CELLULAR RESPONSES OF STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH TOWARDS DIFFERENT CONCENTRATION OF CALCIUM ION

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การเปรียบเทียบการตอบสนองของเซลล์ต้นกำเนิดจากโพรงประสาทฟันน้ำนมต่อความเข้มข้นที่แตกต่างกันของแคลเซียมไอออน

น.ส.ฐานิกา ผลินยศ

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ฐานิกา ผลินยศ : การเปรียบเทียบการตอบสนองของเซลล์ต้นกำเนิดจากโพรงประสาทฟันน้ำนมต่อความเข้มข้นที่แตกต่างกันของแคลเซียมไอออน. (CELLULAR RESPONSES OF STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH TOWARDS DIFFERENT CONCENTRATION OF CALCIUM ION) อ.ที่ปรึกษาหลัก : รศ. ทพญ. ดร.วลีรัตน์ ศุกรวรรณ, อ.ที่ปรึกษาร่วม : ศ. ทพ. ดร.ธนภูมิ โอสถานนท์

วัตถุประสงค์ เพื่อศึกษาผลของความเข้มข้นที่แตกต่างกันของแคลเซียมไอออนต่อการเพิ่มจำนวน การแปรสภาพไปเป็นเซลล์สร้างกระดูก และการเคลื่อนที่ของเซลล์ต้นกำเนิดโพรงประสาทฟันน้ำนมมนุษย์ในห้องปฏิบัติการ

วัสดุและวิธีการ ทำการเพาะเลี้ยงเซลล์ในอาหารเลี้ยงเซลล์และ*อาหารเลี้ยง*เซลล์ที่มีฤทธิ์*เหนี่ยวนำ*ให้แปรสภาพเป็นเซลล์*สร้าง*เนื้อเยื่อแข็งซึ่งมีแคลเซียมไอออน 1.8 มิลลิโมล่าร์ เปรียบเทียบกับอาหารเลี้ยงเซลล์ที่เติมแคลเซียมไอออนจนได้ความเข้มข้นที่ระดับต่างๆ (5.4, 9, 12.6, 16.2 มิลลิโมล่าร์) ศึกษาการเพิ่มจำนวนของเซลล์ด้วยเทคนิคเอ็มทีที และวิธีโคโลนีฟอร์มมิ่ง ศึกษาการเปลี่ยนแปลงไปเป็นเซลล์กระดูกโดยศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างกระดูก และวัดการสะสมแร่ธาตุที่เพิ่มขึ้น และศึกษาการเคลื่อนที่ของเซลล์ด้วยการวัดพื้นที่ที่เซลล์สามารถเคลื่อนที่เข้าหากันหลังจากทำให้เกิดบาดแผล แสดงผลการศึกษาด้วยค่าเฉลี่ย±ส่วนเบี่ยงเบนมาตรฐาน วิเคราะห์ข้อมูลของเทคนิคเอ็มทีทีและการวัดพื้นที่ที่เซลล์สามารถเคลื่อนที่เข้าหากันหลังจากทำให้เกิดบาดแผล ด้วยการวิเคราะห์ความแปรปรวนสองทางและเปรียบเทียบค่าเฉลี่ยภายหลังการทดสอบรวมด้วยวิธีการของทูกี้ เนื่องจากมี 2 ตัวแปรได้แก่ ความเข้มข้นของแคลเซียม และเวลา ส่วนการวิเคราะห์ข้อมูลอื่นใช้การวิเคราะห์ความแปรปรวนทางเดียว

ผลการศึกษา ความเข้มข้นที่แตกต่างกันของแคลเซียมไม่มีผลต่อการเพิ่มจำนวนของเซลล์ต้นกำเนิดโพรงประสาทฟันน้ำนมเมื่อวัดด้วยเทคนิคเอ็มทีที และวิธีโคโลนีฟอร์มมิ่ง การศึกษาการเปลี่ยนแปลงไปเป็นเซลล์กระดูก พบว่าแคลเซียมไอออนที่เพิ่มขึ้นเพิ่มระดับการแสดงออกของยีนออสทีโอแคลซินในวันที่ 7 โดยที่ระดับความเข้มข้นของแคลเซียมที่ 9 และ 12.6 มิลลิโมล่าร์ การแสดงออกของยีนเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) สอดคล้องไปกับการติดสีเข้มเมื่อย้อมสีด้วยอะลิซาลิน เรด และฟอน คอสซา ซึ่งพบว่าเซลล์มีการติดสีเข้มขึ้นเมื่อระดับความเข้มข้นของแคลเซียมสูงขึ้นจนติดสีเข้มที่สุดที่ระดับความเข้มข้นของแคลเซียม 9 มิลลิโมล่าร์ และการศึกษาการเคลื่อนที่ของเซลล์พบว่าภายใน 24 ชั่วโมง พื้นที่ที่เซลล์สามารถเคลื่อนที่เข้าหากันหลังจากทำให้เกิดบาดแผลของกลุ่มที่มีแคลเซียมไอออนมากกว่า 1.8 มิลลิโมล่าร์ มีค่าน้อยกว่ากลุ่มควบคุมซึ่งมีแคลเซียมไอออน 1.8 มิลลิโมล่าร์

สรุป ความเข้มข้นของแคลเซียมไอออนที่เพิ่มขึ้นไม่มีผลต่อการเพิ่มจำนวนของเซลล์ แต่มีผลเพิ่มการแสดงออกของยีนออสทีโอแคลซินและเพิ่มการสร้างแร่ธาตุ นอกจากนั้นยังมีผลในการยับยั้งการเคลื่อนที่ของเซลล์ต้นกำเนิดโพรงประสาทฟันน้ำนม

สาขาวิชา	ทันตกรรมสำหรับเด็ก	ลายมือชื่อนิสิต .....
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KEYWORD: SHEDs, calcium, cell proliferation, osteogenic differentiation, colony forming unit, migration

Thanika Phlinyos : CELLULAR RESPONSES OF STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH TOWARDS DIFFERENT CONCENTRATION OF CALCIUM ION . Advisor: Assoc. Prof. WALEERAT SUKARAWAN, D.D.S., Ph.D. Co-advisor: Prof. Thanaphum Osathanoon, D.D.S., Ph.D.

Objectives: The purpose of this study was to investigate the effect of Ca^{2+} on proliferation, osteogenic differentiation, and migration of stem cells from human exfoliated teeth (SHEDs) in vitro

Materials and methods: SHEDs were seeded in culture media and osteogenic induction media containing 1.8-16.2 mM of Ca^{2+}. SHEDs proliferation was determined using MTT assay and colony forming unit assay. Osteogenic differentiation was evaluated using mineralization assay and osteogenic marker gene expression and cell migration was evaluated using wound healing assay. Values were expressed as mean + S.D. Statistical analysis of MTT assay and wound healing assay were performed using two-way ANOVA followed by Tukey's range test. Other assays were analyzed using one-way ANOVA.

Results: Different Ca^{2+} concentration did not affect cell proliferation and colony forming unit. While, osteocalcin, an osteogenic marker gene, was significantly increased at day 7 in 9.0 and 12.6 mM group ($p<0.05$). The result was also consistent with alizarin red and Von kossa staining, which had the most staining in 9.0 mM group. However, higher Ca^{2+} concentration inhibited SHEDs migration at 24 hours compared to control (1.8 mM).

Conclusion: Ca^{2+} concentration between 1.8 and 16.2 mM did not have any effects on proliferation and colony forming unit. On the other hand, higher Ca^{2+} seemed to induce late stage osteogenic differentiation and mineralization, but migration was inhibited.

Field of Study:	Pediatric Dentistry	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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Best Regards,

Thanika Phlinyos

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Chapter 1

Introduction

Background and rationale

Calcium ion (Ca^{2+}) in pulp capping materials is believed to be the key component that controls osteo/odontoinductive process in vital pulp therapy contributing to tertiary dentinogenesis (1, 2). Recent studies found that elevated extracellular Ca^{2+} in a significant amount can promote an expression of osteogenic markers in various human stem cells such as human adipose-derived, bone-marrow-derived mesenchymal stem cells and dental pulp cell (3-5). The increase of extracellular Ca^{2+} can initiate osteo/odontogenic differentiation and mineralization processes in dental pulp cell via calcium carbonate precipitation in the wound area (6-8), indicating an important role for external Ca^{2+} level in regulating the function of cell.

Ca^{2+} also plays crucial role in other cellular function such as cell proliferation, migration and also apoptosis (9). Change of intracellular Ca^{2+} homeostasis in cells caused apoptosis in many cell types. Recent studies found that high extracellular Ca^{2+} (20 mM) can inhibit bone resorption ability of osteoclasts and induce osteoclast apoptosis (10). The free Ca^{2+} released from pulp capping materials may modulate other cell functions in mineralized tooth tissue as well (11).

Despite being the major constituent in different pulp capping materials, cellular mechanism of Ca^{2+} on dentin regeneration by dental stem cells is largely underexplored. Recent studies about the effect of external Ca^{2+} concentrations were done on dental pulp stem cell (DPSCs) obtained from permanent teeth and found that Ca^{2+} does not affect DPSCs cell proliferation. In fact, Ca^{2+} promotes mineralization but inhibits alkaline phosphatase (ALP) activity, an odontoblastic cell differentiation marker, in a non-linear relationship. Moreover, Ca^{2+} affects osteogenic gene expression by downregulating some gene expressions but upregulating some other genes (6). Surprisingly, study of effect of Ca^{2+} on stem cells from human

exfoliated deciduous teeth (SHEDs) is scarce despite its promising stem cells potential. SHEDs are mesenchymal stem cell obtained from pulp of deciduous teeth, which are easy to collect with little ethical concern. Moreover, SHEDs have high proliferation rate and express high levels of pluripotent markers that make them more immature than DPSCs (12). In addition, SHEDs can differentiate into many types of cell lineages, such as osteogenic/odontogenic, neurogenic, angiogenic, and adipogenic differentiation. For these reasons, SHEDs are an important source of stem cells for dental tissue engineering (12).

Thus, this study aimed to investigate the effect of Ca^{2+} concentrations on SHEDs in many aspects, including cell proliferation, cell migration, and osteogenic differentiation. The results will benefit in determining the optimum Ca^{2+} level suitable for developing SHEDs in dental tissue regeneration and might lead to the new innovation of vital pulp therapy agent in the future.

Research question

Does the Ca^{2+} affect cell proliferation, osteogenic differentiation, and cell migration of SHEDs?

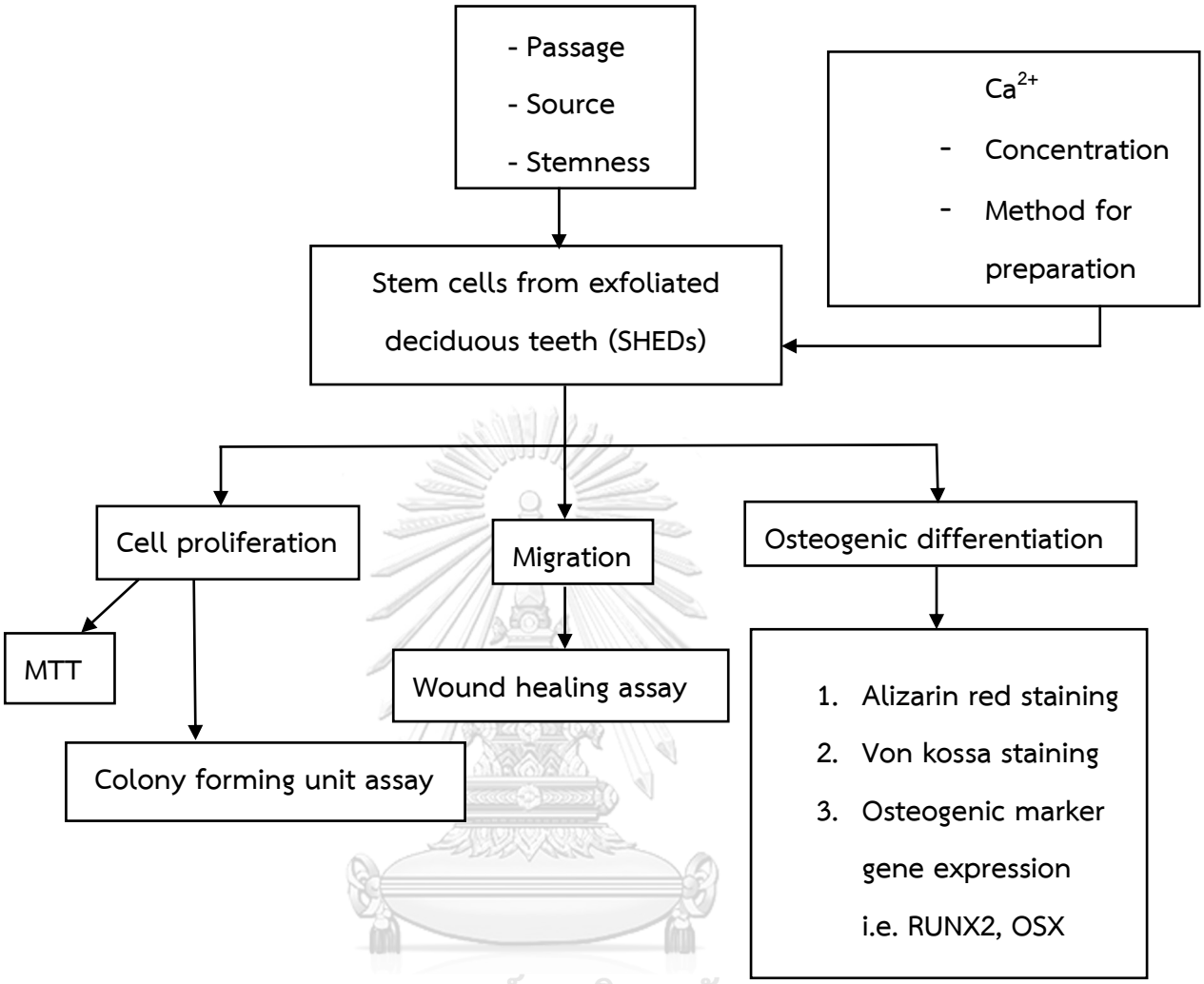
Research Objectives

1. To investigate the effect of Ca^{2+} concentration on proliferation of SHEDs
2. To investigate the effect of Ca^{2+} concentration on osteogenic differentiation of SHEDs
3. To investigate the effect of Ca^{2+} concentration on migration of SHEDs

Hypothesis

The optimal Ca^{2+} concentration can provide desirable outcomes on proliferation, osteogenic differentiation, and migration of SHEDs.

Conceptual framework



Research design

Laboratory Experimental Research

Keywords

SHEDs, calcium, cell proliferation, osteogenic differentiation, colony forming unit, migration

Definitions

1. **SHEDs** : mesenchymal stem cells obtained from dental pulp tissue of human exfoliated deciduous teeth, which are not invasive and easy to acquire. Cells will be isolated from the tissue explants and used in the experiments without immortalization.
2. **Cell proliferation** : the process of growing and dividing of cell to produce two daughter cells. In this study, MTT assay will be used to determine an increasing number of cells.
3. **Osteogenic differentiation** : the process of becoming an osteoblast of unspecialized cells under induced condition. In this study, osteogenic differentiation will be assessed by Alizarin red staining, Von Kossa staining, and osteogenic marker gene expressions.
4. **Colony forming unit** : unit commonly used to estimated the number of cells in a test samples
5. **Migration :** the process of moving of cell from one location to another. In this study, wound healing assay will be used to examine cell migration.

Expected benefits of the study

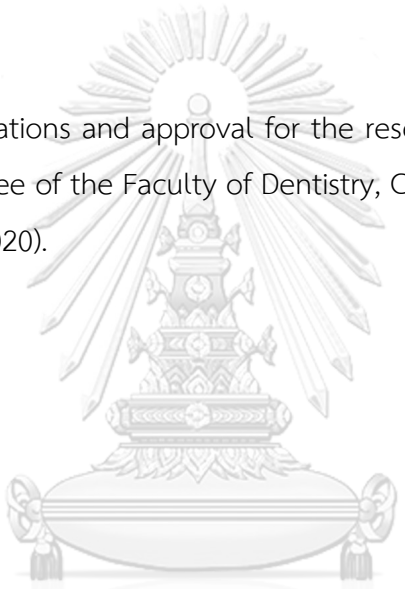
The results provided data suggesting the effect of various concentrations of Ca^{2+} on SHEDs. The information was useful for inducing mineralized tissue formation from SHEDs in tissue engineering in the future.

Limitation

This study was performed in *in vitro* condition. Some human conditions could not be imitated due to several limitations.

Ethical consideration

Ethical considerations and approval for the research protocol were approved by the Ethical Committee of the Faculty of Dentistry, Chulalongkorn University (Ethics code: HREC-DCU 2021-020).



Chapter 2

Literature review

Stem cells

Stem cells are an essential part of tissue engineering, a combination of engineering and bioscience that focuses on developing biological substances that can restore, maintain, or improve tissue function. Tissue engineering requires two additional components, scaffolds and growth factors, which are chemical and physical stimuli. The principles of tissue engineering are to either stimulate regeneration of target tissue from the inside or isolate human stem cells and culture them in a finely replicated in vitro environment of the native tissue scaffolds, aiming to produce biological replacements for previously damaged tissue of the host. Moreover, growth factors must also be provided to maintain stem cells because various molecular factors can affect many properties of stem cells, including their shape, proliferation capability, and death (13-15).

Stem cells are unspecialized cells with excessive proliferation and self-renewal potential capable of differentiation into various cell types of an organism. Stem cells can be classified based on their differentiation ability starting from totipotent, with the most differentiation ability, pluripotent, multipotent, oligopotent, and unipotent, with the least potential. Totipotent stem cells, such as zygotes, can differentiate into any cell type of an organism, both embryo and extraembryonic structure. Pluripotent stem cells are capable of forming into cells of all germ layers; endoderm, mesoderm, and ectoderm, except for extraembryonic structures, such as the placenta. Embryonic stem cells (ESCs) and induced pluripotent stem cells are examples of pluripotent stem cells. Multipotent stem cells, such as mesenchymal stem cells and hematopoietic stem cells, have less proliferative ability than pluripotent stem cells, mostly limited to each germ layer or specific cell lineages. Oligopotent stem cells differentiation potential is more restricted to specific cell lineage. An example of oligopotent stem cells is a myeloid

stem cell, which can develop into only white blood cells but not red blood cells. Unipotent stem cells have the least differentiation capabilities of only one cell type. However, they are a promising candidate for regenerative therapy (16-18).

Another classification of stem cells is based on their origin, which can be divided mainly into ESCs and adult stem cells. Human ESCs are obtained from the inner mass of blastocytes, which have high differentiation potential. Despite their promising features, the use of ESCs is avoided because of ethical issues in harvesting, which result in the destruction of blastocytes. Thus, using adult stem cells is more practical for clinical practice. Adult stem cells can be harvested from numerous tissue such as bone marrow, skeletal muscle, neural tissue, and various oral regions (19).

Human dental stem cells

Uses of human dental stem cells in tissue engineering are becoming more popular because many dental stem cells show promising differentiation abilities and can be harvested without causing much harm. Human dental stem cells can be obtained from various oral tissue, including SHEDs, dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells (DFSCs), and stem cell from dental apical papilla (SCAPs). Despite their similarities in the expression of various gene markers, their proliferation and differentiation potential are quite different (15, 20).

Stem cells from human exfoliated deciduous teeth

Stem cells from human exfoliated deciduous teeth (SHEDs) are multipotent mesenchymal stem cells discovered by Miura et al. in 2003. SHEDs have been reported to show an expression pattern similar to mesenchymal stem cell properties, such as CD13, CD44, CD73, CD90, CD146, CD166, and STRO-1 but not CD34, CD45, which are hematopoietic lineage markers. Since then, SHEDs have gotten more and more attention because of their high expression of proliferation rate and population doublings number compared to dental pulp stem cell (DPSC) and bone marrow stromal stem cell (BMSSC). Moreover, SHEDs also show higher potential for proliferation than DPSC from their expression of various markers such as CD117 (stem cell factor I receptor, typical for pluripotent stem cell), CD71 (a proliferating cells marker), and CD105 (an endothelial cell marker), and also remain more undifferentiated than DPSC (12, 21, 22).

SHEDs are widely known and studied for their odontogenic and osteoblastic lineage differentiation ability because SHEDs express osteogenic markers, such as RUNX2, DSP, bone morphogenetic protein 2 (BMP2), osteocalcin (OCN), osteopontin (OPN), osteonectin, and type 1 collagen (Col-1). SHEDs can differentiate into osteoblasts both in vitro and in vivo with bone-forming and regeneration abilities. This regeneration capability even exists in non-immunosuppressed mice, repairing large bone defects. SHEDs odontoblastic lineage differentiation is also confirmed both in vivo and in vitro. When induced, SHEDs can differentiate into odontoblast-like cells that can generate dental pulp-like tissue, in vivo. However, most of these studies were done in an ectopic implantation model in mice or rats, which are not similar to clinical situations. Thus, further studies under a more real-clinical situation, such as tooth socket or jaw bone of large animals, might be needed (22).

SHEDs are also capable of differentiation into other cell lineages, such as neurogenic, angiogenic, and adipogenic differentiation. SHEDs can be induced to

become a variety of neural cells. Studies have shown that significant recovery improvement was seen in rats with spinal cord injury that had SHEDs transplanted, and further improvement is seen when SHEDs were pre-induced to differentiate into neural-like cells before transplant. SHEDs also express various endothelial differentiation markers when cultured in tooth slice combine with VEGF and can differentiate into endothelial cells when transplants into immunodeficient mice (12, 22).

Harvesting SHEDs is very simple and convenient, with little trauma to none. Moreover, using one's stem cell decrease risks of immune rejection and eliminate risks of getting diseases from donor cells. After extraction, the teeth are transferred to a container and then cultured before cryo-preserved. The preserved SHEDs can be kept for 9 months without losing their properties (23).

Ca^{2+} and osteogenic differentiation of stem cells

Ca^{2+} plays an important role in regulating cell functions such as cell differentiation, proliferation, and apoptosis through cell signaling pathways. Primary sources of Ca^{2+} in most cells, including stem cells, are from an influx of extracellular Ca^{2+} through the plasma membrane and Ca^{2+} release from the internal storage of endoplasmic reticulum (ER) by inositol 1,4,5-trisphosphate receptor (InsP3R). Most of the influx of Ca^{2+} through the plasma membrane is from the stored-operated Ca^{2+} (SOC) channels, which are voltage-independent pathways, with additional help from the voltage-operated Ca^{2+} channels (VOCCs). Changes in intracellular Ca^{2+} levels have been exhibited to begin specific processes of cell behavior in many conditions, for example, in osteogenic differentiation of mesenchymal stem cells (9, 24, 25).

Recent studies found that elevated extracellular Ca^{2+} ranging from 0.3 to 5.0 mM can promote an expression of various osteogenic markers in human adipose-derived stem cells, including ALP activity as well as the mRNA expressions of Runx2 and osteocalcin. The elevation of extracellular Ca^{2+} also activates the calcium

sensing receptor (CaSR), increasing the intracellular Ca^{2+} along with the expression of BMP-2 mRNA. These results suggest that an elevation of extracellular Ca^{2+} can enhance osteogenic differentiation and mineralization of human adipose-derived stem cells (3).

Ca^{2+}, at different concentration, also affect the osteogenic differentiation of bone-marrow-derived mesenchymal stem cells. At 1.8 mM of Ca^{2+}, bone-marrow-derived mesenchymal stem cells show the highest osteogenic differentiation capability, which can be seen from the highest expression of Col-1 and OCN as well as ALP activity. Further increases or decreases in Ca^{2+} concentration result in a significant decreases of these osteogenic markers (4).

Ca^{2+} and apoptosis of stem cell

Apoptosis is a programmed cell death process that happens when cells face stress or damage (26). Change of Ca^{2+} homeostasis in cells is also one of the factors in the apoptosis regulation as increasing intracellular Ca^{2+} induces apoptosis in stem cells. Recent studies found that high extracellular Ca^{2+} (20 mM) can inhibit bone resorption ability of osteoclasts and induce osteoclast apoptosis (10). Furthermore, increasing extracellular Ca^{2+} influx induces mitochondrial reactive oxygen species (ROS) production that leads to oligodendrocyte cell toxicity and apoptosis (27). However, the apoptosis of bone-marrow-derived mesenchymal stem cells does not seem to be affected by the changes in extracellular Ca^{2+} level between 0.0 mM to 7.2 mM, as no apoptotic cells were seen in any of these groups. Nevertheless, Ca^{2+} concentrations used in this study seem to be lower than in other studies. Thus, further studies about the effect of Ca^{2+} on the apoptosis of stem cells might be needed (4).

Effect of Ca^{2+}on pulp cell

It has been proven that calcium is necessary for inducing pulp cells to form reparative dentin, but the mechanism cannot be clearly identified yet (28). Two studies on the effect of various Ca^{2+} concentrations ranging from 1.8 mM to 16.2 mM to DPSC found that increasing levels of Ca^{2+} slightly decrease DPSC proliferation rate in a not significant manner. Both of these studies also state that osteopontin (OPN), a noncollagenous protein that is produced by pulp cells, which helps in mineralization of reparative dentin, gene expression were highest in 5.4 mM Ca^{2+} concentration group (6, 29). However, another study at a lower concentration of Ca^{2+} (1.1 – 1.8 mM) found that OPN level was highest at 1.8 mM Ca^{2+} concentration (30). Both studies also found that mineralization rate of DPSC is not linear to Ca^{2+} concentration, but Ca^{2+} concentrations that induce the most mineralization are different between these studies (5.4 & 9.0 vs. 12.6 mM). There is also an association between the mineralization and the apoptosis rate of DPSC in a linear manner. Therefore, more studies are needed to confirm the relationship between Ca^{2+} to OPN expression and mineralization. ALP gene expression, an odontoblastic differentiation marker, was also found to be lower with higher Ca^{2+} level but may also be in a non-linear relationship (6, 29). Thus, it can be seen that only a few studies about direct effect of various Ca^{2+} concentrations are available, and further studies are needed.

Table 1 Summarize of effects of Ca^{2+}on different stem cells

Cell type	Ca conc used (mM)	Proliferation	Mineralization	Apoptosis	Osteogenic differentiation marker (Highest)						
					ALP	OPN	OCN	RunX2	BMP-2	BMP-4	Col I
APSC (3)	0.3, 1.8, 5.0	X	X	X	5.0 (D7, 14, 21)	X	5.0 (D7, 14, 21)	5.0 (D3)	5.0 (24, 48h)	X	X
BMSC (4)	0, 0.9, 1.8, 3.6, 7.2	Ca < 1.8 inhibit proliferation	Increase with Ca level	No effect	1.8	X	1.8	X	X	X	1.8
DPSC (6)	1.8, 5.4. 9.0, 12.6, 16.2	No effect *except lowest in 16.2(D2)*	Highest at 12.6 (D14, 21)	X	1.8	5.4 (D14, 21)	5.4 (D14, 21)	No sig diff **	X	X	1.8 (D14, 21)
DPSC (29)	1.8, 5.4. 9.0, 12.6, 16.2	No effect	Fastest & highest at 5.4, 9	5.4, 9.0 > 1.8	X	5.4	X	X	X	X	X
DPSC (30)	1.1, 1.3, 1.5, 1.8	X	X	X	1.8 Lowest	1.8	X	X	1.8	1.8 Lowest	X

(except for lowest in 5.4 mM (D21)), **X = no test were done

Calcium staining

Calcium mineralization can be detected by various methods. Two of the most used methods are Von Kossa and alizarin red S staining methods. Von Kossa staining method is a histochemical method frequently used to detect calcium both in vitro and in vivo. This technique uses silver nitrate to treat samples. However, what this technique really detect is not the calcium itself but the anions like phosphate and carbonate that usually bind to calcium instead. Only the reacted phosphate or carbonate will show coloration. Thus, lack of specificity is a major disadvantage for Von Kossa method because it cannot detect other calcium ions that do not bind to phosphate or carbonate. The stained anions will give off grey and black coloration. Nevertheless, these colors are not the direct results of silver nitrate. Originally, when calcium phosphates were treated with silver nitrates, yellow deposit appear at first, but we normally found them as black because silver nitrate was later reduced by organic components in the cells and UV light exposure. Moreover, it is impossible to separate organic materials from calcium deposits in the samples completely. Thus, traditional histological procedures record black coloration instead of yellow that only appear in the early stage (31, 32).

Alizarin red S, a derivative of hydroxy anthra-quinone, is also another frequently used method for detecting calcium with some advantages over Von Kossa because it has more specificity toward calcium. Thus, more coloration was usually seen with alizarin red S compared to Von Kossa. Alizarin red S reacts with calcium ion via its sulfonate and hydroxyl group, creating coloration. Alizarin red S itself is not a dye, but it forms visible colored precipitation with calcium under pH around 4 – 8 with colors ranging from yellow to violet. Alizarin red S also capable of binding with calcium-binding proteins and proteoglycans which may result in misinterpretation. However, alizarin red S specifically bind with calcium at pH of 4.2, which can prevent false-positive coloration from binding with other substances (33-36). It is

recommended to use both of these methods for calcium staining, which can help in confirming each other results. Moreover, additional tests for specific marker expressions should also be used in correlation with the results (37).

Chapter 3
Materials and methods

Patients selection

Stem cells from human exfoliated deciduous teeth (SHEDs) were obtained from primary teeth with no carious lesions or pathologic lesion of healthy pediatric patients from Pediatric dentistry department, Faculty of dentistry, Chulalongkorn University. The teeth were extracted following the treatment plan such as prolong retention teeth and stored in a culture medium. Ethical approval was submitted by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. The study protocol and informed consent were provided to the child's parent.

Sample size

Exfoliated deciduous teeth were obtained from at least 4 different patients for biological replication

The study process

Cell Isolation and culture

Primary dental pulp cells were explanted from exfoliated deciduous teeth. Briefly, extracted teeth were washed with sterile phosphate buffer saline (PBS) 2-3 times; then the pulp tissues were carefully removed from the teeth and cut into small pieces using a surgical blade, and placed on 35-mm tissue culture plate (Corning, New York, NY, USA) with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 2mM L-glutamine (Gibco, USA), 100 Units/ml Penicillin (Gibco, USA), 100 μg/ml Streptomycin (Gibco, USA), and 5 μg/ml Amphotericin B (Gibco, USA). The cell explants were incubated at 37°c in a humidified atmosphere of 5 %CO_2 in the air. The media were changed every two days. When cell confluence was achieved, cells were detached with 0.25 % trypsin-EDTA (Gibco, USA) and subcultured at a 1:3 ratio to first passage on 60-mm tissue culture plates. Cells from passages 6-9 were used for the experiments.

Flow cytometry

The flow cytometry analysis was applied to quantify the expression of specific surface antigens in SHEDs cells. Single cell suspensions were obtained by detaching cells with 0.25 % trypsin-EDTA solution. Cells were centrifuged and the supernatant culture media were discarded. Then, cells were rinsed with PBS and stained with primary antibodies conjugated to fluorescent dye, including anti-human CD44 (BD Bioscience Pharmingen, USA), PerCP-CyTM5.5-conjugated anti-human CD90 (BD Bioscience Pharmingen, USA), and PerCP-conjugated anti-CD45 (BD Bioscience Pharmingen, USA). Stained cells were analyzed using a FACSCalibur flow cytometer using the CellQuest software (BD Bioscience, USA).

Ca^{2+} supplements

Culture media and osteogenic induction media (culture media with 50 ug/mL ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone) containing various concentrations of Ca^{2+} were prepared by dissolving calcium chloride ($CaCl_2$; Sigma-Aldrich, USA) in distilled water and added into the media which regularly contained 1.8 mM Ca^{2+}. The Ca^{2+} concentrations used in this experiment were 1.8 mM (control), 5.4 mM (threefold), 9.0 mM (fivefold), 12.6 (sevenfold) and 16.2 (ninefold).

Cell proliferation assay

SHEDs were seeded at a density of 12,500 cells/well in 24-well plates in culture media, with four repeats for each Ca^{2+} concentration. After 1, 3, and 7 days, changes in cell growth viability were analyzed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The existing media in every well were removed and replaced with 300 ul MTT solution (USB Corporation, USA) for 15 minutes at 37°C in a humidified atmosphere of 5 %CO_2 to allow formazan crystal formation. Next, the formazan was dissolved using eluting agents that contained

dimethylsulfoxide and glycine buffer. The solutions were determined for optical density (OD) by a microplate reader (ELx800; BIO-TEK®) at 570 nm.

Osteogenic/odontogenic induction

SHEDs were seeded at a density of 12,500 cells/well in 24-well plates. After 24 hr, culture media were replaced with osteogenic induction media containing various concentrations of Ca^{2+}, with four repeats for each concentration. Cell-free controls corresponding to each group tested were conducted to determine whether spontaneous precipitation occurred following calcium supplementation under the same conditions as the cell culture. The media were changed every 48 hr. Mineral deposition was analyzed using alizarin red S staining and von kossa staining, as described below. Osteogenic marker gene expression was determined using quantitative real-time polymerase chain reaction, as described below.

Alizarin red S staining

At day 14 of culture, SHEDs were washed one time with PBS and fixed with 4% formalin in PBS for 10 min. After that, cells were washed two times with deionized water. Subsequently, cells were incubated with alizarin red S solution for 3 min at room temperature under gentle agitation. Then, cells were washed three times with deionized water to remove excess staining. The precipitated dye was solubilized in 10% w/v cetylpyridinium chloride solution, and the absorbance was measured at 570 nm

Von kossa staining

At day 14 of culture, SHEDs were washed one time with PBS and fixed with 4% formalin in PBS for 10 min. After that, cells were washed two times with deionized water. Subsequently, cells were incubated with 1% silver nitrate solution for 10 min under ultraviolet light. Then, cells were washed three times with deionized water to remove excess staining and dried overnight. The plates were photographed with a digital camera, and the amount of staining was visually quantified.

Quantitative real-time polymerase chain reaction

The expression levels of osteogenic marker genes after 3 and 7 days in osteogenic induction media with various Ca^{2+} concentrations were determined by quantitative RT-PCR (qRT-PCR). Total cellular RNA was extracted using Trizol Reagent according to the manufacturer's instructions. Then, the complementary DNA (cDNA) was synthesized using reverse transcriptase ImPromII kit (Promega, USA). After that, QPCR was performed on MiniOpticon real-time PCR system (Bio-Rad, USA) using FastStart Essential DNA Green Master kit (Roche Diagnostic, USA). The values of Gene expression were normalized to GAPDH expression values and further normalized to controls. The primer sequences for RT-PCR are listed in Table 1.

Table 2 Primer sequences used in this study

Gene	Primer sequence
GAPDH	Forward: 5’ GAAGGTGAAGGTCGGAGTC 3’ Reverse: 5’ GAAGATGGTGATGGGATTTC 3’
RUNX2	Forward: 5’ ATGATGACACTGCCACCTCTG 3’ Reverse: 5’ GGCTGGATAGTGCATTCGTG 3’
OSX	Forward: 5' GCCAGAAGCTGTGAAACCTC 3' Reverse: 5’ GCTGCAAGCTCTCCATAA 3’
ALP	Forward: 5’ GACCTCCTCGGAAGACACTC 3’ Reverse: 5’ TGAAGGGCTTCTTGTCTGTG 3’
OCN	Forward: 5’ CTTTGTGTCCAAGCAGGAGG 3’ Reverse: 5’ CTGAAAGCCGATGTGGTCAG 3’

Colony forming unit assay

SHEDs were seeded into 6-well culture plates at a density of 500 cells. After 24 hr, the culture media with various Ca^{2+} concentrations were replaced. After 14 days, cells were fixed with 10% buffer formalin (MERCK, Germany) for 10 minutes, washed SHEDs with PBS twice, and stained with methylene blue (Sigma, USA). Then the colony area and intensity were determined using ColonyArea plugin in ImageJ software on the individually taken photo of each well. The ColonyArea plugin then quantified the colony area and intensity based on the difference in color threshold between the colony and the background (38).

Migration assay

Wound healing assay were used to examine cell migration. SHEDs were seeded into 6-well culture plates and allowed to reach full confluence. A vertical line was scratched through the cell monolayer to make a wound gap with a 10 µL pipette tip; the maximum differences in the width of the wound between each well must not exceed 5 percent. Then, culture media with various Ca^{2+} concentrations were replaced. Microphotographs of the scratch were taken at 0, 24, and 48 hours post-wounding. The cultured cells were observed with a phase-contrast microscope (Olympus, Tokyo, Japan). Wound closure rate was determined by image analysis using ImageJ (National Institute of Health, Bethesda, MD, USA).

Statistical analyses

All experiments were repeated from cells obtained from at least four different donors (n=4). All data were expressed as mean ± standard deviation (SD). The results of MTT assays and wound healing assays were statistically analyzed using two-way Anova and Tukey's multiple comparison test of concentration and time. Otherwise, one-way Anova were used in all other experiments. All statistical analyses will be performed using Prism 8 (GraphPad Software, CA, USA). A p-value of < 0.05 will be considered to indicate a significant difference.

Places of Study

Research unit of mineralized tissue, Faculty of Dentistry, Chulalongkorn University

Chapter 4

Results

SHED cell isolation and characterization

Cells isolated from primary teeth exhibited a fibroblast-like and spindle-shaped morphology (figure 1). To quantify the expression of specific surface antigens of SHEDs, SHEDs were analyzed using flow cytometry. SHEDs expressed stem cells markers; CD44 (92.65%) and CD90 (77.87%), which were the known mesenchymal stem cell markers, whereas they were negative for hematopoietic markers, CD45 (0.23%) (figure2).

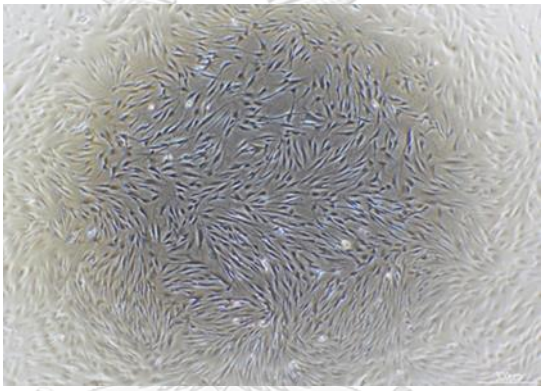


Figure 1 Cell morphology of the isolated cells at day-1 cultured in 10%DMEM (original magnification x4). Cells were spindle and fibroblast like in shape.

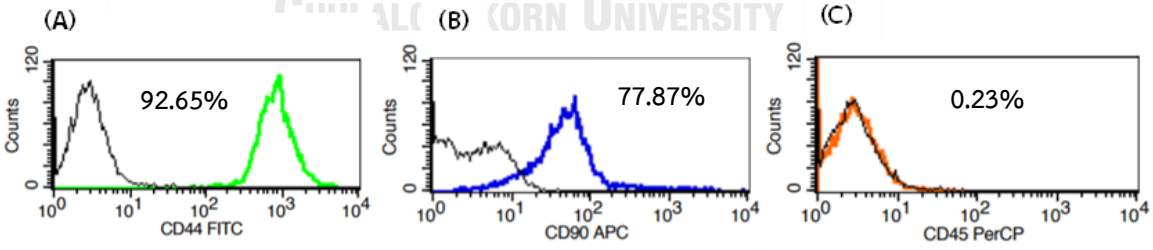


Figure 2 Flow cytometric analysis of surface antigens on SHEDs. Isolated cells expressed mesenchymal stem cells surface markers including CD44 (A), CD90 (B) whereas cells did not express hematopoietic markers, CD45 (C).

Effects of the Ca^{2+} concentrations on the proliferation of SHEDs

To evaluate the effect of Ca^{2+} concentrations on the proliferation of SHEDs, SHEDs were maintained in culture medium with various Ca^{2+} concentration. After 1, 3 and 7 days, cell proliferation were analyzed using MTT assay. The results indicated that there was no statistically significant difference in cell proliferation ability between the control group (1.8 mM Ca^{2+} concentrations) and experimental groups (5.4, 9, 12.6 and 16.2 mM Ca^{2+} concentrations) ($P > 0.05$) (Figure 3).

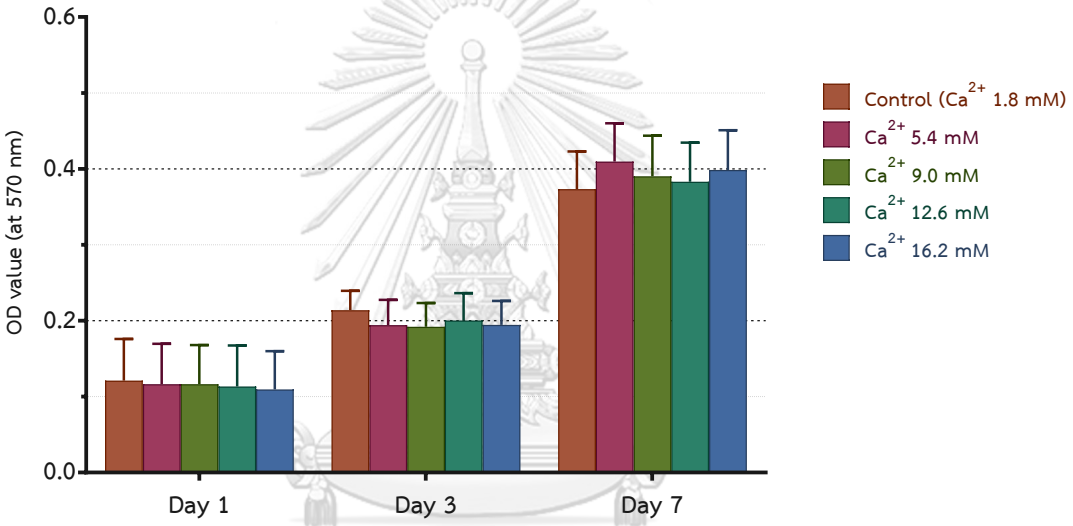
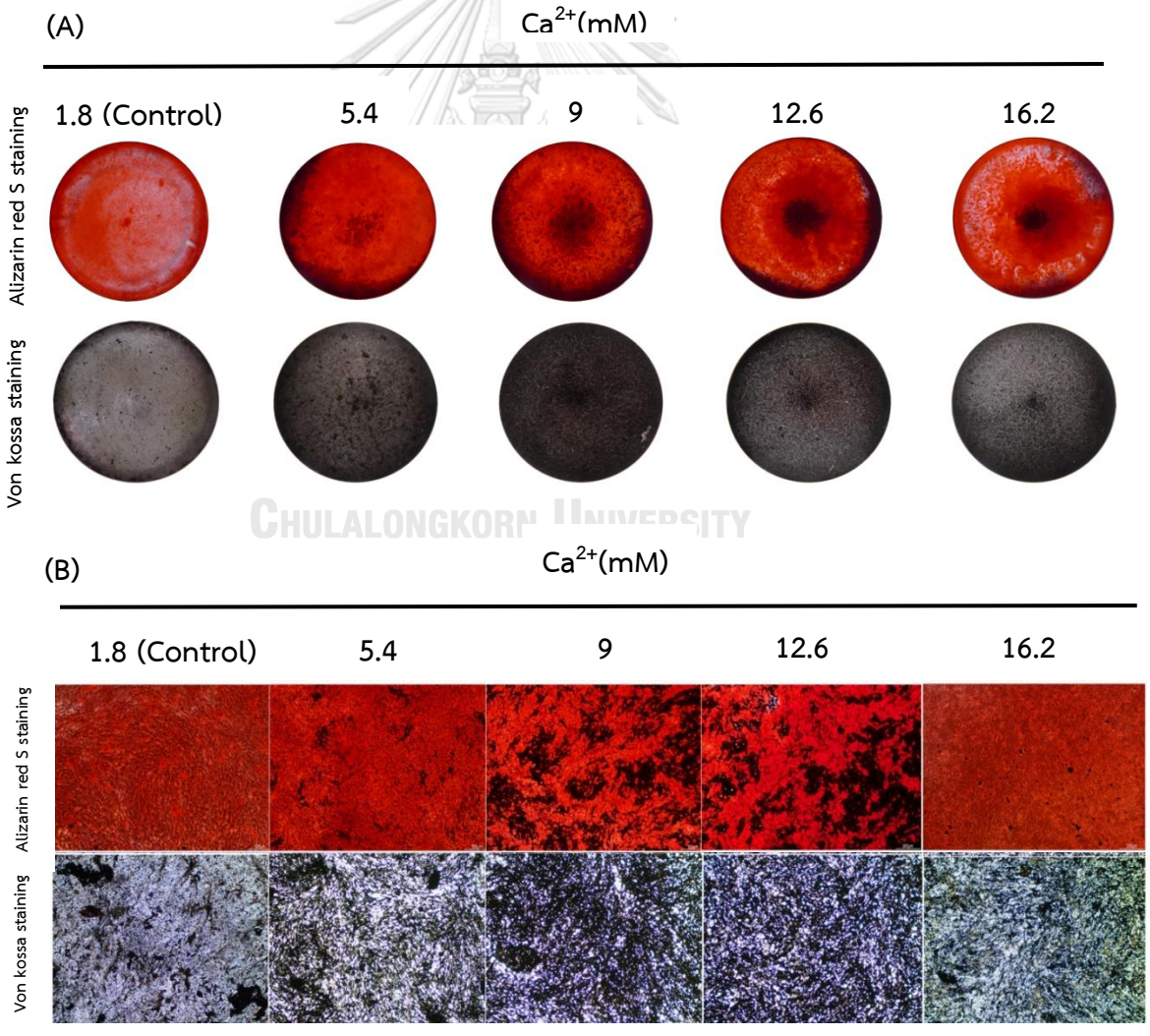


Figure 3 Ca^{2+} concentrations do not affect the proliferation of SHEDs. Increased Ca^{2+} concentrations showed no statistically significant difference in cell proliferation ability between the control and experimental groups ($P > 0.05$).

Effects of the Ca^{2+} concentrations on the mineralization of SHEDs

Increased Ca^{2+} concentrations accelerated mineralization of SHEDs

Cells were cultured in osteogenic induction medium containing various concentrations of Ca^{2+} for 14 days. Then, cells were stained with Alizarin red S and von kossa to evaluate mineralized deposition. As shown in Figure 4, mineralized deposition in SHEDs gradually increased with increasing Ca^{2+} concentrations. The maximal staining was observed at 9 mM Ca^{2+} concentrations. The calcium content in the mineralized nodules was significantly higher in the experimental groups compared to the control group ($P < 0.0001$), except for the 16.2 mM Ca^{2+} concentrations that had less significant difference ($P < 0.05$).



(C)

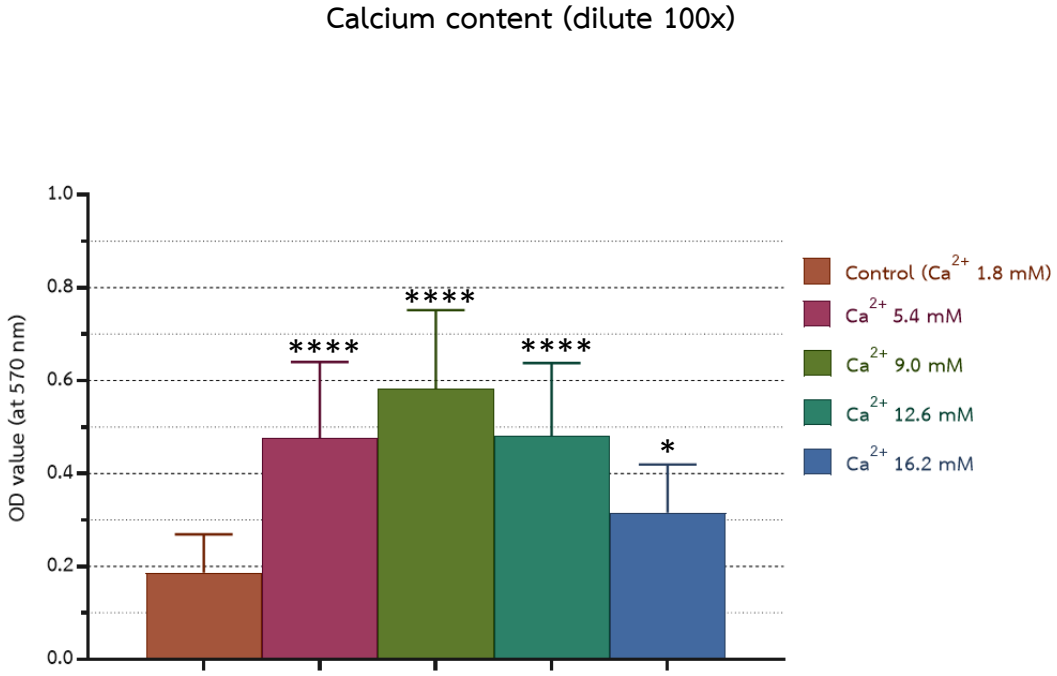


Figure 4 Increased Ca^{2+} concentrations significantly increased mineralization of SHEDs at 14 days. (A) Mineralization was determined using Alizarin red staining and Von Kossa staining. The maximal staining was observed at 9 mM Ca^{2+} concentrations. (B) Images showed representative photographs of 1.8 mM Ca^{2+}- 16.2 mM Ca^{2+} concentration groups at days 14 (4x magnification). (C) Relatively quantified calcium contents of alizarin red staining using optical density at 570nm were presented as mean + SD. Calcium content was significantly higher in the experimental groups compared to the control group (****P < 0.0001, *P < 0.05).

Effects of the Ca^{2+} concentrations on SHEDs colony forming unit ability

To evaluate the effect of Ca^{2+} concentrations on colony forming unit ability of SHEDs, SHEDs were maintained in culture medium with various Ca^{2+} concentration for 14 days. Then, cells were stained with methylene blue. The results indicated that the Ca^{2+} concentrations have no effect on colony forming ability of SHED (Figure 5).

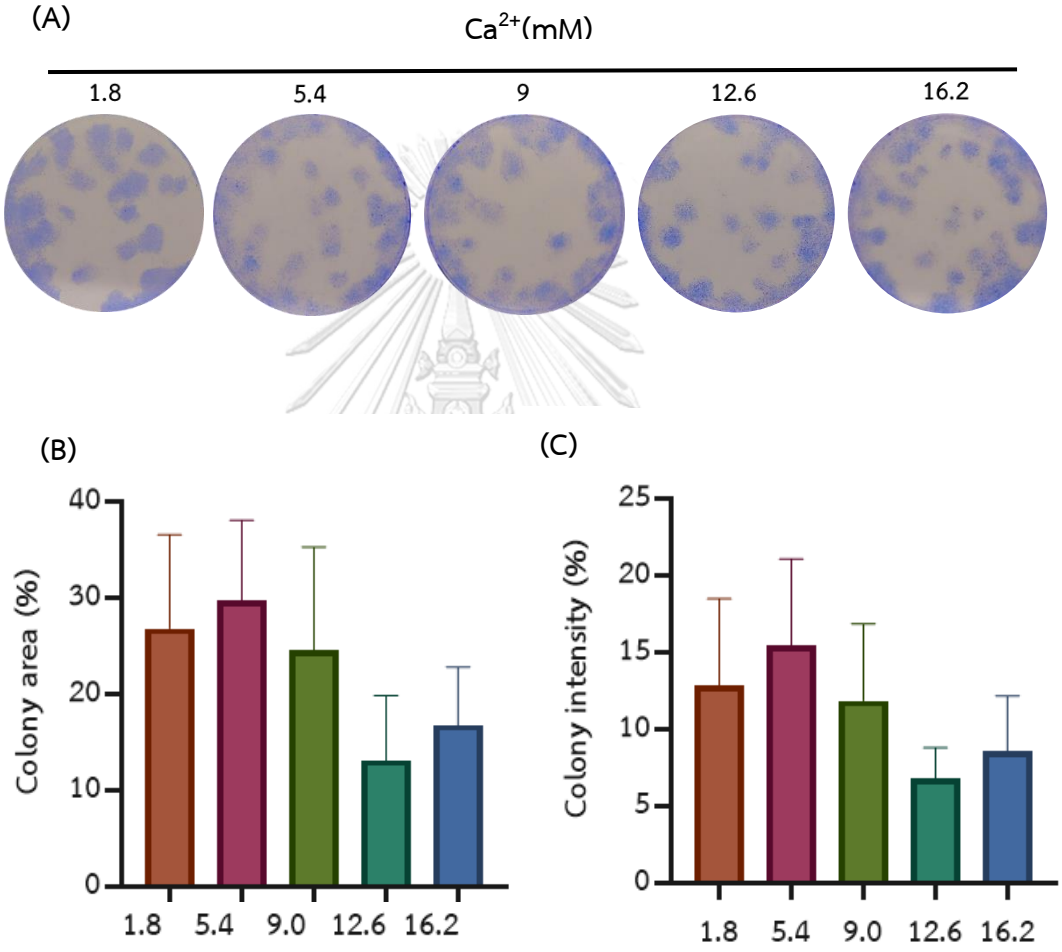


Figure 5 Ca^{2+} concentrations have no effect on the colony forming unit ability of SHEDs. (A) Images showed representative colonies were evaluated using methylene blue staining. (B,C) No statistically significant difference on colony area and colony intensity were found between the control group and experimental groups ($P > 0.05$) when Ca^{2+} concentrations were increased.

Effects of the Ca^{2+} concentrations on the osteogenic gene expression in SHEDs

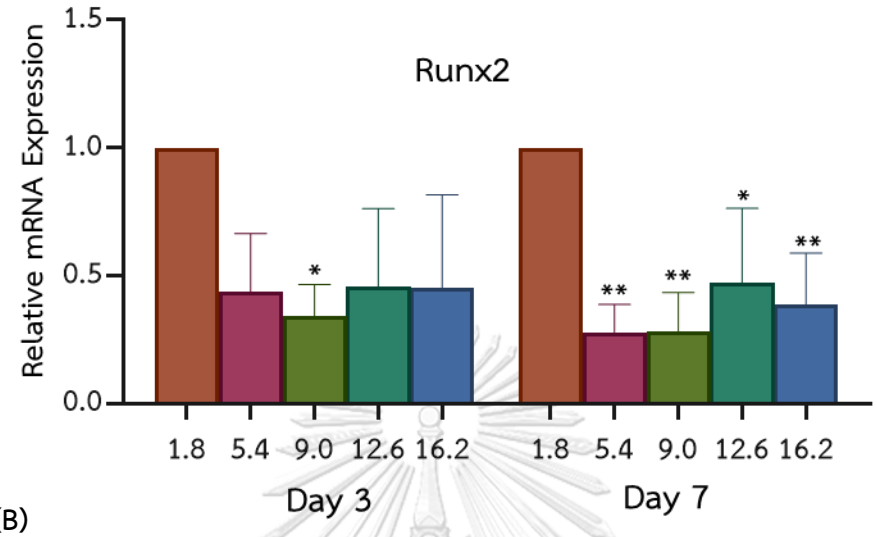
Increased Ca^{2+} concentrations in osteogenic induction medium affects osteogenic gene expression in SHEDs

Cells were cultured in OM containing various concentrations of Ca^{2+} for 3 and 7 days, and the relative mRNA expression of osteogenic gene markers was evaluated using real-time PCR.

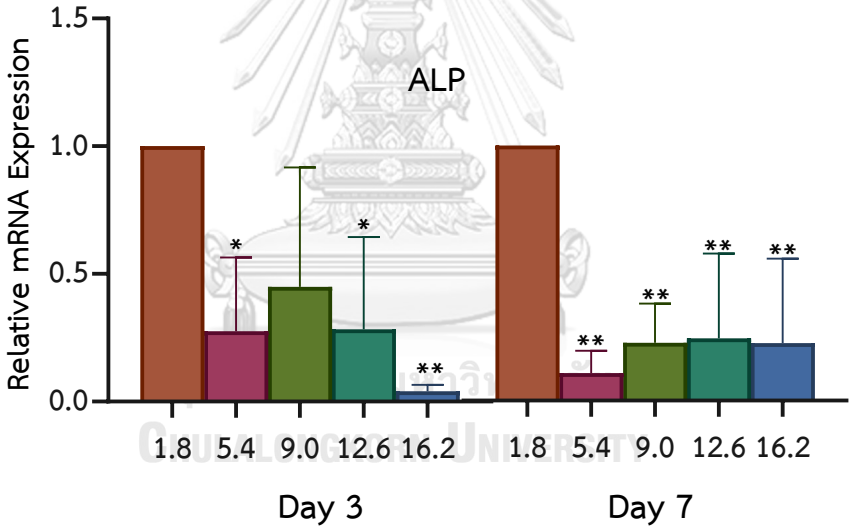
At day 3, downregulation of the early osteogenic markers, Runx2 and ALP was observed, whereas OSX remained unchanged compared to the control. Runx2 expressions were lower than control in every group, but a significant difference was seen only in 9.0 mM group ($p<0.05$). Meanwhile, ALP expressions were downregulated in 5.4 and 12.6 mM groups ($p<0.05$) and significantly lower in 16.2 mM group ($p<0.01$). On the other hand, OSX was slightly upregulated compared to control, but the differences were not significant. At day 7, the difference became more apparent in every group. Runx2 expressions were downregulated in 12.6 mM group ($p<0.05$), and further downregulated in 5.4, 9.0, and 16.2 mM group ($p<0.01$). Lower expressions were also seen in every group of ALP compared to control ($p<0.01$). For OSX, only 12.6 mM group showed significantly higher expression compared to control ($p<0.05$).

The expression of OCN, which represented the late osteogenic marker, was comparable to the control at day 3, with slightly higher expression in 16.2 mM group, but the difference was still not significant. At day 7, OCN expressions were significantly higher in both 9.0 and 12.6 mM group ($p<0.05$). In 5.4 and 16.2 mM group, the expressions were also increased, but the difference were not significant.

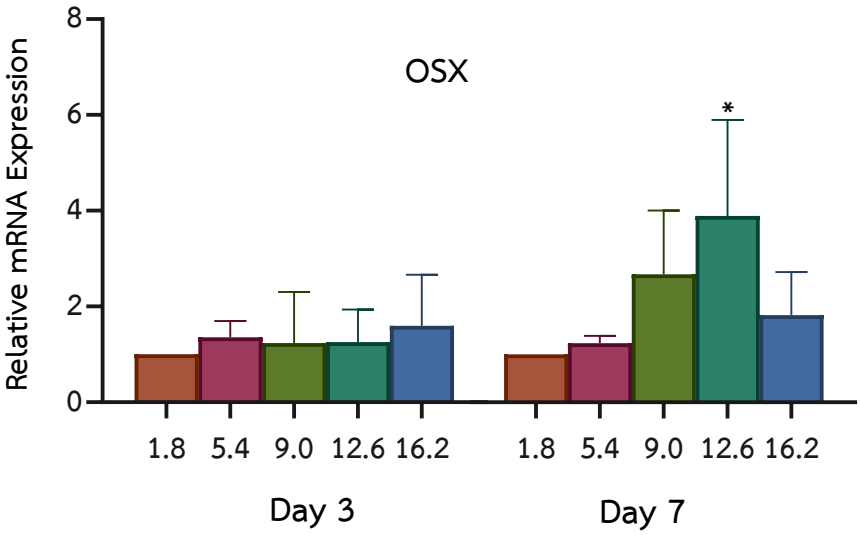
(A)


Runx2
Relative mRNA Expression
1.5
1.0
0.5
0.0
1.8 5.4 9.0 12.6 16.2
1.8 5.4 9.0 12.6 16.2
Day 3
Day 7

(B)


ALP
Relative mRNA Expression
1.5
1.0
0.5
0.0
1.8 5.4 9.0 12.6 16.2
1.8 5.4 9.0 12.6 16.2
Day 3
Day 7

(C)


OSX
Relative mRNA Expression
8
6
4
2
0
1.8 5.4 9.0 12.6 16.2
1.8 5.4 9.0 12.6 16.2
Day 3
Day 7

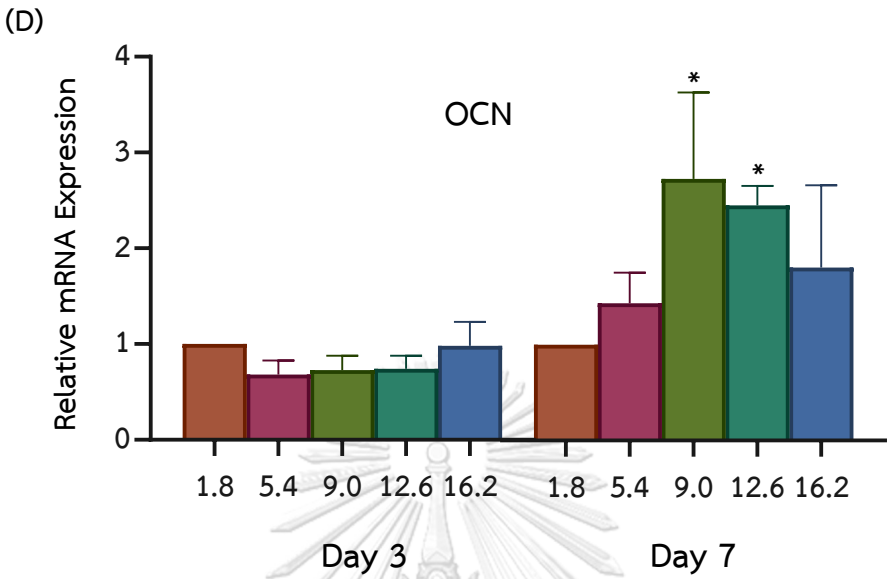
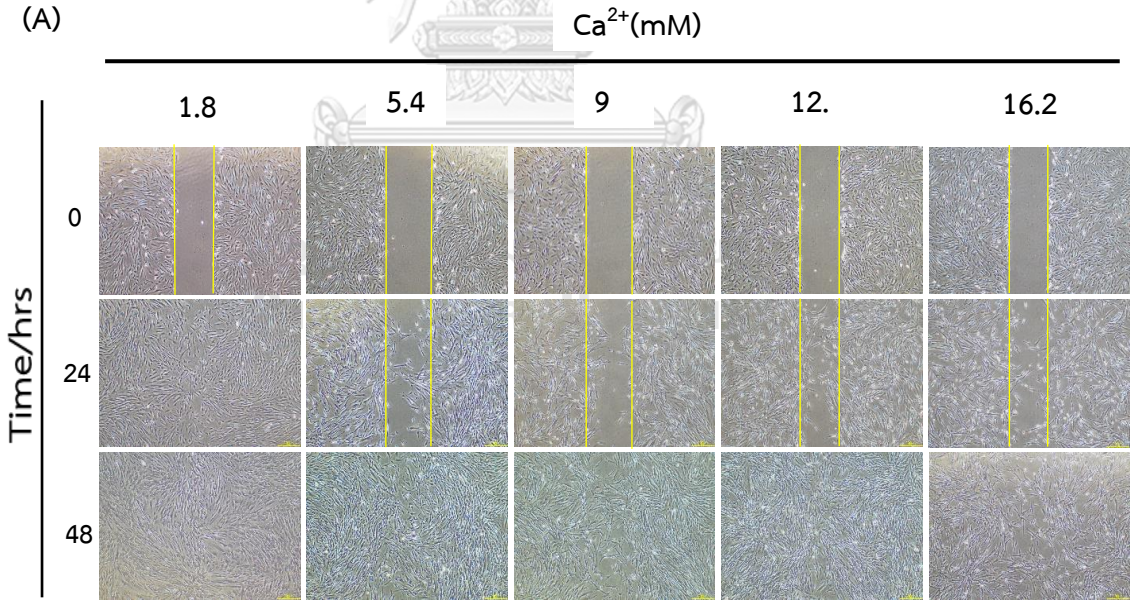


Figure 6 Effect of Ca^{2+} concentrations in OM on mRNA expression of osteogenic gene markers. (6C, 6D) OSX and OCN expression were upregulated on day 7, but only in 9.0 mM in OCN and 12.6 mM in both groups that show statistically significant difference (*P < 0.05). (6A, 6B) Runx2 and ALP expression were down regulated at almost every concentration on day 3 and 7 (**P < 0.01), except for the 9.0 mM of day 3 and 12.6 mM of day 7 of Runx2, and 5.4 mM and 12.6 mM of day 3 of ALP, that the expressions were less down regulated (*P < 0.05).

Effects of the Ca^{2+} concentrations on the migration of SHEDs

Increased Ca^{2+} concentrations inhibited migration of SHEDs

To evaluate the effect of Ca^{2+} concentrations on the migration of SHEDs, SHEDs were assessed using a wound healing assay. The cell monolayer was scratched with a 10 µl pipette tip and maintained in culture medium with various Ca^{2+} concentrations. Cell migration into the wounded area was observed at 0, 24, and 48 hours post-wound infliction. Wound closure area was calculated using Image J software. The wound healing at 24 hours was significantly impaired in the 5.4, 9.0, and 16.2 mM Ca^{2+} concentrations group compared with the control group ($P < 0.05$), but the 12.6 mM Ca^{2+} concentrations group showed non-significant impairment. However, at 48 hours, the wound healing of all elevated Ca^{2+} concentrations groups was impaired compared with control group, but the significant difference was not observed (Figure 7).



(B)

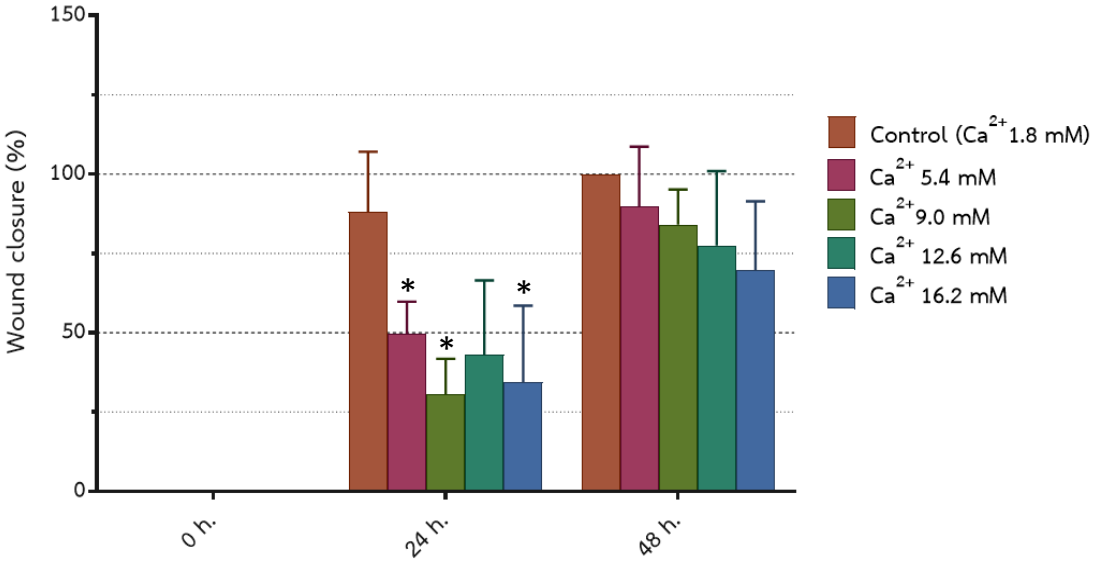


Figure 7 Increased Ca^{2+} concentrations significantly inhibited migration of SHEDs. (A) Scratched cell monolayer in culture medium containing different Ca^{2+} concentrations at 0, 24, 48 hours (original magnification x4). (B) Percentage of wound closure area of culture medium containing different calcium concentrations at 0, 24, and 48 hours as calculated by Image J software (*P < 0.05).

Chapter 5
Discussion and Conclusion

Uses of human dental stem cells in tissue engineering are becoming more popular because many dental stem cells show promising differentiation abilities and can be harvested without causing much harm. Human dental stem cells can be obtained from various oral tissue, including SHEDs that were isolated from primary teeth (15, 20). SHEDs are multipotent mesenchymal stem cells that express various gene markers which can be characterized by their surface antigens using flow cytometry. We found that SHEDs expressed CD44 and CD90 but not CD45 which were hemopoietic markers. Our result was consistent with many previous studies showing that SHEDs can express CD13, CD44, CD73, CD90, CD146, CD166, and STRO-1, but not CD34 and CD45. Moreover, SHEDs can also express CD117, CD71, and CD105, which shows that SHEDs are more undifferentiated and have more promising proliferation capabilities than DPSCs (12, 21, 22).

Ca^{2+} involves in regulating many important cell functions such as cell proliferation, differentiation, and migration through calcium signaling pathways. For most cells, including stem cells, primary sources of Ca^{2+} are from an influx of extracellular Ca^{2+} through the plasma membrane and Ca^{2+} release from the internal storage of the endoplasmic reticulum (9). Calcium dynamic has been exhibited to begin specific processes of cell behavior in many conditions (9, 24, 25). The stimulating effects of extracellular Ca^{2+} on proliferation were stated in numerous previous studies with various results in different cell types (4, 39, 40). Ca^{2+} induced proliferation of BMSCs at 84 hours when cultured in medium containing Ca^{2+} concentration between 0-5 mM in a dose-dependent manner (40). In osteoblast, a similar result was found in low doses as Ca^{2+} 5 mM significantly promoted proliferation, whereas higher doses as 20 mM decreased osteoblast cell proliferation lower than without Ca^{2+}. However, MSCs reacted differently with Ca^{2+} less than 1.8 mM., the proliferation of MSCs was inhibited instead, and no effect was found when the Ca^{2+} concentration was raised between 1.8 to 7.2 mM (4).

Most calcium-based dental pulp capping materials such as Dycal®, MTA, and Biodentine™ often claimed that Ca^{2+} is the major ingredient to induce or promote dental pulp healing or regeneration after in closed contact with the residing dental pulp stem cells (28). It was hypothesized that the high basic environment created by Ca^{2+} ion release from the materials either influence the dental pulp stem cells proliferation or induced osteo/odontoblastic differentiation (1, 41). In our study, when the effect of different Ca^{2+}concentration on cell proliferation and colony forming unit capabilities of SHEDs were examined, no significant differences were found, but colony area and colony intensity tended to decrease in higher Ca^{2+}concentration. These results were also similar to another study in DPSCs, showing no significant differences in proliferation at the same level of Ca^{2+} concentration (6).

However, when tested with various calcium-based pulp capping materials, the results were still controversial. Both ProRoot® MTA and Biodentine™ seemed to promote proliferation of DPSCs (statistically significant differences were not tested in this study) at every concentration of material ranging from 10% to 100% when compared to control at day 7 (42). Results in SHEDs are more conflicting, since one study found that both Biodentine™ and MTA Angelus promoted SHEDs proliferation at 48 hours, with significantly higher proliferation rate in Biodentine™ than MTA Angelus (43). On the other hand, another study also stated that only Biodentine™ showed influence on SHEDs proliferation at day 7 but not MTA (44). We speculated that the differences in the results among the studies may come from the other additional ingredients in the pulp capping materials rather than the Ca^{2+} itself since we observed no influence of Ca^{2+} concentrations on SHEDs cell proliferation.

Runx2 is the master regulator of osteogenic differentiation and is upregulated at the early stage of osteoblastic differentiation to induce the proliferation of osteoblast progenitors (45, 46). Interestingly, no significant change in Runx2 expression was observed at day3 in our study; therefore, this suggested the irrelevance of Ca^{2+} and SHEDs cell proliferation. Moreover, Ca^{2+} concentration is more likely to have the influence on the late stage of osteoblastic differentiation rather than the early process. Runx2 and OSX, the two major early osteogenic markers,

remained unchanged at day 3 compared to the control. Meanwhile, the significantly decreased expression of Runx2 and ALP along with the upregulation of OCN at day 7 indicated the role of Ca^{2+} during the osteoblast maturation process. Both ALP and Runx2 were early osteogenic marker genes, which were normally decreased during the differentiation from immature to mature osteoblast (46, 47). Ca^{2+} concentrations tended to have most effect on ALP since increasing Ca^{2+} significantly inhibited ALP expression in most conditions (except for the 9.0 mM group on day 3) compared to control. Although ALP expressions were strongly inhibited on day 7 ($P < 0.01$), the dose dependent effect was still inconclusive. Our results were also consistent with the study in DPSCs, suggesting that Ca^{2+} also significantly inhibit ALP gene expression at late stage of osteoblast differentiation (day 14 and 21) (6).

Significant upregulation of OCN at day 7 when Ca^{2+} level was increased also indicated the function of Ca^{2+} during extracellular matrices (ECM) production and mineralization since high expression of OCN is expected at the final stage of osteoblast differentiation (48). Since only OCN was examined in this study, other ECM production-related genes such as OPN and COL1A1 should be further investigated for confirmation.

Moreover, an increased in mineralization as examined by Alizarin red and von kossa staining also coincided with the high OCN expression. The Ca^{2+} level correspondence was noted in a specific range between 5.4 and 12.6 mM. The declined in mineralization when the dose exceeded 9.0 mM might be due to the negative feedback that inhibit osteogenic differentiation (49) but did not seem to be affected by cell death since the proliferation rate remained unchanged at high Ca^{2+} concentration. Likewise, the calcium-based pulp capping materials tended to affect the late stage of osteogenic differentiation. Biodentine™ seemed to inhibit ALP gene expression on day 1 to 7 compared to control (50). Meanwhile, OCN expression was promoted by both MTA Angelus and Biodentine™ on day 7 (51). Moreover, Proroot® MTA and Biodentine™ also had the ability to induce mineralization significantly on day 14 (52).

Cell migration is another crucial process in tissue regeneration. Recruitment of remoted progenitor cell to the injury site occurred at the early step of tissue healing

(53). However, there were very few studies about the effects of different Ca^{2+} levels on migration of cells, and none of them were studied on SHEDs. A study on MSCs found that $CaSO_4$ between 3 to 5 mM can induce migration; otherwise, there was no significant difference in higher Ca^{2+} concentration (54). Nevertheless, when $CaCl_2$ was tested with MSCs in another study, the concentration that promoted migration of MSCs the most was 10 mM followed by 8 mM, while 4 and 6 mM did not have any effect on migration (55). Ca^{2+} between 1.8 to 5.0 mM promoted migration in a dose-dependent manner of some cell lines but had no effect on others (56). These dose-dependent results were also similar to the study in dermal fibroblasts, using Ca^{2+} concentration between 0.1 to 3.5 mM. This study also found that the concentration that promoted migration the most was 2.5 mM followed by 3.5, 1.25, and 0.5 mM, respectively (57). It can be seen that the effect of Ca^{2+} on migration of cells was different on several types of cells and the effective dose was varied. Surprisingly, we found that Ca^{2+} higher than 1.8 mM seemed to inhibit migration of SHEDs and the effect was eminent at 24 hours. The less inhibition was seen in 48 hours without any statistical significance.

On the contrary, the effect of pulp capping materials on migration had also been studied on dental pulp stem cells. Many studies had found that Biodentine™ can promote migration more than other pulp capping materials (58, 59). According to the study of Omidi et al. (2019), Biodentine™ can significantly enhance migration compared to control (DPSCs with DMEM), but MTA Angelus and Theracal™ LC seem to inhibit migration. The authors suggested that the migration enhancing capability of Biodentine™ may come from its ability to induce DPSCs to secrete MCP-1 cytokines, a cytokine that played a role in migration of macrophage cells which is associated with initial moderate inflammatory process, a stage followed by and pulp repair respectively (58). Moreover, another study found that at 0.2 mg/ml of Biodentine™, migration rate was higher than control (no Biodentine™) and 2 mg/ml of Biodentine™ (60). However, few studies found that Proroot® MTA and Biodentine™

did not affect migration at all, but most of them still agreed that Theracal™ LC and Dycal® seemed to inhibit migration, with a greater inhibiting effect in Dycal® (42, 61, 62). The differences between each study may come from the difference in the preparation of pulp capping materials, and the effective Ca^{2+} concentration may be restricted and specific to each cell type. Thus, further studies about the optimal concentration of either pulp capping materials or Ca^{2+} for migration on SHEDs and other types of cells were also needed before making any clear conclusions.

The results indicated that the optimum dose range of Ca^{2+} concentration for promoting late stage osteoblast differentiation were between 9.0 and 12.6 mM. However, the concentrations of Ca^{2+} released from various pulp capping materials at day 1 start from 0.45 to 0.978 mM and slowly rise to 0.315 to 1.377 mM at day 7 (63). It can be seen that the amount of Ca^{2+} released from pulp capping materials was still far from achieving late stage osteoblast differentiation promotion. Thus, the invention of high Ca^{2+}-release pulp capping material may be another option for newer pulp capping materials.

According to our results, it seemed that Ca^{2+} did not enhance the proliferation or migration of SHEDs. Thus, using calcium-based pulp capping materials should be limited to the tooth with a small pulp exposure site because these materials cannot induce tissue regeneration. So, further studies on biomolecules focusing on tissue regeneration while maintaining the vitality of the compromised pulp may help improve the success rate of the current vital pulp therapy treatment.

In conclusion, our study showed that Ca^{2+} concentration between 1.8 to 16.2 mM did not alter proliferation and colony forming unit ability of SHEDs. Instead, when Ca^{2+} level was raised to a certain level, SHEDs migration was inhibited. Concurrently, high Ca^{2+} concentration appeared to induce the late stage of osteogenic differentiation, especially ECM production and mineralization.

To our knowledge, this study was the very few first that reported direct effect of Ca^{2+} on SHEDs. So further studies using a broader range of Ca^{2+} concentration on SHEDs are needed before a solid conclusion can be drawn. Furthermore, investigation on other predominant proteins related to mineralization and ECM production, such as fibronectin and OPN, with immunocytochemistry and enzyme linked immunosorbent assay (ELISA), whereas other late osteogenic genes with qRT-PCR (64, 65), may also help in developing better pulp capping materials in the future.

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APPENDIX

APPENDIX A

เอกสารข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย
(Patient/Participant Information Sheet)
สำหรับอาสาสมัครเด็ก

1. โครงการเรื่อง การเปรียบเทียบการตอบสนองของเซลล์ต้นกำเนิดจากโพรงประสาทฟันน้ำนมต่อความเข้มข้นที่แตกต่างกันของแคลเซียมไอออน
2. ชื่อผู้วิจัยหลัก ทพญ. ฐานิกา ผลินยศ
   ชื่อผู้วิจัยร่วมหรืออาจารย์ที่ปรึกษา รศ. ทพญ. ดร. วลีรัตน์ ศุกรวรรณ
   ศ. ทพ. ดร. ธนภูมิ โอสถานนท์
   สถาบันที่สังกัด คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
   แหล่งทุนวิจัย ทุนบัณฑิตศึกษา
3. วัตถุประสงค์ของโครงการ
   - เพื่อศึกษาผลของระดับความเข้มข้นที่แตกต่างกันของแคลเซียมไอออนต่อการเพิ่มจำนวน การพัฒนาไปเป็น
   เซลล์สร้างเนื้อเยื่อแข็ง และการเคลื่อนที่ของเซลล์ต้นกำเนิดจากโพรงประสาทฟันน้ำนม
4. สถานที่ดำเนินการวิจัย หน่วยปฏิบัติการวิจัยเนื้อเยื่ออินทรีย์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
5. วิธีการที่ใช้ในการวิจัย
   งานวิจัยนี้พี่หมอซึ่งเป็นหมอฟันสำหรับเด็กจะทำในห้องทดลอง โดยพี่หมอจะนำฟันน้ำนมที่ไม่ผุที่ถอนจากน้องๆ ไปทำการแยกเอาเซลล์จากโพรงประสาทฟันน้ำนมออกมา แล้วนำมาทดสอบโดยใส่แคลเซียมไอออนที่ความเข้มข้นที่แตกต่างกันออกไป 5 ความเข้มข้น แล้วดูการเพิ่มจำนวน การสร้างกระดูก และการเคลื่อนที่ของเซลล์ต่อความเข้มข้นที่แตกต่างกัน
6. เหตุผลที่เชิญเข้าร่วมเป็นอาสาสมัครในโครงการ
   เนื่องจากน้องๆมีสุขภาพที่แข็งแรงดี และมีฟันที่ไม่ผุ ซึ่งมีลักษณะตามเกณฑ์ที่พี่หมอกำหนดไว้
7. ความรับผิดชอบของอาสาสมัคร และระยะเวลาที่อาสาสมัครจะอยู่ในโครงการ
   ขอให้น้องๆมาตามนัดในวันที่ถอนฟันน้ำนม หากมีปัญหาใด ๆ ให้ติดต่อพี่หมอตามเบอร์โทรศัพท์ 081-921-4121

8. ประโยชน์ของการวิจัยที่อาสาสมัครและ/หรือผู้อื่นอาจได้รับ

ถึงแม้ว่าน้องๆอาจจะไม่ได้รับประโยชน์โดยตรงจากงานวิจัยนี้ แต่ผลการวิจัยที่พี่หมอได้นั้นจะทำให้มีข้อมูลเกี่ยวกับผลของความเข้มข้นของแคลเซียมไอออนระดับต่างๆต่อเซลล์ที่มาจากโพรงประสาทฟันน้ำนม และพี่หมอจะสามารถนำข้อมูลเหล่านี้ไปใช้ประโยชน์ในการทดลองเกี่ยวกับการพัฒนาให้เซลล์ที่มาจากโพรงประสาทฟันน้ำนมสร้างกระดูกได้ในอนาคต

9. ความเสี่ยงหรือความไม่สะดวกที่อาจจะเกิดขึ้นแก่อาสาสมัคร

งายวิจัยของพี่หมอไม่มีความเสี่ยงหรือความไม่สะดวกใดๆที่จะเกิดขึ้นกับน้องๆ

10. ค่าใช้จ่ายที่อาสาสมัครจะต้องจ่าย หรืออาจจะต้องจ่าย

น้องๆไม่ต้องเสียค่าใช้จ่ายในการเข้าร่วมงานวิจัยของพี่หมอ

11. การชดเชยใด ๆ และการรักษาที่จะจัดให้แก่อาสาสมัครในกรณีที่ได้รับอันตรายซึ่งเกี่ยวข้องกับการวิจัย

หากน้องๆได้รับอันตรายหรือเกิดความผิดปกติจากการเข้าร่วมทำวิจัยกับพี่หมอ น้องๆจะได้รับการรักษาโดยพี่หมอจะเป็นผู้รับผิดชอบค่าใช้จ่ายของการรักษาทั้งหมด

12. การจ่ายค่าเดินทาง ค่าเสียเวลา แก่อาสาสมัครที่เข้าร่วมในการวิจัย

น้องๆไม่ต้องจ่ายค่าเดินทางหรือค่าเสียเวลาในการเข้าร่วมงานวิจัย

13. เหตุการณ์ที่อาจจะเกิดขึ้น หรือเหตุผลซึ่งผู้วิจัยจะต้องยกเลิกการเข้าร่วมในโครงการวิจัยของอาสาสมัคร

ฟันน้ำนมของน้องๆที่ถอนออกมาไม่มีเนื้อเยื่อโพรงประสาทฟันหลงเหลืออยู่

14. มีการเก็บชิ้นตัวอย่างที่ได้มาจากอาสาสมัครเอาไว้ใช้ในโครงการวิจัยในอนาคตหรือไม่ เก็บจำนวนเท่าไหร่ อย่างไร และที่ไหน

เมื่อพี่หมอทำการแยกเซลล์จากโพรงประสาทฟันน้ำนมออกจากฟันน้ำนมที่ถอนแล้ว ฟันจะถูกทำลายทันที ส่วนเซลล์จะเก็บไว้ในตู้เย็นอุณหภูมิ -80 องศาเซลเซียส ที่หน่วยปฏิบัติการวิจัยเนื้อเยื่ออนินทรีย์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เพื่อใช้ในการทำโครงการวิจัยต่อไป

15. การกำกับดูแลและควบคุมการดำเนินโครงการ

ผู้กำกับดูแลการวิจัย ผู้ตรวจสอบ คณะกรรมการพิจารณาจริยธรรม และคณะกรรมการที่เกี่ยวข้อง สามารถเข้าไปตรวจสอบการดำเนินโครงการ รวมทั้ง ตรวจสอบบันทึกข้อมูลของอาสาสมัคร เพื่อเป็นการยืนยันถึงขั้นตอนในการวิจัยทางคลินิกและข้อมูลอื่นๆ โดยไม่ล่วงละเมิดเอกสิทธิ์ในการปิดบังข้อมูลของอาสาสมัคร ตามกรอบที่กฎหมายและกฎระเบียบได้อนุญาตไว้ นอกจากนี้ โดยการลงนามให้ความยินยอม อาสาสมัครหรือ ผู้แทนตามกฎหมายจะมีสิทธิตรวจสอบและมีสิทธิที่จะได้รับข้อมูลด้วยเช่นกัน

16. จริยธรรมการวิจัย

การดำเนินการโครงการวิจัยนี้ ผู้วิจัยคำนึงถึงหลักจริยธรรมการวิจัย โดย

1. หลักความเคารพในบุคคล (Respect for person) โดยการให้ข้อมูลจนอาสาสมัครเข้าใจเป็นอย่างดีและตัดสินใจอย่างอิสระในการให้ความยินยอมเข้าร่วมในการวิจัย รวมทั้งการเก็บรักษาความลับของอาสาสมัคร
2. หลักการให้ประโยชน์ไม่ก่อให้เกิดอันตราย (Beneficence/Non-Maleficence) ซึ่งได้ระบุในข้อ 8 และ 9 ว่าจะมีประโยชน์หรือความเสี่ยงกับอาสาสมัครหรือไม่
3. หลักความยุติธรรม (Justice) คือมีเกณฑ์คัดเข้าและคัดออกชัดเจน มีการกระจายความเสี่ยงและผลประโยชน์อย่างเท่าเทียมกัน โดยวิธีสุ่มเข้ากลุ่มศึกษา

17. ข้อมูลที่อาจนำไปสู่การเปิดเผยตัวของอาสาสมัครจะได้รับการปกปิด ยกเว้นว่าได้รับคำยินยอมไว้โดยกฎระเบียบและกฎหมายที่เกี่ยวข้องเท่านั้น จึงจะเปิดเผยข้อมูลแก่สาธารณชนได้ ในกรณีที่ผลการวิจัยได้รับการตีพิมพ์ ชื่อและที่อยู่ของอาสาสมัครจะต้องได้รับการปกปิดอยู่เสมอ และอาสาสมัครหรือผู้แทนตามกฎหมายจะได้รับแจ้งโดยทันท่วงทีในกรณีที่มีข้อมูลใหม่ซึ่งอาจใช้ประกอบการตัดสินใจของอาสาสมัครว่าจะยังคงเข้าร่วมในโครงการวิจัยต่อไปได้หรือไม่

18. หากท่านมีข้อสงสัยต้องการสอบถามเกี่ยวกับสิทธิของท่านหรือผู้วิจัยไม่ปฏิบัติตามที่เขียนไว้ในเอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในการวิจัย ท่านสามารถติดต่อหรือร้องเรียนได้ที่ฝ่ายวิจัย คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ตึกสมเด็จย่า 93 ชั้น 10 หรือที่หมายเลขโทรศัพท์ 02-218-8866 ในเวลาทำการ

19. หากท่านต้องการยกเลิกการเข้าร่วมเป็นอาสาสมัครในโครงการนี้ ให้ท่านกรอกและส่งเอกสารขอยกเลิกมาที่ ทพญ.ฐานิกา ผลินยศ 99/144 ซ.ไทรม้า ถ.รัตนาธิเบศร์ ต.บางรักน้อย อ.เมือง นนทบุรี 11000

20. อาสาสมัครสามารถติดต่อผู้วิจัยได้**ตลอด 24 ชั่วโมง** ที่: ทพญ.ฐานิกา ผลินยศ โทร. 081-921-4121 อาจารย์ที่ปรึกษางานวิจัย รศ.ทพญ.ดร. วลีรัตน์ ศุกรวรรณ โทร. 085-123-1762

ลงนาม.....

(ทพญ.ฐานิกา ผลินยศ)

ผู้วิจัยหลัก

วันที่...../...../.....

หมายเหตุ - ให้พิมพ์ข้อความโดยละเอียดลงในช่องว่าง โดยใช้ตัวอักษร TH SarabunPSK ขนาด 16

- หลังจากกรอกข้อความครบถ้วน พิมพ์เอกสารทั้งหมด แล้วให้ผู้วิจัยหลักลงนาม
- ทำสำเนาเอกสารข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย (Patient/Participant Information Sheet) มอบให้อาสาสมัครแต่ละคนๆ ละ 1 ชุด

APPENDIX B

เอกสารยินยอมเข้าร่วมการวิจัย

(Consent Form)

การวิจัยเรื่อง การเปรียบเทียบการตอบสนองของเซลล์ต้นกำเนิดจากโพรงประสาทฟันน้ำนมต่อความเข้มข้นที่แตกต่างกันของแคลเซียมไอออน

ข้าพเจ้า (นาย/ นาง/ นางสาว).....

อยู่บ้านเลขที่.....ถนน.....ตำบล/แขวง.....

อำเภอ/เขต.....จังหวัด.....รหัสไปรษณีย์.....

ผู้ปกครองของ (เด็กชาย/ เด็กหญิง).....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้

1. ข้าพเจ้าได้รับทราบรายละเอียดข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย รวมทั้งได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการทำวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจากการทำวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว
2. ผู้วิจัยได้ตอบคำถามต่าง ๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ
3. ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าและบุตรหลานเป็นความลับและจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุป ผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าและบุตรหลานต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็นด้วยเหตุผลทางวิชาการเท่านั้น
4. ข้าพเจ้ามีสิทธิที่จะตรวจสอบหรือแก้ไขข้อมูลส่วนตัวของตนและบุตรหลาน สามารถยกเลิกการให้สิทธิในการให้ข้อมูลส่วนตัวของตนและบุตรหลานได้ และสามารถถอนตัวออกจากการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้ตามความสมัครใจ โดยไม่ต้องแจ้งให้ผู้วิจัยทราบ

ข้าพเจ้าจึงสมัครใจให้บุตรหลานเข้าร่วมโครงการวิจัยนี้ตามที่ระบุในเอกสารข้อมูลคำอธิบายสำหรับอาสาสมัครและได้ลง นามในใบยินยอมนี้ด้วยความเต็มใจ และได้รับสำเนาเอกสารใบยินยอมที่ข้าพเจ้าลงนามและลงวันที่ และเอกสารยกเลิกการเข้าร่วมวิจัย อย่างละ 1 ฉบับ เป็นที่เรียบร้อยแล้ว

ลงนาม..... (อาสาสมัคร) (.....) วันที่...../...../.....	ลงนาม..... (ผู้ปกครอง) (.....) วันที่...../...../.....
ลงนาม.....(ผู้วิจัยหลัก) (ทพญ.ฐานิกา ผลินยศ) วันที่...../...../.....	ลงนาม.....(พยาน) (.....) วันที่...../...../.....

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนาม หรือประทับลายนิ้วหัวแม่มือขวาของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม..... (อาสาสมัคร) (.....) วันที่...../...../.....	ลงนาม..... (ผู้ปกครอง) (.....) วันที่...../...../.....
ลงนาม.....(ผู้วิจัยหลัก) (ทพญ.ฐานิกา ผลินยศ) วันที่...../...../.....	ลงนาม.....(พยาน) (.....) วันที่...../...../.....

APPENDIX C

เอกสารยกเลิกการเข้าร่วมวิจัย (Withdrawal Form)

การวิจัยเรื่อง การเปรียบเทียบการตอบสนองของเซลล์ต้นกำเนิดจากโพรงประสาทฟันน้ำนมต่อความเข้มข้นที่แตกต่างกันของแคลเซียมไอออน

ข้าพเจ้า (นาย/ นาง/ นางสาว/ เด็กชาย/ เด็กหญิง).....

บ้านเลขที่.....ถนน.....ตำบล/แขวง.....

อำเภอ/เขต.....จังหวัด.....รหัสไปรษณีย์.....

ผู้ปกครองของ (เด็กชาย/เด็กหญิง).....

ลงนาม.....ผู้ยกเลิก

(.....)

วันที่.....เดือน.....พ.ศ. .....

ลงนาม.....พยาน

(.....)

วันที่.....เดือน.....พ.ศ. .....

ลงนาม.....ผู้วิจัยหลัก

(นางสาว ฐานิกา ผลินยศ)

วันที่.....เดือน.....พ.ศ. .....

ที่อยู่สำหรับส่งเอกสาร ชื่อ นางสาว ฐานิกา ผลินยศ

บ้านเลขที่ 99/144 ซอย ไทรม้า ถนนรัตนาธิเบศร์ ตำบล/แขวง บางรักน้อย

อำเภอ/เขต เมือง จังหวัด นนทบุรี รหัสไปรษณีย์ 11000

หมายเหตุ - สำเนาเอกสารยกเลิกการเข้าร่วมวิจัย แล้วมอบให้อาสาสมัครแต่ละคนๆ ละ 1 ชุด

VITA

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