Title
Ex-Vivo Characterization of Minced Pulp Tissue Explants

Author
Khouqeer, Noor

Publication Date
2019

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Ex-Vivo Characterization of Minced Pulp Tissue Explants

A thesis submitted in partial satisfaction of the

requirement for the degree Master of Science

in Oral Biology

by

Noor Fareed Khouqeer

2019

ABSTRACT OF THE THESIS

Ex-Vivo Characterization of Minced Pulp Tissue Explants

By

Noor Fareed Khouqeer

Master of Science in Oral Biology

University of California, Los Angeles, 2019

Professor Mo K. Kang, Chair

Regenerative endodontics techniques have recently become one of the most investigated areas in the field of endodontics. Utilizing tissue engineering concepts enable us to restore teeth vitality and health after the pulp tissue damage. Dental pulp stem cells (DPSC) transplantation faced several step-backs due to cell culture requirement and regulatory hurdles for *in vitro* expansion of cells. Our group demonstrated previously dental pulp tissue explants generate mesenchymal stem cells, named MP-MSCs, that exhibited stem cell properties such as differentiation capacities and mineralization potential *in vitro* which was comparable to DPSCs. We established the fundamental rational for the usage of pulp tissue grafting in the field of tissue engineering and regenerative endodontics. In this study, we aimed to characterize the dental pulp tissue explants and MP-MSCs *in vitro* through serial tissue passages, comparing their

odontogenic differentiation potential. This study demonstrated that cells migrating out from dental pulp tissue explants at later passages retained their growth potential as well as odontogenic differentiation capacity. Minced-pulp MSCs also expressed stem cells marker, CD146, at late tissue passage (T_{10}). This study indicates that dental pulp tissue explants can potentially yield unlimited source of mesenchymal stem cells which builds up on our novel approach of utilizing direct pulp tissue grafting as a regenerative endodontic therapy.

The thesis of Noor Fareed Khouqeer is approved.

Yangpei Cao

Alireza Moshaverinia

Shane White

Mo K. Kang, Committee Chair

University of California, Los Angeles

2019

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

The work presented in this thesis has been performed at the Shapiro Family Laboratory of Viral Oncology and Aging at the UCLA, School of Dentistry.

I would like to thank my mentor Dr. Mo Kang for his continuous guidance and scientific support. I also want to thank Dr. Shane White, Dr. Alireza Moshaverinia, and Dr. Yangpei Cao for their valuable insights and guidance as the thesis committee members.

Many thanks to my lab members, especially Dr. Wei Chen, Dr. Abdullah Alshaikh and Dr. Jaeyong Lee, and Dr. Drake Williams, for the help and guidance throughout my postgraduate work period. As well as my co-resident Dr. Chaehwan Lee. I thank them for being open-minded to my questions and sharing valuable knowledge and experience that allowed me to complete my study at UCLA.

Lastly, I would like to express my utmost gratitude to my husband, parents, and siblings. Thank you for your support and encouragement throughout my postgraduate training at UCLA for the last three years.

INTRODUCTION

Conventional root canal treatments performed on mature teeth aim to eliminate the infected dental pulp tissue and bacteria colonizing the dental pulp tissue space. Teeth that received non-surgical root canal treatment was shown to have long-term survival rates that are comparable single-tooth implants (Torabinejad *et al.* 2007). The presence of preoperative apical periodontitis was shown negatively impact treatment outcomes. Mature teeth with preoperative normal periapical tissues yielded a success of 93%; whereas, teeth with preoperative apical periodontitis yielded success 82% (De Chevigny et al. 2008).

In contrast to mature teeth, management of immature teeth diagnosed with pulp necrosis aim to eliminate bacteria colonizing the dental pulp space and promote further root growth. Apexification therapies have shown to be a predictable treatment option for immature teeth. Traditionally, calcium hydroxide was used to carry out apexification therapy with success rates ranging from 87% to 100% (Lin et al. 2016). More recently, Mineral Trioxide Aggregate (MTA) became the material of choice in apexification therapy due to the disadvantages of using calcium hydroxide. These include: increased treatment duration with risk of re-infection, weakening of root dentin, and increased risk of cervical root fracture. Apexification therapies had a clinical success of 93% to 100% when MTA was used (Lin et al. 2016).

Regenerative endodontic therapies were introduced as an alternative to apexification therapies. They are intended to restore the pulp-dentin complex, stimulate further root growth and development and overcome the drawbacks of apexification therapies. The current American Association of Endoodntics (AAE) guidelines for Regenerative Endodontic Procedures (REPs) recommend disinfection with 20 mL of 1.5% sodium hypochlorite followed by 20 mL of 17% EDTA per canal in the first visit and placement of an intra-canal antibacterial medicament. In the

second visit, it is recommended to irrigate with 20 mL of 17% EDTA per canal, and stimulate bleeding in the canal, before placing MTA in the cervical third of the root. Bleeding from the apical area was found to increase Mesenchymal Stem Cells (MSC) markers in the canal above that in circulating blood (Hargreaves et al. 2012). Therapeutic success is measured by elimination of patient's signs and symptoms, radiographic evidence of healing, and continued root growth, and a positive response to tooth vitality tests. A retrospective study revealed that 75% of teeth that received REPs showed complete healing after 3 years (Bukhari 2016). Although the term "Regenerative Endodontics" has been used widely, studies have shown that only cementum-like and bone-like tissues were deposited following REPs in dog teeth (Wang 2010). Hence, the term "Pulp Revascularization" is recommended as a more accurate term for such therapies. Although a recent comprehensive characterization of on tooth that received REPs showed true pulp regeneration 3.5 years post-REP (Austah et al. 2019), tissue engineering methods are still being investigated to ensure 100% de novo pulp regeneration.

Different types of post-natal stem cells were isolated from human teeth, such as DPSCs and stem cells from human-exfoliated deciduous teeth (SHEDs) (Gronthos et al. 2000). It was first demonstrated by Iohara *et al*., that transplantation of fractionated side-population (SP) cells enriched with CD31-/CD146- immunophenotype led to successful pulp-dentin complex regeneration (Iohara et al. 2011). Thus, DPSCs could be a viable source for pulp regeneration in endodontic treatment could be possible. It was previously shown that pulpal MSCs undergo a finite number of cell division when cultured *in vitro*. It eventually reaches a terminal state called "replicative senescence" where it loses essential odontogenic and osteogenic differentiation capacities due to $P16^{INK4A}$ induction and loss of Bmi-1 (Kang et al. 2004 and Mehrazarin et al. 2011). Thus, the preparation of pulpal MSCs for cell transplantation may result in loss of integral

properties for pulpal regeneration. Also, such cell-based approach may not be feasible in a chairside manner or dental office due to the requirements of good manufacturing practice (GMP).

An isolation method of GMP- grade DPSC by granulocyte colony-stimulating factor (G-CSF)-induced mobilization was developed (Murakami et al.2016). In a clinical pilot study, the same group later demonstrated complete pulp regeneration following autologous mobilized dental pulp stem cells (MDPSCs) with G-CSF in teeth diagnosed with irreversible pulpitis (Nakashima et al. 2017). Another clinical trial revealed that autologous implantation of DPSCs from deciduous teeth allowed regeneration of dental pulp in teeth with pulp necrosis due to previous trauma (Xuan et al. 2018). The safety and efficacy of pulp regeneration following allogenic transplantation of MDPCSs was demonstrated in canines (Iohara et al 2018). Despite the success of the MDPSCs, it necessitates GMP *in vitro* expansion of the cells.

In order to overcome the mentioned drawbacks of cell-based procedures, we previously proposed pulp tissue grafting for REPs. Direct pulp transplantation allows bypassing the *in vitro* cell expansion of DPSCs, which make it feasible in a day-to-day endodontic practice. We previously demonstrated that human minced pulp tissue yielded MSCs (Liang et al. 2018). Minced dental pulp tissue retained the odontogenic and osteogenic differentiation capacities of pulpal MSCs (Liang et al. 2018). In the pulp tissue grafting model, pulp tissue and MP-MSCs will be a part of the recipient tooth and might undergo time-dependent changes.

Here, we hypothesize that minced human dental pulp tissue yield finite number of MP-MSCs that retain osteogenic and odontogenic differentiation capacities during serial passages of the tissues. This hypothesis addressed minced human dental pulp tissue harvested from non-

carious human teeth that were extracted. Mesenchymal stem cells migrated from different dental pulp tissue explants at serial tissue passages were compared by various experiments in order to characterize the phenotype. To address this, we propose the following specific aims:

1. To characterize the morphological and stem characteristics within minced pulp tissue through serial tissue passages *ex-vivo*.

2. To investigate the change in the odontogenic differentiation potential of MP-MSCs through serial tissue passages *ex-vivo*.

MATERIALS AND METHODS

Sample collection and primary culture

Freshly extracted healthy non-carious third molars or premolars extracted for orthodontic purposes were collected from Oral Surgery Clinic at School of Dentistry, UCLA. Patients' ages ranged between 16-23 years. Teeth were stored sterile Falcon tubes containing primary culture medium, α-MEM medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Invitrogen), 15 μg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA), and 20 mmol/L L-glutamine (Invitrogen). The sterile tubes were kept on ice until being transferred to the biosafety cabinet for dental pulp isolation, which was completed within 12 hours of extraction.

First, gingival tissue attached to the tooth were removed using a scalpel. Second, the tooth was washed with PBS supplemented with 15 μg/mL gentamicin sulfate for three times. After that, the tooth was broken at the cemento-enamel junction using an orthodontic wire-cutter. The pulp was carefully retrieved and placed onto a 60mm petri dish with primary culture medium for at least 30 minutes using sterile tweezers. Next, using sterile micro-scissors, the pulp was minced into fine pieces ($<1\ mm^3$) and transferred to a new 60mm petri dish with fresh primary culture medium. Dishes were incubated in 80% humidity, 5% carbon dioxide atmosphere at 37 degrees Celsius. Once the tissue was attached (approximately 3-5 days), the primary culture medium was changed every other day.

When 80% confluency was reached, the finely minced dental pulp tissues were transferred to new dish with fresh primary culture medium. MP-MSCs were then maintained in basal medium, α-MEM (Intvitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) and 5 μg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA). Herein, we name MSCs migrated from dental

DPTEs at the first tissue passage **(MP-MSCs T_1),** such cells are obtained from tissues at post-explantation day 7-12. Additionally, **(MP-MSC T_5)** are MSCs that migrated from tissues at the fifth tissue passage, often 70-80 days post-explantation. Finally, MSCs obtained from DPTE explants at the tenth passage **(MP-MSC T_{10}),** usually harvested at post-explantation day 140-150.

MTT Cell Proliferation assay

Different passages of MP-MSCs were seeded in 96-well plates at $1x10^5$, $2x10^5$, $3x10^5$ and incubated for at least 24 hours to allow the cells to reach 80% confluency. The MTT assay was used to investigate cells proliferation assay, (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). The MTT Reagent (ATCC) was then added following manufacturer instructions. After incubation for 2-4 hours, the Detergent Reagent (ATCC) was added and mixed gently. Following incubation for 4-24 hours, the absorbance was measured with Synergy ^{TM} Microplate Reader at 570 nm. The results were later analyzed using Microsoft Excel. The experiment was performed in triplicates and repeated 3 times.

Cell Counting for Proliferation Assay

Dental pulp tissue explants MSCs from different tissue passages were seeded in 24-well plate with 3,000 cell/well. Each day, basal medium was aspirated from three wells and Trypsin EDTA (0.25%) (Thermofisher) was added and incubated for 5-7 minutes at 37 degrees Celsius. Basal medium was then added and cell suspensions were then transferred to Eppendrof tubes (Fisher Scientific). After mixing the cells, 10 µl of cell suspension was added bellow the cover-slip of the hemocytometer. The cells were counted using a microscope at 10x magnification. A total of eighteen wells were seeded for each cell passage. Triplicates of each passage were counted at each day.

Odontogenic differentiation assay

MP-MSCs from different tissue passages were seeded in 24-well plates at $3x10^5$ per well. Before they reached 80% confluency, odontogenic differentiation was induced using odontogenic induction medium, which is basal medium conditioned with 100 μmol/L L-ascorbic acid 2-phosphate (Sigma, St Louis, MO), 9mmol/L KH2PO4, 10 mmol/L β-glycerolphosphate, and 9.8 nmol/L dexamethasone (Sigma, St Louis, MO). Control group cells were maintained in basal medium. The medium was changed every two days.

At day 7, cells were stained with Alkaline Phostphatase (ALP) Staining Kit (Sigma, St. Louis, MO). Cells were first washed with Dulbecco's phosphate buffered saline (DPBS) (ThermoFisher), they were then fixed with 1:2.5 acetone/citrate for 30 seconds. Following fixation, cells were washed with distilled water for three times. Mixed 1 mL of Fast Violet B Salt with 1 mL of sodium nitrite solution for 2 minutes. Then, 1 mL of Naphtol AS-MX phosphatase alkaline solution was added with 48 mL of distilled water. Then, 2 mL of the mixture was added to each well. The cells were incubated for 10 minutes in the dark. Each group was seeded in triplicates and the experiment was repeated 5 times. The results are then observed under the microscope and photographs or the wells are taken.

Equivalent number of cells were seeded in a 24-well for Alizarin Red Staining. Triplicate of each cell passage were seeded for control group that were maintained in basal medium. Odontogenic induction medium was used to maintain the induction group for 14 days. Both medias were changed every two days. At day 14, cells were washed with DPBS and fixed using 10% naturally buffered formalin for 30 minutes. The samples were later washed with sterile water before staining with 40 mM Alizarin red (pH 4.2) for 45 minutes at room temperature with

gentle rotation. To remove Alizarin Red staining, cells were rinsed for five times with distilled water. The results are then observed under the microscope and photographs or the wells are taken. Each group was seeded in triplicates and the experiment was repeated 3 times.

Western Blot

Once cell cultures reached 80% confluency, whole cell extracts were isolated with cell lysis buffer (1% Triton X-100, 20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 2.5 mM sodium pyrophosphate, 1μM β- glycerophosphate, 1mM sodium orthovanadate, 1mg/ml PMSF). SDS-PAGE was used to fraction the isolated whole cell extracts before transferring it to a polyvinyl difluoride protein membrane (Millpore). They were later blocked using 5% low-fat milk with Phosphate Buffered Saline (PBS) solution with the detergent Tween® 20 (PBST). After that, the membrane was incubated with the primary antibody at 4 degrees Celsius overnight. Following washing the membrane with TBST for three times, the membrane was incubated with the secondary antibody for 1 hour at room temperature. The membrane was later exposed using the chemiluminescence reagent for protein detection. Alkaline phosphatase (ALP) and GAPDH (Santa Cruz Biotech) antibodies were detected. Secondary antibodies utilized horseradish-peroxidase (HRP) conjugate (Santa Cruz Biotech). This experiment was repeated three times.

Reverse transcription and quantitative real-time PCR (qPCR)

MSCs at different tissue passages were allowed to reach 70-80% confluency before total RNA was isolated using TRIzol reagents (Invitrogen). Chloroform was added and the samples were centrifuged for 15 minutes. The aqueous layer containing RNA was preserved. Isopropanol was added to allow formation of RNA precipitate. Total RNA samples were then washed with ethanol twice. NanoDrop Spectrophotometer (ThermoFisher Scientific) was used to measure

total RNA and assess its quality.

Amplification of cDNA was then completed with SYBR Green I Master Mix (Roche), utilizing the LightCycler 480 II real-time PCR system (Roche), following manufacturer's instructions. Thermal conditions were set at 95 degrees Celsius at 10 minutes (initial denaturation), followed by 45 cycles of 95 degrees Celsius for 10 seconds. Lastly, 58 degrees Celsuis for 45 seconds and 72 degrees for 10 seconds (denaturation). The internal control used was GAPDH and all samples were triplicated. Second derivative of the Cq value was determined by comparing the gene of interest to GAPDH for fold-differences of amplification as recommended by manufacturer's protocol (Roche). The experiment was repeated twice.

Tissue processing and staining

DPTE at different tissue passages were washed with PBS before fixation with 10% natural buffered formalin for 24 hours. Tissues were further washed with sterile water and embedded in paraffin. Five nanometer sections were made with a microtome and tissue were mounted onto slides. Histological slides were placed in a conventional oven to for deparaffinization. They were later rehydrated with xylene washing for two times, followed by serial-dilution of ethanol (100% twice, 95% twice, and 70% once). For hematoxylin and eosin staining, slides were washed with running tap water for 5 minutes, stained with hematoxylin for 2.5 minutes. Slides were subsequently rinsed with 95% ethanol and stained with eosin for 1 minutes. Moreover, slides were washed with running tap water then dehydrated with serially-diluted ethanol (70% once, 95% twice, and 100% three times). They were finally washed with xylene for three times before mounting. Masson-Trichome staining was also performed using Sigma-Aldrich staining kit as the protocol recommended.

Nuclear staining was also performed for the DPTE tissues at different tissue passages. Following rehydration of the slides, tissues were permeabilized with Trition X for 30 minutes at room temperature. Tissues were then rinsed with PBST for three times. Subsequently, they were incubated with DAPI nuclear labeling solution for 3 minutes at room temperature in the dark. Nuclear staining was later observed using confocal microscopy at 24x magnification by choosing random fields.

Immunophenotypic characterization

DPTE at different tissue passages were washed with PBS then fixed with 10% natural buffered formalin for 24 hours. They were later embedded into paraffin and sectioned. After rehydration of the slides, tissues were blocked with %5 bovine serum albumin (BSA) for 1 hour in room temperature followed by 0.5% Trition X incubation for 30 minutes. They were then incubated overnight in 4 degrees Celcius with primary antibody, CD 146 (Abcam), which were diluted in 1% BSA and 0.05% Trition X-100/PBS. Samples were then incubated with secondary antibody for 1-2 hours at room temperatures, which were fluorochrome conjugated, were diluted in 1% BSA/0.05% Trition X-100/PBS. They were then counterstained with DAPI nuclear staining and scanned using confocal fluorescent microscopy at 24x magnification by choosing random fields.

MP-MSCs at serial tissue passages (1, 5 and 9) were harvested (5 x 10^5 cells) and resuspended in PBS with 0.1% sodium azide and 1% BSA. They were later incubated in antibody (CD146 and STRO-1 fluorescent-conjugated) for 15 minutes according to manufacturer's instructions. Flow cytometry was then completed at the Flow Core of Jonsson Comprehensive Cancer Research at UCLA.

RESULTS

DPTEs continued to display stem cells characteristics through serial tissue passages.

We collected 5 freshly extracted healthy non-carious third molars from Oral Surgery Clinic at School of Dentistry, UCLA during the period between January 2019 and May 2019. Patients' ages ranged between 18-23 years, 3 female patients and 2 male patient were included. The obtained pulp tissue was minced with micro-scissors to 1 mm^3 cubes that was fixed at different tissue passages, mainly Tissue passage 1 (T_1), Tissue passage 5 (T_5), and Tissue passage (T_{10}). Hematoxylin and eosin staining was performed. It was noted that at T_5, there was some discrete islands of eosinophilic staining. Furthermore, T_{10} revealed almost 80% eosinophilic staining (figure 1). Due to the qualitative changes noted when tissues were cultured, Masson-Trichome staining was performed. It showed no significant difference in the collagen amount, which is colored as blue, between the three groups (figure 2). This implies that the eosinophilic staining could be due to *in vitro* tissue culturing for 140-150 days.

Nuclear staining was performed to visualize cellular abundance within the tissues at different tissue passages. DAPI was used to label nuclei and staining was observed with confocal microscopy at 24 x magnification. Permeabilization of the tissues allows DAPI to enter the cell and bind to its double strand DNA (dsDNA). Cellularity was observed in the human dental pulp tissue at different tissue passages. However, T_1 exhibited the most abundant cellularity compared to T_5 and T_{10}. Increased concentration of cellularity in the periphery of the minced pulp tissue rather than the center was noted at serial tissue passages. Nonetheless, cellularity at the center of the tissues was still observed at T_{10}. The pulp tissue at different tissue passages was also viewed with phase contrast using the confocal microscopy (figure 3).

Immunofluorescence staining of CD146, which is mesenchymal stem cells surface marker, was performed. All tissues examined showed positive CD146 staining. However, tissues at T_{10} passages showed significantly less CD146 positive staining when compared to T_1 and T_5 tissue passages (figure 4). Flow cytometric analysis of MP-MSCs examined two antibodies, CD146 and STRO-1. CD146 was shown to be expressed in both MP-MSCs T_1 and T_{10}. However, the expression levels decrease from 55% to 24% in MP-MSC T_1 and MP-MSC $T_{10,}$ respectively. STRO-1 is also a stem cell marker of mesenchymal stem cells, which was not expressed in both groups (figure 5).

MP-MSCs retained cell proliferation potential during the serial tissue passages.

The minced dental pulp tissue was maintained in primary culture media, until the yielded MSCs reached 80% confluency (approximately 14 days). The yielded MSCs were further maintained in basal culture media. All experiments were performed with MSCs from early passage cells (P_{1-3}) and triplicated within each experiment. Also, every experiment was repeated three times.

To observe cell proliferation rate, MP-MSC $T_{1,}$ $T_{5,}$ and T_9 were seeded into 24-well plates. Cell were counted at day 1-6 of culturing. There was no significant difference in the proliferation rate observed at day 1-4. It is indicated that MP-MSC T_5 had slightly increased cell proliferation rate. At Day 6, MP-MSC T_1 showed increased proliferation rate when compared to MP-MSC T_{10} (figure 6).

Cellular proliferation was also assessed using MTT assay. There was no significant difference between cells from $T_{1,}$ T_5 and T_9, which indicated similar proliferation rates (figure 7).

DPTEs maintain their osteogenic differentiation potential through serial tissue passages.

To characterize MSCs yielded from different tissue passages, ALP staining was performed. It was noted that there was no significant difference between the control group and osteogenic induction group in the three compared tissue passages, MP-MSC T_1 MP-MSC T_5, and MP-MSC T_9. However, there was a marked increase in the MP-MSC T_5 group in both the control and osteogenic induced group when compared to the other samples (figure 8A).

Furthermore, protein expression levels of ALP also revealed no significant difference between the compared groups. Thus, it was suggested that MP-MSCs retains its' osteo/odontogenic differentiation capacities through serial tissue passages, which is an important stem cell characteristic (figure 8B). The OCN, Osteocalcin, mRNA expression levels were significantly upregulated in MP-MSC T_5 and MP-MSC T_9 when cultured with osteogenic induction medium for 7 days. There was no significant difference between MP-MSC T_1 when it was cultured with basal medium and osteogenic induction medium (figure 10).

Alizarin Red Staining was performed to evaluate the mineralization capacity of MP-MSCs at different tissue passages. Cells were cultured in osteogenic medium with medium changed every two days and cells were stained at day 14. Cells from compared tissue passages showed similar mineral deposition when exposed to calcifying conditions. The results suggested that MP-MSCS T_5 and MP-MSCs T_9 retained their mineralization capacities. (figure 10A, B).

DISCUSSION

The aim of this study was to characterize MP-MSCs at serial tissue passages (1, 5, and 10). To this end, we here revealed that minced pulp tissue explants retained its osteo/odontogenic differentiation potentials up to passage 10. It was revealed previously by Couble that dental pulp tissue explant cells (DPTE) showed features of active odontoblasts. Such as, dentin-like collagenous mineralizing matrix synthesis (Couble et al. 2000). The same group discovered that DPTEs exhibited a polarized cell morphology and DSPP expression *in situ*, which is essential for the initiation of odontogenic mineralization. Those preliminary findings firstly validated the feasibility of stem cells isolation from dental pulp tissue explant culture. Previously, we demonstrated that cells migrating from minced pulp tissue is a viable approach to bypass the *in vitro* culture process of DPSC (Liang et al. 2018).

By the utilization of primary culture methods, our study indicated that MP-MSCs maintained an increase in proliferation rates from days 1 to 6. MP-MSC T_5 exhibited slightly higher proliferation rate, which could be attributed to more favorable primary culture conditions. There was also a significant difference between MP-MSCs T_1, T_5, T_9 at day 6. When the MTT cell proliferation rate was performed, no significant difference was found between the three cell groups (MP-MSCs T_1, T_5, and T_9). Cells were cultured for 48 hours before MTT assay was performed, which could explain the contrasting results.

Furthermore, our study investigated the osteogenic and odontogenic differentiation capacities of dental pulp tissue explants at different tissue passages. In a previous study, we showed that DPSCs lost their odonto/osteogenic differentiation capacities at replicative senescence (Mehrazarin et al. 2011). We obtained cells from tissue explants at 170-180 days post-explantation and we compared MP-MSC T_1, T_5 and T_9 cells. The control group and osteogenic

induction groups showed positive ALP staining and no difference between groups. However, there was a marked increased activity in MP-MSC T_5 which could be due to the increased replication demonstrated by the proliferation rate. We additionally performed western blotting of ALP protein and did not find any significant differences between the control and induction groups. The similarity between the control and induction groups could be due to *in vitro* culture and alteration of cells properties due to cryopreservation. The ALP staining experiment will be repeated for each MP-MSC immediately after migration from tissues at each passage. To further understand its' properties RT-qPCR was performed using an osteogenic marker, osteocalcin (OCN), which showed significant difference between osteogenic induction and non-induction groups of MP-MSC T_5 and T_9. We did not find any significant difference between T_1 control and induction group and DPSC group was significantly increased compared to all groups. This could be due to the short induction period, 7 days, and since OCN is secreted at late maturation stages (Boguslawski 2000). Our experiments exhibited dense culture conditions which was shown to activate integrin signaling osteogenesis pathways due to cell-cell contact adhesions in DPSCs (Noda et al. 2019). DPSCs were shown to remain undifferentiated and are influenced by multiple passaging (Suchanek et al. 2007, Lizier et al. 2012). In contrast, MP-MSCs appear to retain its differentiation potential at serial tissue passages.

Nuclear staining with DAPI indicated reduction in the cellular content and migration to the tissue margins. In our previous study, we showed that at day 15 post-explantation there was indeed a reduction in the cellular content when compared to day 0 (Liang *et al*. 2018). Despite this reduction in cellular content of the tissue, we show that the cells migrating from later tissue passages exhibits a significantly high proliferation and growth rate. CD146 is an integral membrane glycoprotein and was found to be upregulated in high proliferative cells (Sorrentino et

al 2008). In a study conducted by Matusi et al., they suggest that CD146+ DPSCs transplantation resulted in increased area of dentin/pulp-like structures (Matusi et al. 2018).

We compared the expression of CD146, a MSC marker, in both MP-MSC T_1 and MP-MSC T_{10}. Although there was a decrease in the expression of CD146, our study showed that the osteogenic/odontogenic differentiation potential was not affected. In contrast to DPSCs, which were shown to lose their differentiation ability when there was no expression of CD146 (Macrin et al. 2019). Our flow cytometric analysis shows 55.2% CD146+ DPTE T_1 cells and 23.7% CD146 DPTE T_{10} cells. The results indicated that after over 150 days of culture, minced pulp tissues still yields CD146+ cells, which is critical to promote dentin-pulp complex regeneration.

CONCLUSION

This study provided evidence that cells migrating out from dental pulp tissue explants through serial tissue passages retained their growth potential as well as odontogenic differentiation capacity. Minced-pulp MSCs, as well as DPTEs expressed stem cells surface marker, CD146, at late tissue passage (T_{10}). Indicating that their stem cells characteristics where maintained through serial tissue passages.

We therefore conclude that minced pulp tissues are able to yield unlimited number of mesenchymal cells which retain their osteo/odontogenic differentiation capacities and demonstrated stem cell characteristics. This newly proven multi-potency of MP-MSCs provide a promising future for regenerative endodontics and our proposed pulp tissue grafting approach.

FIGURES

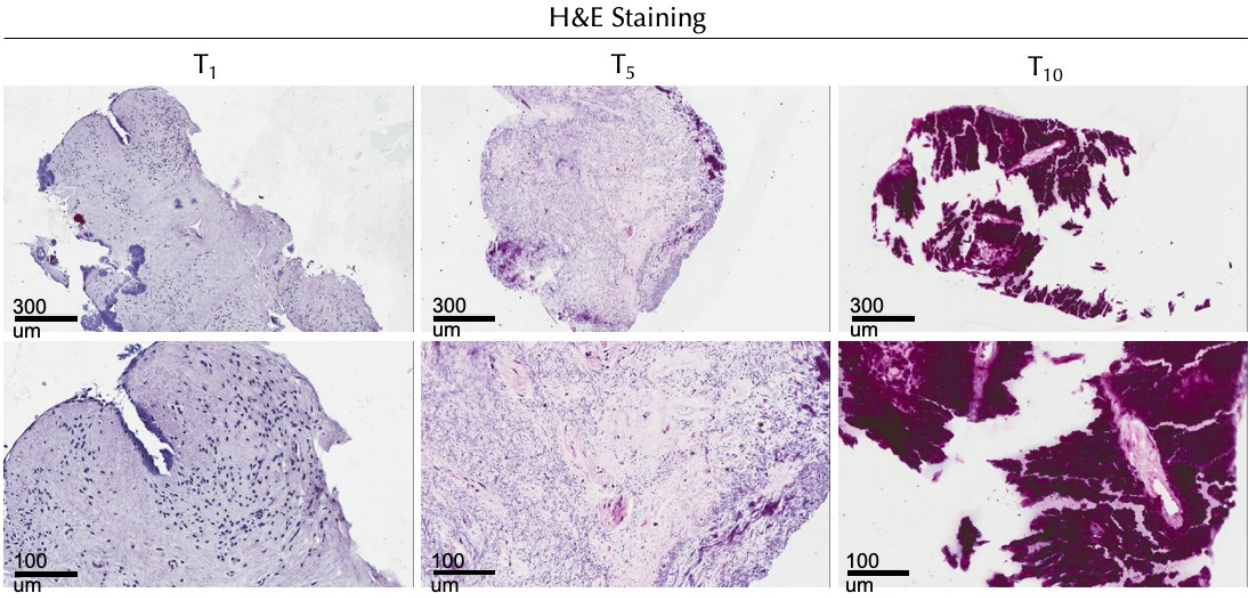

Figure 1. Hematoxylin and Eosin staining of minced pulp tissue at serial tissue passages. Distinct eosinophilic staining is demonstrated at T_{10} when compared to T_1 and T_5. Indicating a fibrotic texture of T_{10}.

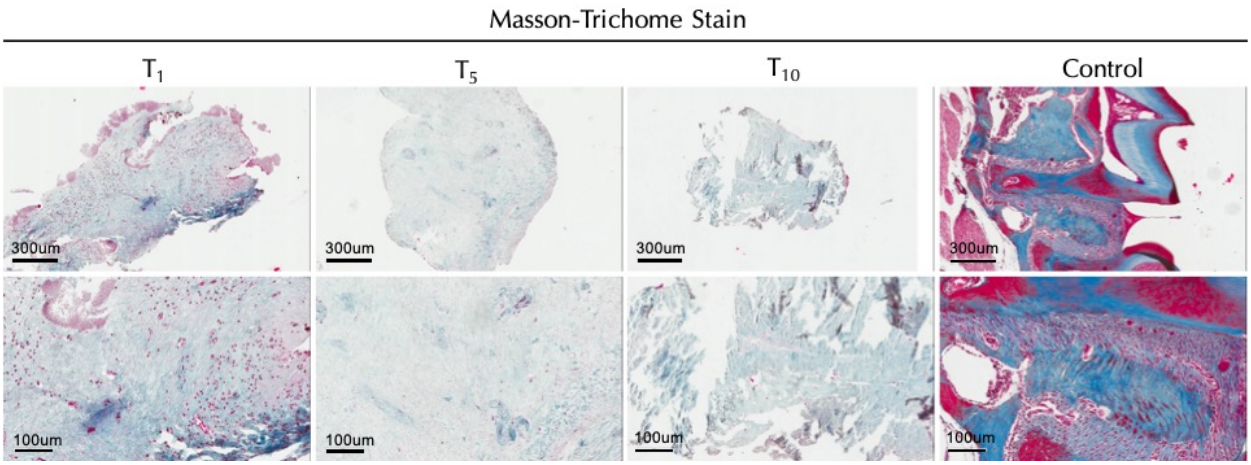

Figure 2 . Masson-Trichome staining of minced pulp tissue at various tissue passages.

Minced pulp tissue at early passage displays more cellular content marked as red staining, which is decreased with multiple tissue passages. T_{10} tissue passages display blue stain which is collagenous content suggesting that pulp tissue maintain their original morphology.

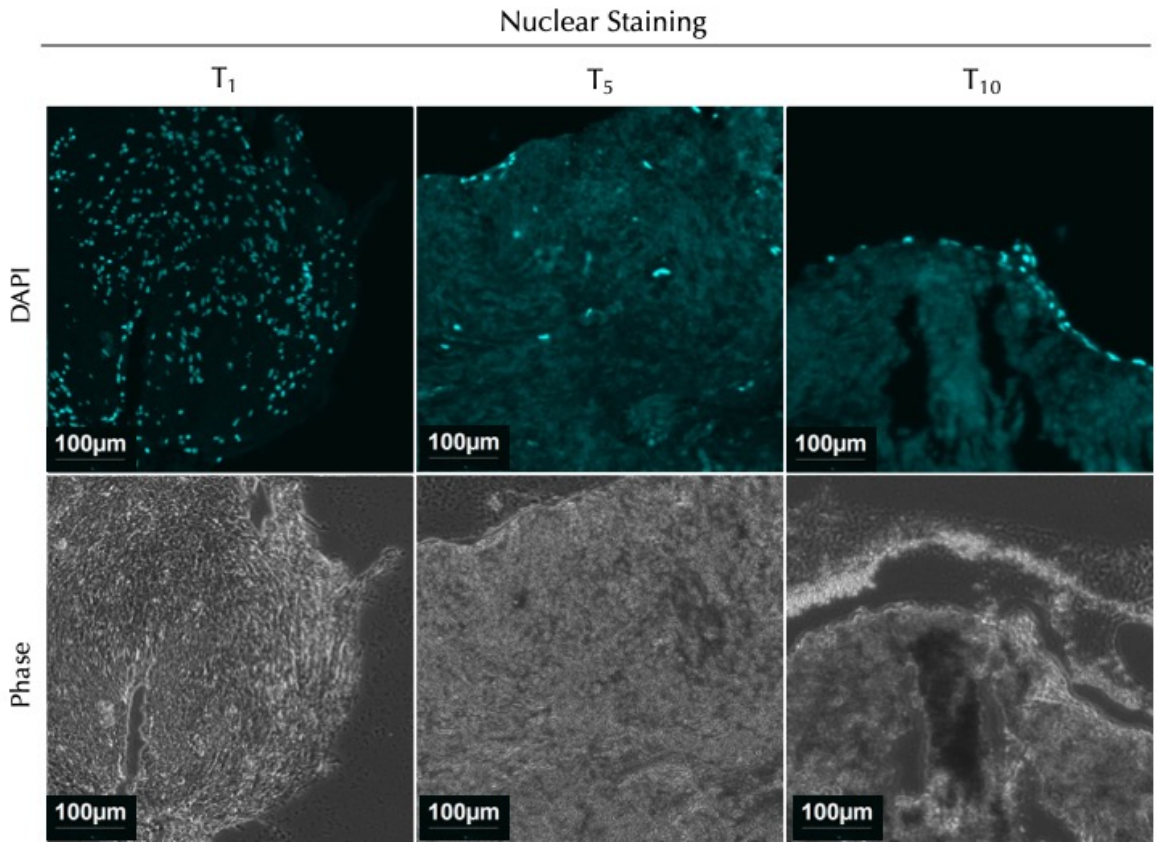

Figure 3. Nuclear and phase-contrast staining of minced pulp tissue at tissue passage 1, 5 and 10. Minced pulp tissues were maintained in primary cultures and scanned at different tissue passages. In the upper row, nuclei stained with DAPI was labelled and scanned them under fluorescence microscopy. In the lower row, phase contrast staining of the tissues was observed.

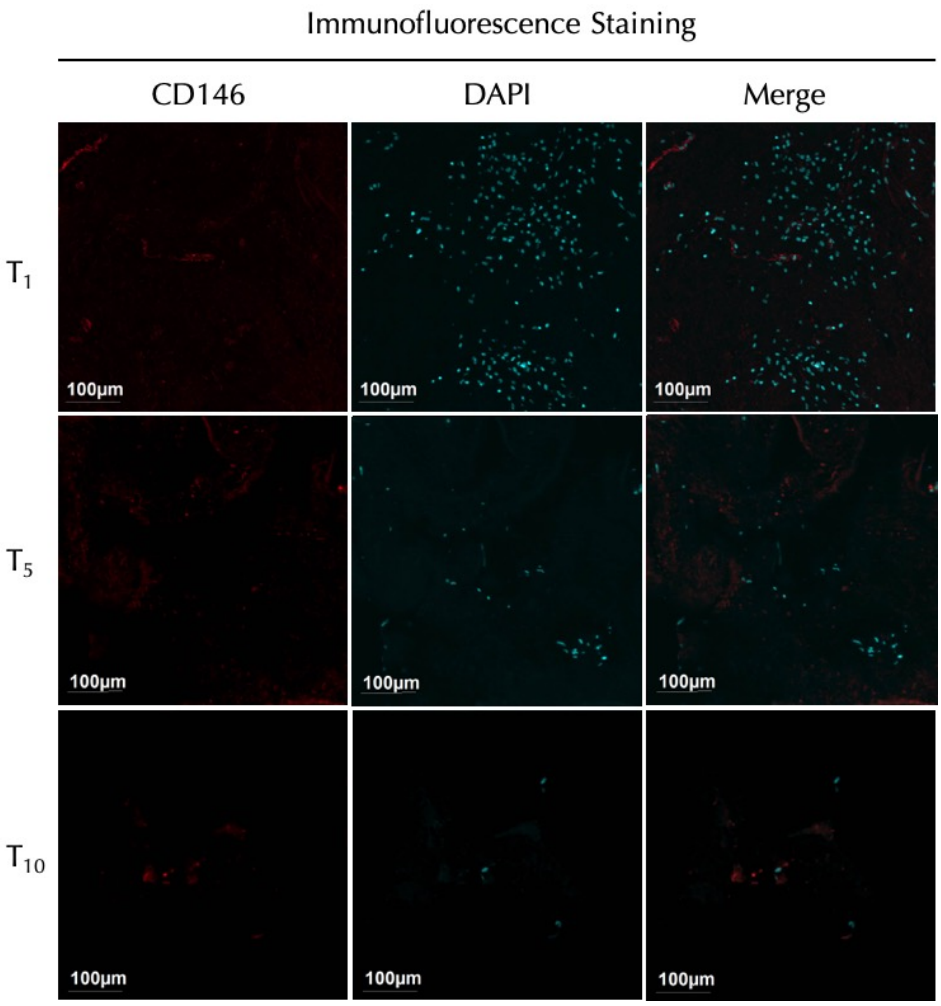

Figure 4. Dental pulp tissue exhibits mesenchymal stem cells characteristics. Pulp tissues at serial tissue passages were stained with CD146 and examined under fluorescence microscopy. In the left panel, CD146+ areas are stained as red and $T_{1,}$ $T_{5,}$ T_{10} all displayed positive staining. DAPI nuclear labeling was also performed as shown in the middle panel as blue. CD146+ staining and DAPI Merge staining was observed in the left panel to observe the relationship of CD146+ staining to nuclei.

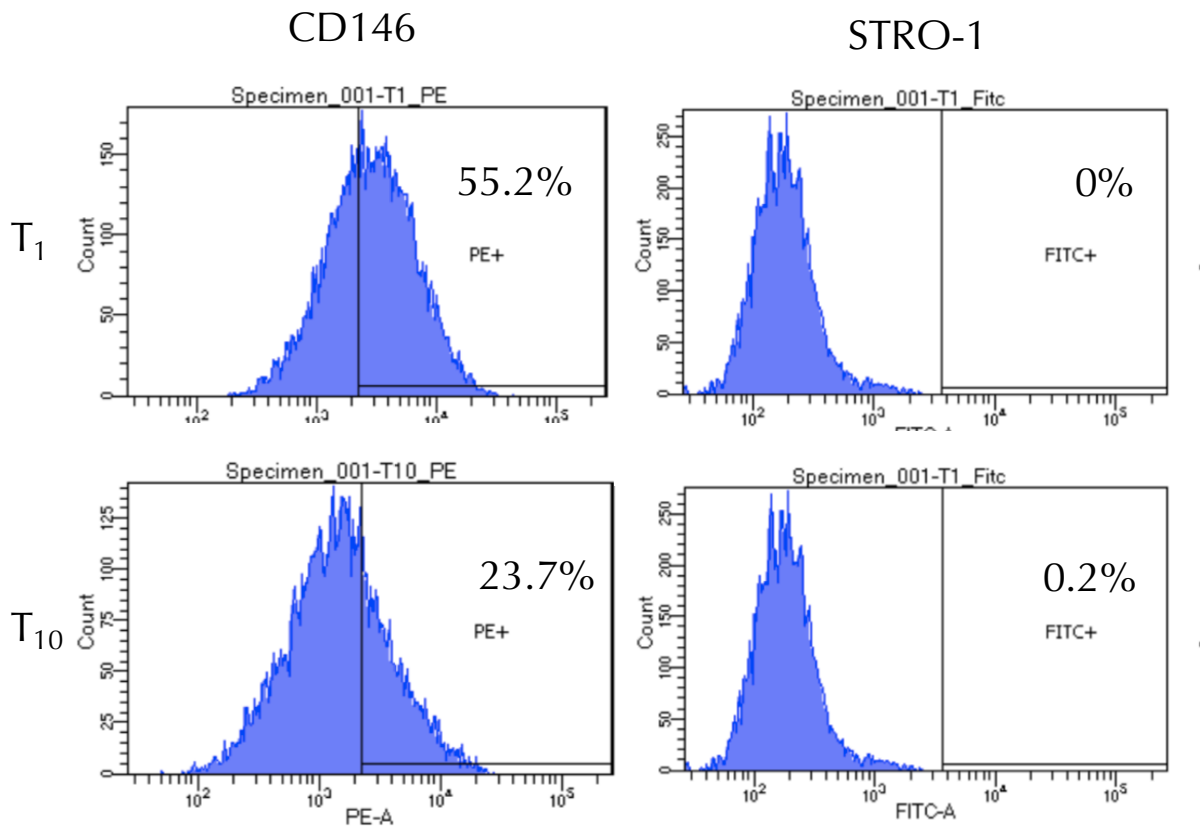

Figure 5. Cells from MP-MSC Tissue passage express stem cells surface marker CD146.

MP-MSC T_1P_3 expressed 55.2% CD146+ cells and MP-MSC $T_{10}P_3$ expressed 23.7% CD146+ cells. No STRO-1+ cells were observed in both groups.

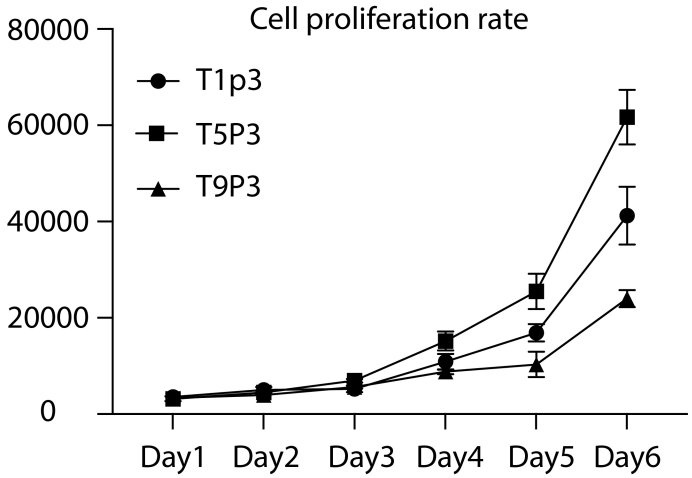

Figure 6. Cell Proliferation rates of compared groups indicated an increase at day 6. No significant difference between the compared groups (MP-MSC T_1, MP-MSC T_5, MP-MSC T_9) was observed until day 5 of culture. From day 5 to 6, DPTE T_5 display the most significant increase.

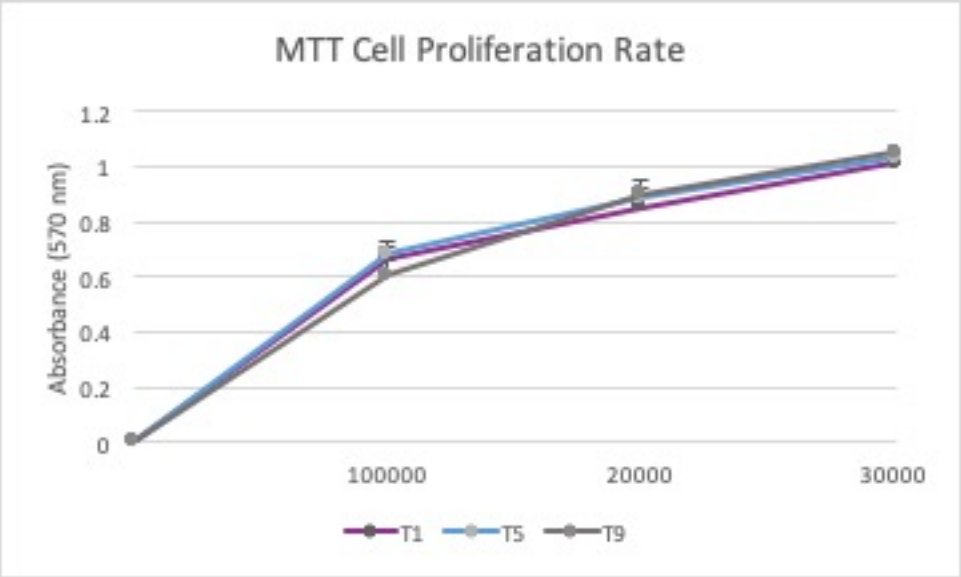

Figure 7. MP-MSC T_1, MP-MSC T_5, and MP-MSC T_9 demonstrated comparable proliferation rates. MTT cell proliferation rate assay compared the three tissue passages using concentrations of 1×10^5, 2×10^5 and 3×10^5 Cells. No significant difference was found between the compared groups.

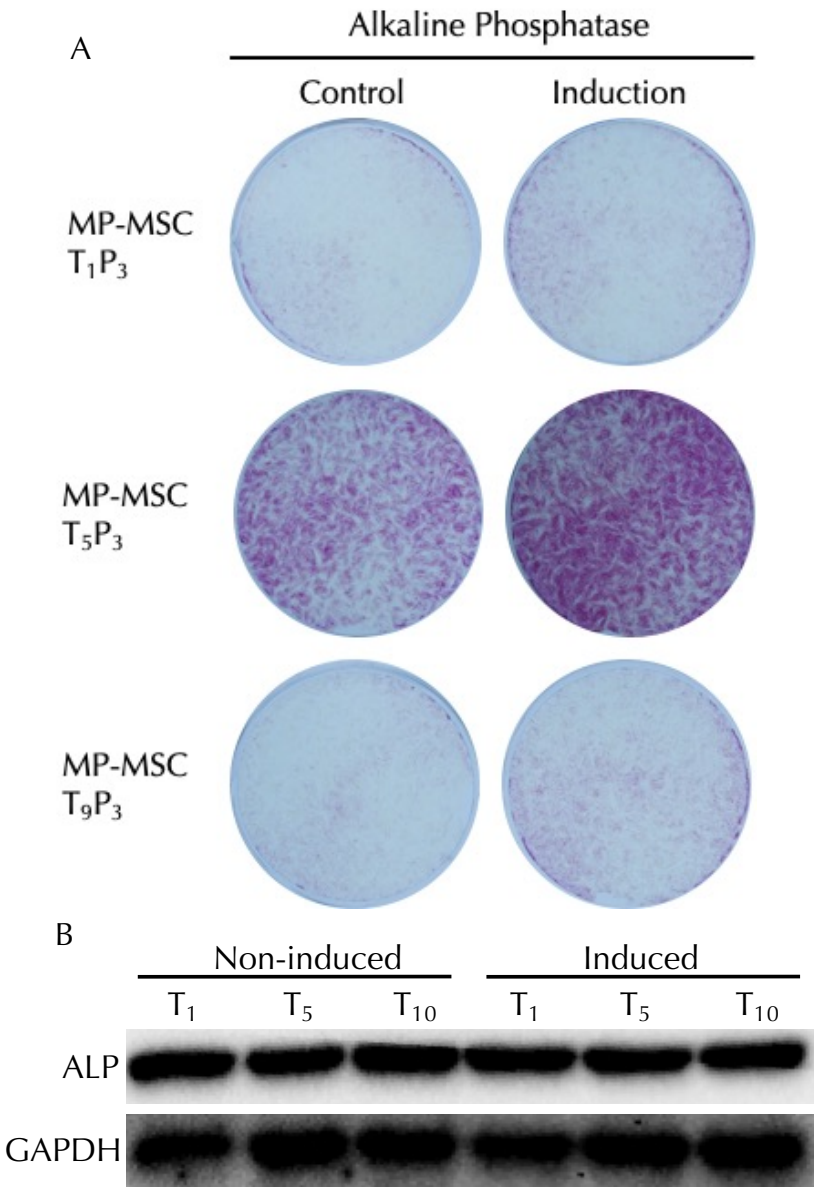

Figure 8. MP-MSCs displayed osteo/odontogenic differentiation potential at serial tissue passages. (A) ALP activity of MP-MSCs at tissue passage 1, 5, and 9 were compared after culturing in osteogenic differentiation media for 7 days. No significant difference was observed between control and osteogenic induction groups. MP-MSC T_5P_3 demonstrated increased ALP activity. (B) Western blotting was performed for ALP in DPTEs after 7 days of culture with and without osteogenic induction media. No significant difference in the ALP protein expression between the compared groups. GAPDH was used as an internal control.

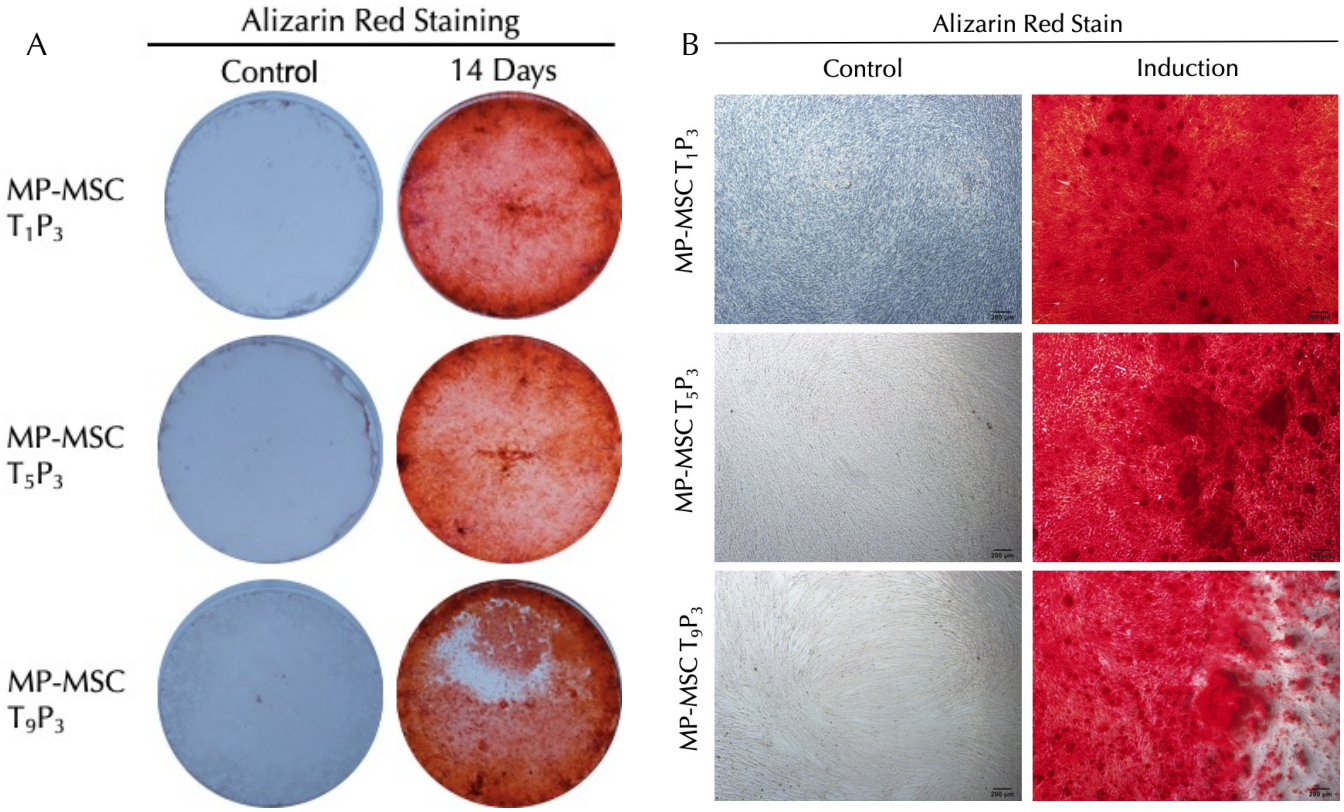

Figure 9. MP-MSCs yielded from various tissue passage 1, 5 and 10 MP-MSC T_1P_3, T_5,P_3, T_9 P_3) demonstrated mineralization capacity. (A) When MP-MSC s were cultured in osteogenic induction medium, Alizaren Red Staining was increased indicating high mineral depositions. (B) The same dishes were observed under light microscopy at a magnification of 4X.

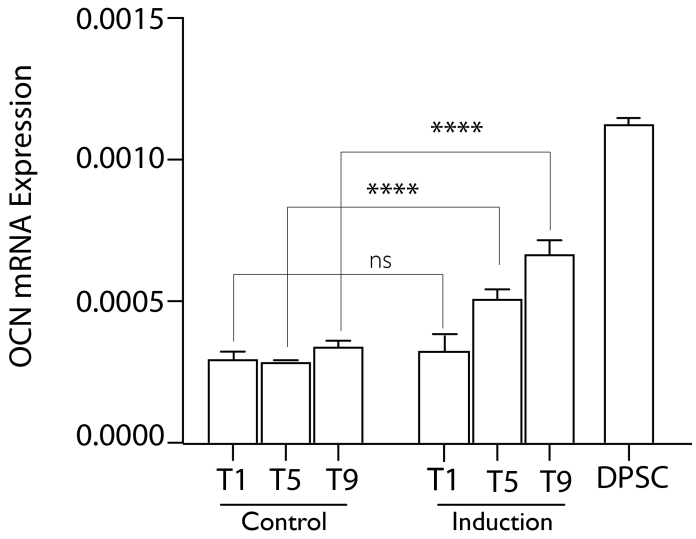

Figure 10. MP-MSC demonstrates increased OCN expression when exposed to osteogenic induction conditions. MP-MSCs at different tissue passages were cultured with and without osteogenic culture medium for 7 days. OCN mRNA expression increased significantly in T_5 and T_9. DPSCs were used as the positive control

REFERENCES

American Association of Endodontists. 2017 “AAE clinical considerations for a regenerative procedure.”

Austah, O., Joon, R., Fath, WM., Chrepa, V., Diogenes, A., Ezeldeen, M., Couve, E., Ruparel, NB. 2018 “Comprehensive Characterization of 2 Immature Teeth Treated with Regenerative Endodontic Procedures”. 44:1802-1811 doi: 10.1016/j.joen.2018.09.007.

Boguslawski, G., Hale, VL., Yu, XP., Miles, RR., Onyia, J., Santerre, R., Chandrasekhar, S. 2000 “Activation of Osteocalcin Transcription Involves Interaction of Protein Kinase A- and Protein Kinase C-dependent Pathways.” Journal of biological chemistry. 357(2):999-1006.

Bukhari, S., Kohli, MR., Setzer, F., Karabucak, B.. 2016 “Outcome of Revascularization Procedure: A Retrospective Case Series. “ *Journal of Endodontics. 42*:1752-1759. https://doi.org/10.1016/j.joen.2016.06.021

Couble, M. L., J. C. Farges, F. Bleicher, B. Perrat-Mabillon, M. Boudeulle, and H. Magloire. 2000. “Odontoblast Differentiation of Human Dental Pulp Cells in Explant Cultures.” *Calcified Tissue International* 66: 129–38. doi:10.1007/s002230010027.

De Chevigny C, Dao TT, Basrani BR, et al. 2008 “Treatment outcome in endodontics: The Toronto study—Phase 4: Initial treatment.” *Journal of Endodontics* 34:258–263.

Gronthos S, Mankani M, Brahim J, et al. 2000 “Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo.” *Proc Natl Acad Sci USA* 97:13625-30.

Hargreaves K. M., Diogenes A., Teixeira FB. 2013. “Treatment Options: Biological Basis of Regenerative Endodontic Procedures.” *Journal of Endodontics.* 3 (39):S30-S43. doi: 10.1016/j.joen.2012.11.025

Iohara et al. 2018. “Allogeneic transplantation of mobilized dental pulp stem cells with the mismatched dog leukocyte antigen type is safe and efficacious for total pulp regeneration.” *Stem Cell Research & Therapy* 9:116.

Iohara, Koichiro, Kiyomi Imabayashi, Ryo Ishizaka, Atsushi Watanabe, Junichi Nabekura, Masataka Ito, Kenji Matsushita, Hiroshi Nakamura, and Misako Nakashima. 2011. “Complete Pulp Regeneration After Pulpectomy by Transplantation of CD105 Stem Cells with Stromal Cell-Derived Factor-1.” *Tissue Engineering Part A* 17 (15-16): 1911–20. doi:10.1089/ten.tea.2010.0615.

Kang, Mo K., Ayako Kameta, Ki Hyuk Shin, Marcel a. Baluda, and No Hee Park. 2004. “Senescence Occurs with hTERT Repression and Limited Telomere Shortening in Human Oral Keratinocytes Cultured with Feeder Cells.” *Journal of Cellular Physiology* 199 (May 2003): 364–70. doi:10.1002/jcp.10410

Liang, Z., Kawano, S., Chen, W., Sadrkhani, MS., Lee, C., Kim, E., Moshaverinia, A., Kim, RH., , Kang, MK. 2018 “Minced Pulp as Source of Pulpal Mesenchymal Stem Cells with Odontogenic Differentiation Capacity.” *Journal of Endodontics*. 44:80-86. DOI: https://doi.org/10.1016/j.joen.2017.08.011

Lin JC, Lu JX, Zeng O, et al. 2016 “Comparison of mineral trioxide aggregate and calcium hydroxide for apexification of immature permanent teeth: a systematic review and meta-analysis.” *J Formos Med Assoc*. 115:523–30

Lizier, N. F. *et al*. 2012 “Scaling-up of dental pulp stem cells isolated from multiple niches.” *PLoS One* 7, e39885.

Macrin D. Algahdeer A., Zhao Y. et al. 2019 “Metabolism as an early predictor of DPSCs

aging." *Scientific Reports.* 9:2195.

Matsu M., Kobayashi T., Tsutsui T. 2018. "CD146 positive human dental pulp stem cells promote regeneration of dentin/pulp-like structures" Human Cell. 31:127-138

Mehrazarin, S., Oh, JU, Chung, Wei Chen, W., Kim, RH., Shi, S., Park, NH., Kang, MK. 2011. "Impaired Odontogenic Differentiation of Senescent Dental Mesenchymal Stem Cells Is Associated with Loss of Bmi-1 Expression." *Journal of Endodontics* 37 (5). Elsevier Ltd: 662–66. doi:10.1016/j.joen.2011.02.009

Murakami M, Horibe H, Iohara K, Hayashi Y, Osako Y, Takei Y, Nakata K, Motoyama N, Kurita K, Nakashima M. 2013 "The use of granulocyte-colony stimulating factor induced mobilization for isolation of dental pulp stem cells with high regenerative potential. Biomaterials." *Biomaterials* 34:9036–47.

Nakashima, M. Iohara, K.; Murakami, M. Nakamura, H. Sato, Y. Ariji, Y. Matsushita, K. 2017 "Pulp regeneration by transplantation of dental pulp stem cells in pulpitis: A pilot clinical study. " *Stem Cell Res. Ther*, 8, 61.

Noda S., Kawashima N., Yamamoto M., Hashimoto M., Nara K., Sekiya I, Okiji T. 2019 " Effect of cell culture density on dental pulp-derives mesenchymal stem cells with reference to osteogenic differentiation". *Nature Scientific Reports*. 9:5430.

Suchánek, J. *et al*. 2007. "Human dental pulp stem cells – isolation and long term cultivation." *Acta. Medica. Cordoba.* 50, 195–201.

Torabinejad, M. *et al* 2007. Outcomes of root canal treatment and restoration, implant-supported single crown, fixed partial dentures, and extraction without replacement: A systematic Review " *Journal of Prosthetic Dentistry, 98 (42):285-311.*

Wang X, Thibodeau B, Trope M, et al. 2010. “Histological characterization of regenerated tissues in canal space after revitalization/revascularization procedure of immature dog teeth with apical periodontitis”. *Journal of Endodontics*. 36:56–63.

Xuan K., Li1 B., Guo H., Sun W., Kou X., He X., Sun YZ., Liu A., Liao L., Liu L., Liu W., Hu C., Shi S., Jin, Y. 2018. “Deciduous autologous tooth stem cells regenerate dental pulp after implantation into injured teeth.” *Sci Transl Med*. 22:10(455).