Zandieh Doulabi, Cornelis M. Semeins, Paul I.J.M. Wuisman, Jenneke Klein-Nulend. Adipose Tissue-Derived Mesenchymal Stem Cells Acquire Bone Cell-Like Responsiveness to Fluid Shear Stress on Osteogenic Stimulation. Tissue Engineering. 2005;11(11/12):1780-8.
30. Riddle RC, Taylor AF, Genetos DC, Donahue HJ. MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation. Am J Physiol Cell Physiol. 2006;290(3):C776-84.
31. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, et al. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A. 2002;99(20):12600-5.
32. Huang Y, Jia X, Bai K, Gong X, Fan Y. Effect of fluid shear stress on cardiomyogenic differentiation of rat bone marrow mesenchymal stem cells. Arch Med Res. 2010;41(7):497-505.
33. Arnsdorf EJ, Tummala P, Jacobs CR. Non-canonical Wnt signaling and N-cadherin related beta-catenin signaling play a role in mechanically induced osteogenic cell fate. PLoS One. 2009;4(4):e5388.

34. Zhou X, Liu D, You L, Wang L. Quantifying fluid shear stress in a rocking culture dish. Journal of biomechanics. 2010;43(8):1598-602.
35. Delaine-Smith RM, Reilly GC. The effects of mechanical loading on mesenchymal stem cell differentiation and matrix production. Vitam Horm. 2011;87:417-80.
36. Gurkan UA, Akkus O. The mechanical environment of bone marrow: a review. Ann Biomed Eng. 2008;36(12):1978-91.
37. Julia C Chen CRJ. Mechanically induced osteogenic lineage commitment of stem cells. Stem cell research & therapy. 2013;4(107):1-10.
38. Zhen Li BE, Shan-Jing Yao, Ph.D., Mauro Alini, Ph.D., and Martin J. Stoddart, Ph.D. Chondrogenesis of Human Bone Marrow Mesenchymal Stem Cells in Fibrin–Polyurethane Composites Is Modulated by Frequency and Amplitude of Dynamic Compression and Shear Stress. TISSUE ENGINEERING. 2010;16.
39. Sittichokechaiwut A, Edwards JH, Scutt AM, Reilly GC. Short bouts of mechanical loading are as effective as dexamethasone at inducing matrix production by human bone marrow mesenchymal stem cells. European Cells and Materials. 2010;20:45-57.
40. Haudenschild AK, Hsieh AH, Kapila S, Lotz JC. Pressure and distortion regulate human mesenchymal stem cell gene expression. Ann Biomed Eng. 2009;37(3):492-502.
41. Kearney EM, Farrell E, Prendergast PJ, Campbell VA. Tensile strain as a regulator of mesenchymal stem cell osteogenesis. Ann Biomed Eng. 2010;38(5):1767-79.
42. Jang JY, Lee SW, Park SH, Shin JW, Mun C, Kim SH, et al. Combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagents. J Biomed Biotechnol. 2011;2011:860652.
43. Hanson AD, Marvel SW, Bernacki SH, Banes AJ, van Aalst J, Loboa EG. Osteogenic effects of rest inserted and continuous cyclic tensile strain on hASC lines with disparate osteodifferentiation capabilities. Ann Biomed Eng. 2009;37(5):955-65.
44. Rui YF, Lui PP, Ni M, Chan LS, Lee YW, Chan KM. Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. J Orthop Res. 2011;29(3):390-6.
45. Kraft DC, Bindslev DA, Melsen B, Klein-Nulend J. Human dental pulp cells exhibit bone cell-like responsiveness to fluid shear stress. Cytotherapy. 2011;13(2):214-26.
46. Marrelli M, Codispoti B, Shelton RM, Scheven BA, Cooper PR, Tatullo M, et al. Dental Pulp Stem Cell Mechanoresponsiveness: Effects of Mechanical Stimuli on Dental Pulp Stem Cell Behavior. Front Physiol. 2018;9:1685.
47. Kraft DCE BD, Melsen B, Abdallah BM, Kassem M, Klein-Nulend J. Mechanosensitivity of dental pulp stem cells is related to their osteogenic maturity. Eur J Oral Sci. 2010;118:29-38.
48. Han M-J, Seo Y-K, Yoon H-H, Song K-Y, Park J-K. Effect of mechanical tension on the human dental pulp cells. Biotechnology and Bioprocess Engineering. 2008;13(4):410-7.
49. Phumpatrakom P, Srisuwan T. Regenerative capacity of human dental pulp and apical papilla cells after treatment with a 3-antibiotic mixture. J Endod. 2014;40(3):399-405.

50. Vishwanath V, Nadig R, Nadig R, Prasanna J, Karthik J, Pai V. Differentiation of isolated and characterized human dental pulp stem cells and stem cells from human exfoliated deciduous teeth: An in vitro study. Journal of Conservative Dentistry. 2013;16(5):423-8.
51. De Bari C, Dell'Accio F, Vanlauwe J, Eyckmans J, Khan IM, Archer CW, et al. Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. Arthritis Rheum. 2006;54(4):1209-21.
52. Puwanan S. Developing a tissue engineering strategy for cleft palate repair. PhD thesis, University of Sheffield. 2014.
53. Johnson M. Protein Quantitation. 2012.
54. Kaewkhaw R, Scutt AM, Haycock JW. Anatomical site influences the differentiation of adipose-derived stem cells for Schwann-cell phenotype and function. Glia. 2011;59(5):734-49.
55. Castren E, Sillat T, Oja S, Noro A, Laitinen A, Konttinen YT, et al. Osteogenic differentiation of mesenchymal stromal cells in two-dimensional and three-dimensional cultures without animal serum. Stem cell research & therapy. 2015;6:167.
56. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, et al. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell and tissue research. 2013;353(1):65-78.
57. Delaine-Smith RM, MacNeil S, Reilly GC. Matrix production and collagen structure are enhanced in two types of osteogenic progenitor cells by a simple fluid shear stress stimulus. European cells & materials. 2012;24:162-74.
58. Klien B.Y. Gal I. BH. Cell-mediated mineralization in culture at low temperature associated with subtle thermogenic response. Journal of cellular biochemistry. 1996;63:229-38.
59. Orr AW, Helmke BP, Blackman BR, Schwartz MA. Mechanisms of mechanotransduction. Developmental cell. 2006;10(1):11-20.
60. Nauman EA, Satcher RL, Keaveny TM, Halloran BP, Bikle DD. Osteoblasts respond to pulsatile fluid flow with shortterm increases in PGE2 but no change in mineralization. J Appl Physiol. 2001;90:1849-54.
61. Kreke MR, Huckle WR, Goldstein AS. Fluid flow stimulates expression of osteopontin and bone sialoprotein by bone marrow stromal cells in a temporally dependent manner. Bone. 2005;36(6):1047-55.
62. BROWN DCL, R. S. Improvements to parallel plate flow chambers to reduce reagent and cellular requirements. BMC immunology. 2001;2.
63. McCoy RJ, O'Brien FJ. Influence of shear stress in perfusion bioreactor cultures for the development of three-dimensional bone tissue constructs: a review. Tissue engineering Part B, Reviews. 2010;16(6):587-601.
64. Chen HH, Decot V, Ouyang JP, Stoltz JF, Bensoussan D, de Isla NG. In vitro initial expansion of mesenchymal stem cells is influenced by the culture parameters used in the isolation process. Biomed Mater Eng. 2009;19(4-5):301-9.
65. Coelho MJ FM. Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. Biomaterials. 2000;21(11):1095-102.
66. Nakamura S, Yamada Y, Baba S, Kato H, Kogami H, Takao M, et al. Culture medium study of human mesenchymal stem cells for practical use of tissue engineering and regenerative medicine. Bio-Medical Materials and Engineering. 2008;18(3):129-36.

67. Puwanun S, Delaine-Smith RM, Colley HE, Yates JM, MacNeil S, Reilly GC. A simple rocker-induced mechanical stimulus upregulates mineralization by human osteoprogenitor cells in fibrous scaffolds. Journal of tissue engineering and regenerative medicine. 2018;12(2):370-81.
68. Pre D, Ceccarelli G, Visai L, Benedetti L, Imbriani M, Cusella De Angelis MG, et al. High-Frequency Vibration Treatment of Human Bone Marrow Stromal Cells Increases Differentiation toward Bone Tissue. Bone marrow research. 2013;2013:803450.
69. Huber D, Oskooei A, Casadevall ISX, Andrew d, Kaigala GV. Hydrodynamics in Cell Studies. Chemical reviews. 2018;118(4):2042-79.
70. Yourek G, McCormick SM, Mao JJ, Reilly GC. Shear stress induces osteogenic differentiation of human mesenchymal stem cells. Regenerative medicine. 2010;5(5):713-24.
71. James S, Fox J, Afsari F, Lee J, Clough S, Knight C, et al. Multiparameter Analysis of Human Bone Marrow Stromal Cells Identifies Distinct Immunomodulatory and Differentiation-Competent Subtypes. Stem cell reports. 2015;4(6):1004-15.
72. Hruska MWAFCPCDMMKA. Mechanically Strained Cells of the Osteoblast Lineage Organize Their Extracellular Matrix Through Unique Sites of αVβ3-Integrin Expression. Journal of bone and mineral research. 2000;15(9):1731-45.
73. Sodek J, Ganss B, McKee MD. Osteopontin. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 2000;11(3):279-303.
74. DCE K, DA B, B M, BM A, M K, J K-N. Mechanosensitivity of dental pulp stem cells is related to their osteogenic maturity. Eur J Oral Sci. 2010;118:29-38.
75. Ralston RJvTHaSH. Nitric oxide and bone. Immunology. 2001;103:255-61.