The Role of IL-6 in Osteogenic and Neurogenic Differentiation Potentials of Dental Pulp Stem Cells (DPSCs)

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THESIS

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ABSTRACT

The Role of IL-6 in Osteogenic and Neurogenic Differentiation Potentials of Dental Pulp Stem Cells

Introduction: Interleukin 6 (IL-6) is a pleiotropic soluble mediator which is involved with many different parts of inflammatory processes. Studies have shown that there is a significantly higher concentration of IL-6 in inflamed pulp tissues when compared to healthy pulp tissues. However, there are no comprehensive studies on how IL-6 can interact with dental pulp stem cells (DPSCs) from healthy (H-DPSCs) and inflamed pulp tissues (I-DPSCs) and how it can affect the differentiation potential of DPSCs. Therefore, I hypothesized that IL-6 can promote osteogenesis while inhibiting neurogenesis from DPSCs. The aims of this study were to investigate the baseline differences between the H-DPSCs and I-DPSCs, and determine how IL-6 can affect the osteogenic and neurogenic differentiation potentials of DPSCs.

Materials and Methods: H-DPSCs and I-DPSCs were isolated and cultured from 28 teeth consisting healthy impacted third molars (n=14) from patients with 17-27 years of age, and severely decayed molars (n=14) with spontaneous, sharp pain from patients with 24 – 56 years of age, respectively. The levels of IL-6 from H-DPSCs and I-DPSCs were measured by ELISA, and IL-6 and neutralizing IL-6 were added to H-DPSCs and I-DPSCs, respectively. Immunofluorescence and Alizarin Red staining, and western blot were performed to assess the differentiation potentials of DPSCs. The independent unpaired two-tailed Student's t-test were performed following quantification analysis.

Results: H-DPSCs and I-DPSCs showed similar expression of stem cell markers including CD44, CD73, CD90, and CD105, while H-DPSCs showed lower level of IL-6 (p<0.001), lower osteogenic and higher neurogenic (p<0.001) differentiation potentials compared to I-DPSCs. Addition of IL-6 to H-DPSCs increased the osteogenic potentials and decreased the neurogenic potentials (p<0.001), while neutralization of IL-6 for I-DPSCs led to decreased osteogenic potentials and increased neurogenic potentials (p<0.001).

Conclusion: This study showed IL-6 has the capacities to enhance osteogenesis while hindering neurogenesis of DPSCs.

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LITERATURE REVIEW

Stem cells in Oral Cavity

Stem cells are one of the hottest topics in the field of research including both medicine and dentistry in recent years, because they have been identified as a promising source for many clinical applications including cell therapy and tissue engineering.

Stem cells, by their definition, are unique for two distinct characteristics which are the abilities to self-renew and to differentiate into different cell types (Egusa et al., 2012). There are three types of stem cells: adult stem cells, embryonic stem cells and induced pluripotent stem cells. Adult stem cells and embryonic stem cells are naturally found in human body while induced pluripotent stem cells are synthetically made through genetic manipulation of somatic cells (Takahasi, 2006).

Adult stem cells, including dental stem cells, are considered multipotent, meaning they are able to differentiate into a few different types of cells, while embryonic stem cells and induced pluripotent stem cells are considered pluripotent, meaning they can differentiate into all cell types from three germinal layers (Egusa et al., 2012).

Adult stem cells are found in many tissues and organs in our body. Through self-renewal and differentiation, adult stem cells work to keep healthy tissues and fix injured tissues throughout the body. Many adult stem cells are located in certain mesenchymal tissues and they are collectively called mesenchymal stem cells (MSCs) (Hortwitz et al., 2005). Originally, MSCs have been identified in the bone marrow, but similar types of MSCs have also been identified from many different areas of the body, including adipose tissue, skin and dental tissues (Egusa et al., 2007).

Recent studies have identified our oral cavity as a rich niche for many different types of stem cells. These cells include bone marrow-derived MSCs (BMSCs) from orofacial bone, dental pulp stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, dental follicle stem cells, tooth germ progenitor cells, stem cells from the apical papilla, oral epithelial progenitor/stem cells, gingiva-derived MSCs, periosteum-derived stem cells and salivary gland-derived stem cells (Egusa et al., 2012).

Bone marrow stem cells possess a high replicative capacity and have the ability to differentiate into different connective tissue cell types. In addition, they form bone-like structure in vivo, which makes them an attractive target for bone regeneration therapy (Egusa et al., 2012). The orofacial MSCs are functionally different from

other types of BMSCs with higher proliferation and osteogenic differentiation capacity based on several studies (Igarashi et al., 2007).

In 2000, adult dental stem cells were first isolated from the dental pulp, then MSC-like cells were subsequently isolated from the pulp of human deciduous teeth (Gronthos et al., 2000). These cells possess characteristics of stem cell, including the ability to self-renew and differentiate into multiple cell types (Shi et al., 2001).

A few years after the first discovery of adult stem cells from the dental pulp, MSC-like cells were identified again from the periodontal ligament (Seo et al., 2004). They have also demonstrated certain stem cell properties; additionally, they have shown the ability to regenerate periodontal tissues in animal models (Seo et al., 2004).

Based on active on-going research on stem cells, many promising studies are being conducted for stem-cell-based therapy in dentistry, focusing on tissue engineering and chair-side cellular grafting approaches. These studies are heading to more predictable regenerative outcomes in the future using stem cells in oral cavity (Egusa et al., 2012). These studies include alveolar bone augmentation, tooth regeneration, salivary gland regeneration, mandible condyle regeneration, tongue regeneration, cell-based immunotherapy and more (Egusa et al., 2012).

Dental Pulp Stem Cells

As briefly mentioned above, dental pulp stem cells (DPSCs) were first isolated by Gronthos et al., in 2000. These cells were isolated from adult human dental pulp and were clonogenic and proliferative just like human BMSCs (Gronthos et al., 2000). Although DPSCs and BMSCs showed similarities in immunophenotype *in vitro*, they behave differently when transplanted into immunocompromised mice (Egusa et al., 2012). DPSCs generated a dentin-like structure lined with human odontoblast-like cells that surrounded a pulp-like interstitial tissue when transplanted into immunocompromised mice, while BMSCs formed lamellar bone with osteocytes and surface-lining osteoblasts, surrounding a fibrous vascular tissue with active hematopoiesis adipocytes (Gronthos et al., 2000). This showed that DPSCs have the ability to form a dentin/pulp-like complex.

DPSCs are commonly found within the "cell rich zone" of dental pulp tissues in adult teeth (D'aquino et al., 2018). DPSCs have the potential to differentiate into many different cell types *in vitro* such as osteoblasts, chondrocytes, adipocytes, myocytes, neurons, and hepatocytes (Karamzadeh & Eslaminjad, 2013). The current literatures indicate that DPSCs have the potential to differentiate into odontoblast, dentin, dental pulp, bone, cartilage, muscle, hair follicle, neuronal cells, cornea, melanocytes and endothelial cells (Kawashima, 2012). Studies have shown that DPSCs are often inactive, but react quickly when there's an injury. After

an injury, these DPSCs react immediately to repair the tissue by differentiating into osteoblasts, odontoblasts and chondrocytes to produce bone, dentin and cartilage tissues (Potdar & Jethmalani, 2015).

The identification of DPSCs depends on the presence or absence of specific cell surface markers. Unfortunately, there is no specific markers for DPSCs, because they are a heterogeneous population (Kawashima, 2012). Common surface markers for mesenchymal stem cells include STRO-1, CD29, CD44, CD73, CD90, CD105, CD146, CD166 and CD271 (Kawashima, 2012). In addition, the negative markers include HLA-DR and hematopoeitc antigens like CD34 and CD45 (Karamzadeh & Eslaminjad, 2013). DPSCs also contain stemness factors such as OCT-3/4 and NANOG (Kawashima, 2012)

These cells have shown many characteristics that make them an attractive target for research and future clinical applications. It has been reported that DPSCs can be cryopreserved and stored for long periods of time, and they can still keep their stem cell functions (D'aquino et al., 2018). Additionally, DPSCs have been heavily studied for tissue engineering to promote 3-D tissue formation (D'aquino et al., 2018).

From extracted human teeth, dental pulp tissues can be easily isolated and cells can be enzymatically released from these tissues (Karamzadeh & Eslaminjad, 2013). After plating on a culture dish, DPSCs have the capacity to attach to the

dish and form a single-colony cluster usually about two weeks after culture, and these colonies include fibroblast-like cells and they are subjected to secondary ex vivo culture to expand cell numbers (Liu et al., 2006).

The standard protocol for isolating DPSC from dental pulp tissue is very well established from Liu et al., 2006. Steps include the following: 1. Collect freshly extracted human teeth and place them into a sterile container with saline. Human third molars are the most ideal source due to the fact that it's one of the most commonly extracted teeth and its optimal amount for dental pulp tissue. 2. Scrape the tooth surface to remove any gingival or periodontal tissues. Disinfect the tooth surface with iodine and 70% ethanol, and rinse 5 times with 1x phosphate-buffered saline (PBS). 3. Cut around the cementum-enamel junction of the tooth with sterilized high-speed dental burr to separate the crown and root, then the pulp tissue will be exposed. 4. By using sterilized tweezers, pick up the pulp tissue. Mince the pulp tissues with a scalpel with moisture from culture medium like alpha-MEM. By using a mixture of collagenase type I (3mg/ml) and dispase (4mg/ml), incubate the tissues at 37C for about 45 minutes. 5. Add 5 volumes of culture medium, alpha-MEM, with 10% serum. Centrifuge the sample at 1200 rpm for 10minutes, then suspend the cell pellet in culture medium. By using a 70-um pore size strainer, pass the cells to obtain single-cell suspensions. 6. Plate the cells into culture dishes with culture medium containing alpha-MEM with 10% fetal calf serum (FCS), 100 uM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, penicillin

(100 U/ml), and streptomycin (100 mg/ml). Incubate the cells at 37C with 5% CO2.

7. Cells grow slowly in the beginning and it takes about 2 weeks to observe single colonies (Liu et al., 2006).

Pulp Immunology

The dental pulp is a very special tissue in our body because it's enclosed in a hard tissue just like our brain, and as long as this hard barrier is undamaged, the pulp will remain healthy and functional. However, when this barrier is damaged by carious lesions, dental trauma or operative dental procedures, microbial components can be introduced to the pulp (Bergenholtz, 1990).

Pulpal inflammation involves complicated processes and various types of immune cells. Before looking into the details of each process of pulpal inflammatory responses, we will go over the basic immune components of the human pulp.

The classic immunohistochemical analysis of the human dental pulp done by Pulver et al., in 1977 showed the difference between the immune components of normal and inflamed human pulp by looking at immunoglobulin-containing cells (ICC). According to their study, there was virtually no immune components that are necessary for immunoglobulin synthesis in the dental pulp. However, in the inflamed dental pulp tissues, various ICCs were detected including IgG, IgA and

IgE with IgG being the most prominent (Pulver et al., 1977). More recently in 2015, Gaudin et al., collected healthy human third molars from 42 young patients and quantified the immunocompetent cells in the human dental pulp by utilizing fluorescence-activated cell sorting, followed by flow cytometry and immunostaining analyses. According to their studies, leukocytes or CD45+ cells represented about 0.94%+/-0.65% from the whole dental pulp tissues following enzymatic digestion. Granulocytes/neutrophils CD16+CD14+ (50.01%) cells were found to be the most prominent subpopulation within the CD45+ cells. CD3+ T lymphocytes (32.58%), CD14+ monocytes (8.93%), HLA-DR Lin1- dendritic cells (4.51%), CD3-CD56+ natural killer cells (2.63%), CD19+ B lymphocytes (1.65%) were also detected in the healthy human pulp (Gaudin et al., 2015).

The detailed mechanism of pulpal inflammation involves an extensive number of immune cells both from innate and adaptive immune responses. Host immune response to microbial insult to the dentin/pulp complex is a double-edged sword, because if the issue is not resolved, it leads to collateral damage to the tissue, leading to necrosis (Cooper et al., 2014).

When there's a microbial invasion to the pulp, innate immune response is activated. When the innate immune response is not adequate to stop the insult, adaptive immune response is elicited to augment protective mechanisms by cellular and specific antibody responses (Hahn & Liewehr, 2007).

Generally, innate immune response does not require specific antigen recognition; rather, it uses molecular patterns that are commonly found in microbes (Abbas & Lichtman, 2003). Cells that express the major histocompatibility complex (MHC) will first recognize the microbes and react accordingly (Holland & Botero, 2014). There are type I and II MHC cells. Type I MHC cells are essentially all the nucleated cells in the body and they are considered "antigen-recognition" cells, and the type II MHC cells are considered antigen-presenting cells, because they take part of antigen and travel through the lymphatic system to present the antigen to T cells (Holland & Botoero, 2014). Usually, after recognition, these microbes are internalized and killed through the process called phagocytosis by immune cells like neutrophils and macrophages (Hahn & Liewehr, 2007).

In oral mucosa, typical components of innate immunity include epithelial barriers, circulating cells and proteins, and these components recognize microbes or the substances made from microbes to stop the microbial invasion (Hahn & Liewehr, 2007). However, the components of the innate immunity in the dentin/pulp complex are a bit different from those of the oral cavity. According to Hahn and Liewehr 2007, these components include dentinal fluid and intratubular immunoglobulins, odontoblasts, neuropeptides and neurogenic inflammation, innate immune cells including natural killer (NK) cells, T cells, and immature dendritic cells (DCs) and their cytokines and chemokines. According to the literatures, the transition from the innate to adaptive immune response occurs during the progressing stages of

reversible pulpitis (Hahn & Liewehr, 2007).

We will briefly review each component of innate immunity in the dentin/pulp complex. The first unique component of the innate immunity in the human pulp is the dentinal fluid and intratubular immunoglobulins (Hahn & Liewehr, 2007). When there's positive intrapulpal pressure due to initial immune response to carious lesions, the outward flow of dentinal fluid is increased which can decrease the amount of noxious stimuli coming through the dentinal tubules (Matthews & Vongsavan 1994). Additionally, dentinal fluid contains serum proteins and immunoglobulins (Knutsson et al., 1994). Especially, when there's deep carious lesion, the concentration of certain immunoglobulins including IgG1, IgA1, IgA2, and IgM increase in dentinal tubules (Hahn & Best, 2006).

Another unique component of innate immunity in the dentin/pulp complex is odontoblasts. These cells have cellular processes that extend into the dentinal tubules and they are usually the first cells to encounter the microbial invasion from carious lesions (Hahn & Liewehr, 2097). Odontoblasts express Toll Like Receptors (TLRs) which are known to be part of innate immunity (Copper et al., 2014). Odontoblasts also secrete chemokines like CCL2 and CXCL10 which can attract immature Dendritic cells and leukocytes (Hahn & Liewehr, 2007).

Neuropeptides and neurogenic inflammation also has an important role in innate immune response (Hahn & Liewehr, 2007). In human pulp, sensory neuropeptides like calcitonin gene-related peptide (CGRP), neurokinin A (NKA) and substance P

(SP) are often found (Awawdeh et al., 2002). When there is pulpal injury, the concentration of these neuropeptides increase which lead to neurogenic inflammation (Bowles et al., 2003). This leads to the outward flow of dentinal fluid and increase in interstitial tissue pressure (Hahn & Liewehr, 2007). Additionally, when the pulp is inflamed, the concentrations of SP and CGRP are increased which can lead to recruitment of immune cells including T cells and immature DCs (Awawdeh et al., 2002).

The most common innate immune cells in the pulp include neutrophils, mononuclear phagocytes, NK cells, T cells and immature DCs (Hahn & Liewehr, 2007). Neutrophils and macrophages act as phagocytes. NK cells also have an important role because they can promote DC maturation and produce IFN-gamma which activates macrophages and promotes type-I T cells responses in adaptive immune response (Raulet, 2004). The common cytokines secreted in the innate immune response include TNF-alpha, IL-1, IL-12, IL-18, IFN-gamma, IL-6 and IL-10 (Abbas & Lichtman, 2003). The main role of TNF-alpha and IL-1 include promotion of extravasation of phagocytes during inflammation (Hahn & Liewehr, 2007). IL-12, IL-18 and IFN-g are mainly associated with transitioning to the subsequent type 1 adaptive cellular immune response from the innate immune response (Trinchieri 1995). IL-10 acts as an inhibitor of macrophages and DCs to control innate immune responses and cell-mediated immunity (Hahn & Liewehr, 2007).

Lastly, chemokines, that are produced during the innate immunity, have the ability to recruit neutrophils, monocytes, immature DCs, and lymphocytes to the site of infections (Hahn & Liewehr, 2007).

Adaptive immune response is different from innate immunity in a sense that it is antigen specific, but its main purpose is to augment the innate immune response. The main players of adaptive immune response are antigen-specific lymphocytes and their products, such as cytokines, chemokines and antibodies (Hahn & Liewehr, 2007).

Chemokines play an important role; for example, during pulpal inflammation, certain chemokine ligands are upregulated, such as monocyte chemotactic protein-1 (CCL2/MCP-1), macrophage inflammatory protein 3-alpha (CCL20/MIP-3alpha), and IL-8, and these can recruit more immune cells like monocytes, memory T cells, NK cells, and immature DCs to amplify the immune response (Nakanishi et al., 2001).

Antigen presenting cells (APCs) are the main component of adaptive immunity, because they capture, process, and present the antigens to T cells (Holland & Botero, 2014). The common APCs in the inflamed pulp are DCs and macrophages in early pulpitis and B cells in irreversible pulpitis (Hahn & Liewehr, 2007).

Besides lymphocytes, macrophages are crucial in pulpitis. The main roles of macrophages include phagocytosis, antigen presentation and immunomodulation (Fujiwara & Kobayashi, 2005). Macrophages are often activated by IFN-gamma and once activated, they produce many different types of mediators involved with inflammation including IL-1, IL-2, IL-10, TNF-alpha, chemokines, prostaglandins and leukotrienes (Hahn & Liewehr, 2007).

T cells play an important role in adaptive immune response. Once T cells are activated by APCs, they differentiate into different types of effectors cells including CD4+ T helper cells, cytotoxic CD8+ T cells, Regulatory T cells (Treg) or memory cells (Hahn & Liewehr, 2007). CD4+ T helper cells can be classified into type I T helper cells (Th1) and type II T helper cells (Th2). Th1 cells and some non T helper cells including CD8, NK cells or Treg produce type -1 cytokines like IFN-gamma, IL-2, IL-12 and TNF-alpha (Hahn & Liewehr, 2007). All of these lead to stronger cellular immune responses and prevent the production of type-2 cytokines. Th2 cells and some non T helper cells can produce IL-4 and IL-10 to suppress macrophage activation and activate B cell proliferation and differentiation (Lebre et al., 2005). According to the current literatures, concentration of both type-1 and -2 cytokines is elevated during pulpal inflammation (Hahn & Liewehr, 2007).

Cytotoxic CD8+ effector T cells can also be classified into different subtypes including T cytotoxic-1 (Tc1), Tc2 and CD8+ Treg (Noble et al., 2006). Tc1 cells

produce IFN-gamma while Tc2 cells produce IL-5 (Mosmann et al.,1997). CD8 Treg cells act to suppress immune responses by producing IL-10 (McGuirk & Mills, 2002). However, the distribution of these cytotoxic CD8+ effector T cells in reversible pulpitis are not clearly known yet (Hahn & Liewehr, 2007).

Even during early pulpitis, B cells appear to participate in protective mechanism, and their involvement increases significantly as the carious lesions progress toward the pulp (Hahn & Liewehr, 2007). The main functions of B cells include producing antibodies and cytokines like IL-4, IL-10 and IFN-gamma, and functioning as APCs (Bayry et al., 2005). According to immunohistochemical analysis from Pulver et al., 1977, IgG tends to be the most prominent antibodies in inflamed pulp tissues, and IgA and IgE are also commonly found during pulpal inflammation. Although the presence of these antibodies in inflamed pulp has been shown, their protective mechanisms have not been proven yet (Hahn & Liewehr, 2007). Additionally, some suggest that even if they are protective by providing bacterial clearance by macrophages or continuous B-cell activation, it may still be too late for cases of irreversible pulpitis (Hahn & Liewehr, 2007).

This may seem like an extensive review on immunology of pulpal inflammation. However, there are still many unanswered mechanisms even with many attempts being made to further the understanding of pulp immunology. The better

understanding of pulp immunology can lead us to clinically keep the pulp healthy with immunological pulp therapies in the future.

Role of Stem Cells in Pulpitis

Mesenchymal stem cells, especially the ones from bone marrow, are thoroughly examined for their surface markers that are involved in the immune response. The surface markers that are identified on MSCs include various cytokines like IL-1, IL-4, IL-6, IFN-γ, TNF-α, growth factors, platelet-derived growth factor, TGF-β, epidermal growth factor (EGF), insulin-like growth factor (IGF), bone morphogenetic proteins (BMPs), chemokines, various adhesion molecules, and surface molecules for cell to cell interactions (Deans & Moseley, 2000 and Docheva, 2008). DPSCs have not been this extensively studied for their surface markers but many studies have shown them to have similar pattern (Leprince et al., 2011). In addition, the current literature is indicating that there are many cytokines and growth factors that can influence the activity of DPSCs including the recruitment, proliferation and differentiation. Studies have shown that TGF- β1, VEGF, and FGF-2 can act as a positive regulator for chemokines for DPSC recruitment. Numerous factors including platelet-derived growth factor (PDGF-BB), VEGF, GFG-2, insulin-like growth factor (IGF-1) and TGF- β1 have been shown to stimulate the DPSC proliferation (Leprince et al., 2011). Additionally, TGF- β1,

TGF- β3, bone morphogenetic progein-7 (BMP7), BMP2, VEGF, IFN-g-2b, TNF-a, and IL-1 have been identified as factors that can stimulate the differentiation of dental stem cells (Leprince et al., 2011). All these factors are identified as positive regulators of dental stem cells which can eventually lead to healing of the pulp.

However, there are certain factors that can reduce the healing potential of dental stem cells. When there is microbial insult to the pulp, inflammatory responses lead to interruption of DPSC differentiation and pulp homeostasis (Hozhabri et al. 2014). During the pulpal inflammation, there is an increase in proinflammatory cytokines like tumor necrosis factor alpha (TNFa) and IL-1β which can decrease the dentinogenic potential of DPSC and IFN-g which can prevent the proliferation of dental stem cells (Alongi et al. 2010). These lead to decreased collagen synthesis and odontogenesis, degradation of collagen, and interruption of pulp tissue regeneration (Hozhabri et al. 2014). With an increase in concentrations of TNFa and IL-1β, nuclear factor-kB (NF-kB) becomes activated, which leads to higher inflammatory response (Bonizzi & Karin, 2004). A study done by Hozhabri et al. 2014 showed that downregulation of the transcription factor NF-kB in the presence of TNFa and IL-1β lead to enhancement of odontoblastic differentiation and collagen matrix formation of DPSCs.

In addition to responding to the inflammatory signals during pulpal inflammation, many current literatures have shown that DPSCs have anti-inflammatory and

immunomodulatory properties. DPSCs have been shown to inhibit activated T-cell response and suppress the proliferation of peripheral blood mononuclear cell proliferation (Leprince et al. 2011). The immunomodulatory functions of DPSCs are currently being thoroughly studied for their clinical application. For example, a recent study by Omi et al. 2016 showed that transplantation of DPSC suppressed inflammation in sciatic nerves for diabetic polyneuropathy in rats. Rats with diabetes typically show extremely reduced sciatic nerve conduction velocities and sciatic nerve blood flow; however, when DPSCs were transplanted into the unilateral hindlimb skeletal muscles for these rats, there was a significant increase in sciatic nerve conduction velocities and sciatic nerve blood flow (Omi et al., 2016). In addition, DPSC transplantation lead to significantly lower concentration of monocytes/macrophages and decreased tumor necrosis factor-alpha mRNA expression, and increased expression of IL-10 (Omi et al. 2016). In addition, currently, MSCs are being used for the clinical application for the treatment of graft-versus-host-disease (GVHD) for their anti-inflammatory capacities (Leprince et al., 2011). A study conducted by Yazid et al. showed that DPSCs isolated from inflamed pulp tissues showed significantly decreased immunomodulatory functions compared with DPSCs isolated from healthy pulp tissues. Healthy DPSCs were able to suppress various inflammatory mediators including TNFa, TNFB, and IL-2, and a significantly higher level of IL-10 was also detected in healthy DPSCs compared with DPSCs from inflamed tissues (Yazid et al. 2013).   The

immunosuppressive properties of dental stem cells can definitely influence the future treatment of pathogenesis of the pulp.

IL-6 in Immunology

Interleukin 6 (IL-6) is a pleiotropic soluble mediator that's involved with various steps throughout inflammatory processes, because it induces synthesis of acute phase proteins like CRP, fibrinogen, serum amyloid A, and it stimulates antibody production and effector T cell development (Tanaka et al. 2014). IL-6 is generally not produced in the steady state with no inflammation (Snick, 1990). IL-6 can coordinate the activity of the innate and adaptive immune responses and regulate B and T cell responses (Garbers et al., 2018). During early inflammation, the release of IL-6 is promoted by proinflammatory cytokines like IL-1, TNFa, IFN-g, platelet-derived growth factor, and IL-3 (Barnes et al., 2011). However, in general, IL-6 can be secreted by many different cell types including monocytes and macrophages, fibroblasts, keratinocytes, astrocytes, endothelial cells, activated B cells and T cells (Garbers et al., 2018). IL-6 is produced during the acute phase of inflammation and it alters the nature of leucokyte infiltrate from neutrophils to monocyte/macrophages; thus controlling the transition from acute to chronic inflammation (Gabay, 2006). The expression of endothelial leukocyte adhesion

molecules including VCAM-1 and ICAM-1 increases with IL6 production which leads to more recruitment of leukcoytes (Barnes et al., 2011).

IL-6 binds to IL-6 Receptor (IL-6R) and they bind to glycoprotein 130 (gp130) which initiates intracellular signaling (Schaper & Rose-John, 2015). IL-6R is expressed only a few cell types including hepatocytes, epithelial cells and some leukocytes (Scheller et al., 2011). IL-6R also exists in a soluble form as sIL-6R, and some cells can be stimulated by the complex of sIL-6R-IL-6, and this process has been called IL-6 trans-signaling (Riethmueller et al., 2017, and Rose-John & Heinrich, 1994).

IL-6 has three very important biological roles which include macrophage differentiation, bone metabolism and acute phase response (Garber et al., 2018). IL-6 classic signaling induces the increased expression of IL-4 receptor which leads to a gene expression toward M2 differentiation (Braune et al., 2017). The M2 state of macrophage is associated with anti-inflammatory and is very important for regenerative functions (Garber et al., 2018). The classic signaling of IL-6 can also activate osteoblasts by increasing the expression of RANKL, and osteoblasts can secrete IL-6 which can lead to osteoclastic differentiation through trans-signaling (Garber et al., 2018). Lastly, IL-6 expression leads to the production of acute phase proteins. During inflammation, IL-6 binds to IL-6R on hepatocytes in the liver which leads to activation of the Janus kinase (JAK) which leads to signal transducer and activator of transcription (STAT) signaling and acute phase proteins including C-

reactive protein, serum amyloid A2 protein, haptoglobin and fibrinogen (Garber et al., 2018).

According to many studies, IL-6 is basically involved in almost every aspect of innate immune response, ranging from accumulating neutrophils at the site of infection to hematopoiesis (Hunter & Jones, 2017). IL-6 is known to regulate chemokines that activate neutrophils and is also involved with neutrophil apoptosis (Hunter & Jones, 2017). IL-6 also has protective roles during innate immune response by preventing excessive tissue damage through enhancing the transition from the recruitment of neutrophils to that of monocytes/macrophages (Jones, 2005). In addition, IL-6 inhibits the activation of the transcription factor NF-kB, and enhances the expression of the IL-1 receptor antagonist; thus it has an important role in healing and resolution of inflammation (Hunter & Jones, 2017)

During the acute phase of inflammation, IL-6 stimulates the production of most acute phase proteins (Gabay, 2006). During the initial phases of acute inflammation, transcription factor NF-kB gets activated and it promotes the transcription of the mRNA of inflammatory cytokines like tumor necrosis factor-a, IL-1B and IL-6 (Tanaka et al., 2014). However, IL-6 plays another important role by developing specific cellular and humoral immune responses like end-stage B cell differentiation, immunoglobulin secretion and T cell activation (Gabay, 2006). Additionally, IL-6 may play a role in chronic inflammation by rescuing T cells from apoptosis; thus enhancing a chronic inflammatory cell infiltrate (Barnes et al.,

2011). IL-6 also has pivotal roles in adaptive immunity and one of which includes the induction of the maturation of B cells into plasma cells (Hunter & Jones, 2017). This relationship has been heavily studied in many studies and in Castleman's disease, where there's an increased concentration of IL-6 followed by B cell hyperplasia (Yoshizaki et al., 1989). IL-6 interacts with CD4+ T cells by inducing the transcriptional repressor Bcl-6 which enhances these cells to be committed to the follicular helper T cells which are the cells that promote B cell proliferation and immunoglobulin class switching (Hunter & Jones, 2017).

Current literatures have shown that IL-6 has various roles in acute and chronic phases of inflammation, autoimmunity, endothelial cell dysfunction and fibrogenesis, and IL-6 has the two completely opposite roles during inflammation (Gabay, 2006). During acute phase of inflammation, it has anti-inflammatory functions by suppressing the concentration of proinflammatory cytokines like IL-1, while it has a detrimental effect during chronic inflammation because it promotes accumulation of mononuclear cells at the site of injury (Gabay, 2006).

In addition, IL-6 is deeply associated with proliferation, survival and commitment of T cells and their cytokines (Hunter & Jones, 2017). IL-6 is involved in development of commitment to specific effector subsets including Th17, Th22 and Tfh subsets (Hunter & Jones, 2017). IL-6 binds to IL-6R on naïve T cells which leads to promotion of retinoic acid receptor-related orphan nuclear receptor-gamma (ROR-

gamma) expression which is the main transcription factor for Th17 cells (Garbers et al., 2018). IL-6 also controls the differentiation of naïve T cells to the T follicular helper cells (Garbers et al., 2018).   Additionally, IL-6 can also determine the T cell recruitment by expressing certain chemokine receptors or IL-6 trans-signaling on stromal tissues that controls inflammatory chemokines. IL-6 also regulates the proliferation and survival of Th1 and Th2 cells (Hunter & Jones, 2017).

IL-6 is very deeply involved with B cells because not only it promotes B cell differentiation and proliferation and induction of immunoglobulins by plasma, but it also becomes expressed by B cells (Garbers et al., 2018). IL-6 induces STAT3 activation which induces BLIMP1 expression which is important for the terminal differentiation of plasma cells (Shapiro-Shelef, 2005). IL-6 also leads to expression of IL-7R which leads to induction of recombinase genes RAG1 and RAG2, and these genes are required for the B cell receptor editing (Hillion, et al., 2007). Additionally, IL-6 has immunomodulatory function by inducing IL-10 producing B regulatory cells (Rosser et al., 2014).

Since IL-6 also has an important role as a transitioning molecule from innate to adaptive immune response, the impaired IL-6 activity leads to compromised immune response to infection (Hunter & Jones, 2015)

IL-6 has been shown to be involved with its elevated concentration in various inflammatory diseases including rheumatoid arthritis, juvenile idiopathic arthritis, systemic lupus erythematosus, psoriasis, ankylosing spondylitis, and Crohn's disease (Gabay, 2006)

When IL-6 was first identified, it was known for its abilities to regulate the acute-phase immune response, activate T cells, and differentiate B cells (Hunter and Jones, 2015). Recently, IL-6 is being heavily studied for its hormone-like traits which can be associated with lipid metabolism, vascular disease, mitochondrial activities, insulin resistance, neuropsychological behavior and neuroendocrine system (Hunter and Jones, 2015).

IL-6 in Pulpitis

The presence of IL-6 in human dental pulp and periapical lesions was detected by Barkhordar et al. in 1999. According to their study, the concentration of IL-6 from the periapical samples from lesions and inflamed pulpal tissues was significantly higher than the control group by using ELISA. Many studies following Barkhordar et al. in 1999 showed very similar results where there is a significantly higher concentration of IL-6 in inflamed pulp tissues when compared with that of in healthy pulp tissues (Sabir & Sumidarti, 2016 and Park, 2002). As we have reviewed so far, IL-6 is one of the key mediators that are involved in the inflammatory responses. The increased concentrations of IL-6 have been detected in various oral diseases, and the prolonged excessive IL-6 production can lead to chronic inflammation, followed by tissue damage and loss of periodontal ligament and alveolar bone (Nibali et al., 2011).

Although IL-6 is being thoroughly studied currently, there is no extensive review on the exact function of IL-6 in the human pulp tissues. Many attempts are being made to investigate the role of IL-6 in pulpal inflammation in relation to stem cells.

IL-6 and Osteogenesis

IL-6 has been found to have both pro- and anti-inflammatory functions; therefore, many studies have been performed to investigate their role in inflammatory bone destruction following pulpal inflammation/infection. IL-6 has the ability to stimulate the formation of osteoclast precursors which leads to increased concentration of osteoclast, then to bone resorption (Kurihara et al., 1990). However, other studies have shown that IL-6 also has the opposite role by upregulating tissue inhibitor of metalloproteianses-1 and inducing acute-phase proteins that have anti-inflammatory properties (Akira et al., 1993). A study done by Balto et al., in 2001 conducted experiments to investigate the net effect of IL-6 in inflammatory bone destruction by using IL-6-/- (knockout) mice. According to the analysis on the samples from the periapical lesions caused by pulpal infection, bone destruction was significantly increased in IL-6 knockout mice, meaning IL-6 has anti-inflammatory functions and can modulate bone destruction in vivo (Balto et al., 2001).

Studies have shown that the levels of IL-6 and IL-6R are increased during osteogenic differentiation in bone marrow mesenchymal stem cells (BM-MSCs)

(Xie et al., 2018). The IL-6 and IL-6R complex activate the downstream STAT3 signaling pathway which leads to osteogenic differentiation in BM-MSCs (Chalaris et al., 2011). The neutralizing antibodies against IL-6 or IL-6R have shown to decrease the osteogenic differentiation of BM-MSCs to confirm their role (Xiet et al., 2018).

There are many other studies that indicate the role of IL-6 in promoting osteogenic differentiation through many different pathways. For example, a study done by Huang et al. in 2018 showed that IL-6 and soluble IL-6R can increase BMP-2 induced osteogenic and adipogenic differentiation of human BMSCs through increasing BMPR1A-mediated BMP/Smad and p38 MAPK pathways (Huang et al., 2018).

IL-6 and Neurogenic Differentiation

Neural stem cells (NSCs) are known to have a very high neurogenic differential potential which can replace damaged cells, and regenerate and re-myelinate host axons through paracrine effects (Jung et al., 2016). Similar to NSCs, DPSCs also have the capacity to differentiate into neural-like cells; therefore, they are a good candidate for neural regeneration (Ibarretxe et al., 2012). For an example, a study done by Jung et al. in 2016 showed that when DPSCs were transplanted into the

brain and injured spinal cord in rats, they were able to survive and express neuronal markers including nestin and Sox2. Therefore, DPSCs, especially with their cranial neural crest lineage, are an attractive source for neural regeneration and repair in nerve-related diseases in addition to their immunomodulatory factors (Luo et al., 2018).

IL-6 also plays an important role in neurogenic inflammation. IL-6 can induce the generation of neurogenic inflammation and pain generation (Ebbinghaus et al., 2015). However, there is no studies conducted on its role on neurogenic differentiation of mesenchymal stem cells.

MANUSCRIPT

<u>Introduction</u>

Interleukin 6 (IL-6) is a pleiotropic soluble mediator that is involved in various steps throughout inflammatory processes (Tanaka et al. 2014). IL-6 can coordinate the activity of the innate and adaptive immune responses and regulate B and T cell responses (Garbers et al., 2018). IL-6 is produced during the acute phase of inflammation and it alters the nature of leukocyte infiltrate from neutrophils to monocyte/macrophages; thus controlling the transition from acute to chronic inflammation (Gabay, 2006). Although IL-6 has been thoroughly investigated for its role and relationships with various types of immune cells, there is not yet an extensive review on its relationship with dental pulp stem cells (DPSCs).

DPSCs were first isolated from adult human dental pulp, and their clonogenic and proliferative properties were similar to human bone marrow mesenchymal stem cells (BMSCs) (Gronthos et al., 2000). DPSCs have the potential to differentiate into many different cell types *in vitro* such as osteoblasts, chondrocytes, adipocytes, myocytes, neurons, and hepatocytes (Karamzadeh & Eslaminjad, 2013). Furthermore, recent studies have shown DPSCs have immunomodulatory effect on immune cells (Lee et al., 2016 & Zhao et al., 2012). In addition, a subset of MSCs also has been isolated from inflamed human pulps (I-DPSCs) and shown the full

capability of proliferation and multi-potent differentiation as compared to those from healthy pulps (H-DPSCs) (Alongi et al., 2010 & Pereira et al., 2012).

Studies have shown that the level of IL-6 is increased during osteogenic differentiation in BMSCs (Xie et al., 2018). IL-6 can activate the downstream STAT3 signaling pathway which leads to osteogenic differentiation of BMSCs (Chalaris et al., 2011). The neutralizing antibodies against IL-6 have shown to decrease the osteogenic differentiation of BM-MSCs to confirm their role (Xie et al., 2018). IL-6 also plays an important role in neurogenic inflammation. IL-6 can induce the generation of neurogenic inflammation and pain (Ebbinghaus et al., 2015). However, there is no extensive study conducted on its role on neurogenic differentiation of MSCs.

Including the study done by Barkhordar et al., in 1999, many studies have shown that there is a significantly higher concentration of IL-6 in inflamed pulp tissues when compared with that of in healthy pulp tissues (Sabir & Sumidarti, 2016, Park, 2002). However, there are no studies to demonstrate the interaction of IL-6 with DPSCs in healthy and inflamed pulp tissues and the effect of IL-6 on the differentiation potential of DPSCs.

Therefore, I hypothesized that IL-6 can promote osteogenesis while inhibiting neurogenesis from DPSCs. The aims of this study were to investigate the baseline differences between the H-DPSCs and I-DPSCs, and determine how IL-6 can affect the osteogenic and neurogenic differentiation potentials of DPSCs.

Materials and Methods

Subjects and Primary Culturing

Healthy impacted third molars from patients with 17-27 years of age, and severely decayed molars with no restorability and spontaneous, sharp shooting pain from patients with 24-56 years of age were collected at the Oral Surgery Clinic at the University of Pennsylvania School of Dental Medicine after informed consent was obtained following the protocol approved by the Institutional Review Board of the University of Pennsylvania.

A total of 20 patients with ages, that range from 17 to 56 years of age, with no systemic diseases were seen at the Oral Surgery Clinic at the Penn Dental Medicine, and a total of 28 molars were extracted from these patients. Eight healthy impacted third molars from four patients were used for four cultures of primary culturing of H-DPSCs (two teeth from each person per culture); two healthy impacted third molars for H & E staining; four healthy impacted third molars for Toluidine Blue staining. Eight severely decayed molars from four patients were used for four cultures of primary culturing of I-DPSCs (two teeth from each person per culture); two severely decayed molars for H & E staining; four severely decayed molars for Toluidine Blue staining.

Tooth surfaces were scraped to remove gingival tissues and they were disinfected with 70% ethanol and rinsed with 1x phosphate-buffered saline (PBS). By using a sterilized dental bur, each tooth was cut at the cementum-enamel junction, and the pulp tissue was gently taken out with sterilized tweezers. Only the coronal portion

of pulp tissue from severely decayed teeth was used for I-DPSC culturing to ensure only the inflamed areas are being affected. The pulp tissues were minced with a scalpel and were digested in a solution of 3mg/ml collagenase type I (Worthington Biochem, Freehold, NJ) and 4mg/ml dispase (Boehringer Mannheim) for 1 hour at 37 °C. The single-cell suspensions were obtained by using a 70-μm pore size strainer (Falcon) to pass the cells. Cells were plated into culture dishes with culture medium containing α-MEM (Invitrogen) with 10% fetal bovine serum (FBS) (Gemini), 100 μM L-ascorbic acid 2-phopshate (Wako Pure Chemicals, Osaka) / 2mM L-glutamine/ 100 units/ml penicillin, 100 μg/ml streptomycin (Biofluids, Rockville, MD), and they were incubated at 37°C in 5% CO_2.

Colony-Forming Unit-Fibroblasts (CFU-F) Assay and Proliferation Assay

After 14 days of culturing, cells were fixed with 4% formalin and then stained with 0.1% toluidine blue for the assessment of colony-forming efficiency. A colony was defined as aggregates that are consisted of more than 50 cells. The proliferation rate of subconfluent cultures of healthy and inflamed DPSCs was investigated with bromodeoxyuridine (BrdU) incorporation for 10 hours by using a BrdU Staining Kit (Invitrogen) following the manufacturer's instructions. By using ten image areas per subject, BrdU-positive and total cell numbers were counted. The BrdU-positive cells were quantified as a percentage of the total number of cells.

In order to investigate the healthy and inflamed pulp tissues, the samples were fixed with 4% paraformaldehyde (Sigma-Aldrich), decalcified with 5% EDTA, then

embedded in paraffin. These paraffin sections (6μM) were then stained with hematoxylin and eosin (H&E). The ImageJ software was used to analyze the stained pulp tissues.

Flow Cytometric Analysis

In order to analyze the baseline stem cell markers for DPSCs, healthy and inflamed DPSCs were incubated with various antibodies, including CD44, CD73, CD90, CD105, CD34, and CD45. CD44, CD73, CD90, and CD105 were used as a positive marker while CD34 and CD45 were used as a negative marker for DPSCs. The FACS^{Calibur} with CellQuest software was used for the analysis.

Immunofluorescence Staining

In order to perform immunofluorescence staining, frozen sections were prepared. The slides were stained with CD3, CD105 and IL-6, followed by secondary antibody staining.

For the assessment of neurogenic differentiation of healthy and inflamed DPSCs, the samples were cultured on 4-well chamber slides (Nunc, Rochester, NY, USA) (2 x 10^3/well). They were incubated with neurogenic markers including MAP2 and NeuN before and after the treatment with IL-6, followed by neurogenic differentiation. The secondary antibody staining was done for all the slides, then they were mounted with Vectashield mounting medium containing DAPI.

Enzyme-linked Immunosorbent (ELISA) Assay

The levels of IL-6 from the culture supernatant from healthy and inflamed DPSCs were measured with the Human IL-6 ELISA MAX^{TM} Deluxe kit purchased from Biolegend, following the manufacturer's instructions.

IL-6 Treatment to Healthy and Inflamed DPSCs

In order to examine the role of IL-6 in osteogenic and neurogenic potentials of DPSCs from the healthy and inflamed pulp tissues, IL-6 was added. Since the level of IL-6 in the culture supernatant from healthy DPSCs was lower than that of inflamed DPSCs, IL-6 molecules (R&D Systems**)** were added to the culture medium. In contrast, neutralizing IL-6 antibodies (Biolegend) (10µL per 1mL medium) were added to the culture medium of inflamed DPSCs to reduce the effects of IL-6.

Osteogenic Differentiation Assay

For the assessment on the difference between the healthy and inflamed DPSCs and on the role of IL-6 on DPSCs, four different sets of DPSCs were used. Healthy DPSCs, Inflamed DPSCs, healthy DPSCs treated with additive IL-6 and inflamed DPSCs with neutralizing IL-6 were loaded at 1 x 10^6 per well into a 6-well plate. Osteogenic differentiation was induced when cells reached 100% confluence. The osteogenic medium contained 2mM ß-glycerophosphate (Sigma), 100 µM L-ascorbic acid 2-phosphate (Sigma) and 10 nM dexamethasone (Sigma). In order to

assess the osteogenic markers including OCN and Runx2, total protein was isolated from cultured DPSCs after 14 days of osteogenic induction, and then the Western blot analysis was performed. After 2 weeks of osteogenic induction, the cells were stained with 1% alizarin red-S (Sigma) and the ImageJ software was used for the analysis.

Neurogenic Differentiation Assay

Similar to the osteogenic differentiation assay, four different sets of DPSCs were used including healthy DPSCs, inflamed DPSCs, healthy DPSCs treated with additive IL-6 and inflamed DPSCs with neutralizing IL-6. These cells were then loaded at 1×10^6 per well into a 6-well plate. Neurogenic differentiation was induced when cells reached 100% confluence and the neurogenic medium contained DMEM (Invitrogen) with 10% FBS, 1 x N-2 supplement (Life Technologies), 100 U/mL penicillin, 100 µg/mL streptomycin, 10 ng/mL fibroblast growth factor 2, and 10 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN, USA). After 14 days of neurogenic induction, the neurogenic markers including NeuN and MAP2 were assessed through the Western blot analysis after total protein was isolated from the cultured DPSCs.

Western Blot Analysis

The M-PER mammalian protein extraction reagent (Thermo, Rockford, IL, USA) was used to extract total protein from the samples. The concentrations of proteins

were measured by using protein concentration assay (Bio-Rad Laboratories). 20 µg of proteins were applied and separated by SDS PAGE gel and they were transferred to 0.2 µm nitrocellulose membranes (Millipore). The membranes were then blocked with 5% non-fat dry milk and 0.1% tween-20 for 1 hour. The membranes were incubated overnight with the primary antibodies diluted in blocking solution following manufacturer's instructions. Antibodies to human Runx2, ALP, NeuN, and MAP2 were purchased from Santa Cruz Biotechnology, Inc., and antibodies to mouse ß-actin were purchased from Abcam. For the secondary binding, the membranes were incubated for 1 hour in HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). The SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and BioMax film (Kodak) were used to detect the immunoreactive proteins. With the ImageJ software, the bands were analyzed with ß-actin as a control.

Statistical Analysis

The independent unpaired two-tailed Student's *t*-tests were used when comparing between two groups. P-values less than 0.05 were considered statistically significant.

Results

Baseline characteristics of Healthy and Inflamed Pulp Tissues

Although there are many studies on analyzing the differences between the DPSCs from healthy pulp tissues (H-DPSCs) and the ones from inflamed pulp tissues (I-DPSCs), functions of these DPSCs vary slightly from each studies. Therefore, we used our own samples to investigate the baseline characteristics of H-DPSCs and I-DPSCs first.

As mentioned above, the results come from four different sets of H-DPSCs and I-DPSCs for culturing, two different sets of pulp tissues for healthy and inflamed pulp tissues for H&E staining, and four different sets of H-DPSCs and I-DPSCs for Toluidine Blue staining for assessment of proliferation rate.

Figure 1. H & E staining of healthy and inflamed pulp tissues
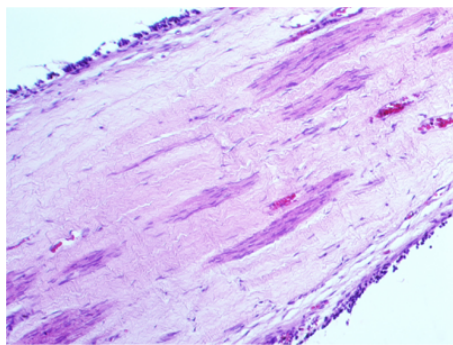
Figure 1a. Healthy pulp tissue   Figure 1b. Inflamed pulp tissue
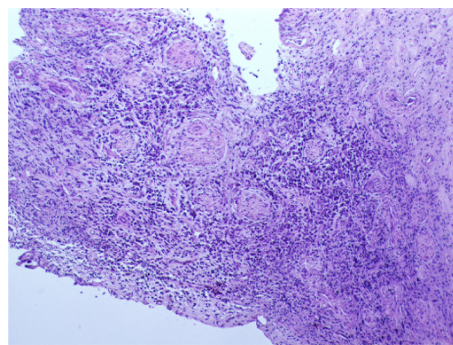
The H & E staining of healthy and inflamed pulp tissues serve two purposes. Firstly, it is to confirm that diagnosis on healthy and inflamed teeth are correctly made by examining the infiltrates of the pulp tissues. Secondly, it is used for general characterization of healthy and inflamed pulp tissues. As seen in figure 1, normal

pulp tissue is made up with blood vessels, connective tissues, red blood cells, small number of lymphatic cells and nervous elements**,** while inflamed pulp tissue is filled with immune cells, indicative of severe inflammation.

**Figure 2.** Immunofluorescence staining of healthy and inflamed pulp tissues

Healthy DPSCs　　　　　　　　　　　　　**Inflamed DPSCs**

2a.　H-DPSC with CD105 (g)　　　　　　2b. I-DPSC with CD105(g)

Healthy DPSCs　　　　　　　　　　　　　**Inflamed DPSCs**

2c. H DPSC with CD3 (g)　　　　　　2d. I-DPSC with CD3 (g)

Healthy DPSCs　　　　　　　　　　　　　**Inflamed DPSCs**

2e. H-DPSC with IL-6 (g)　　　　　　2f. I-DPSC with IL-6 (g)
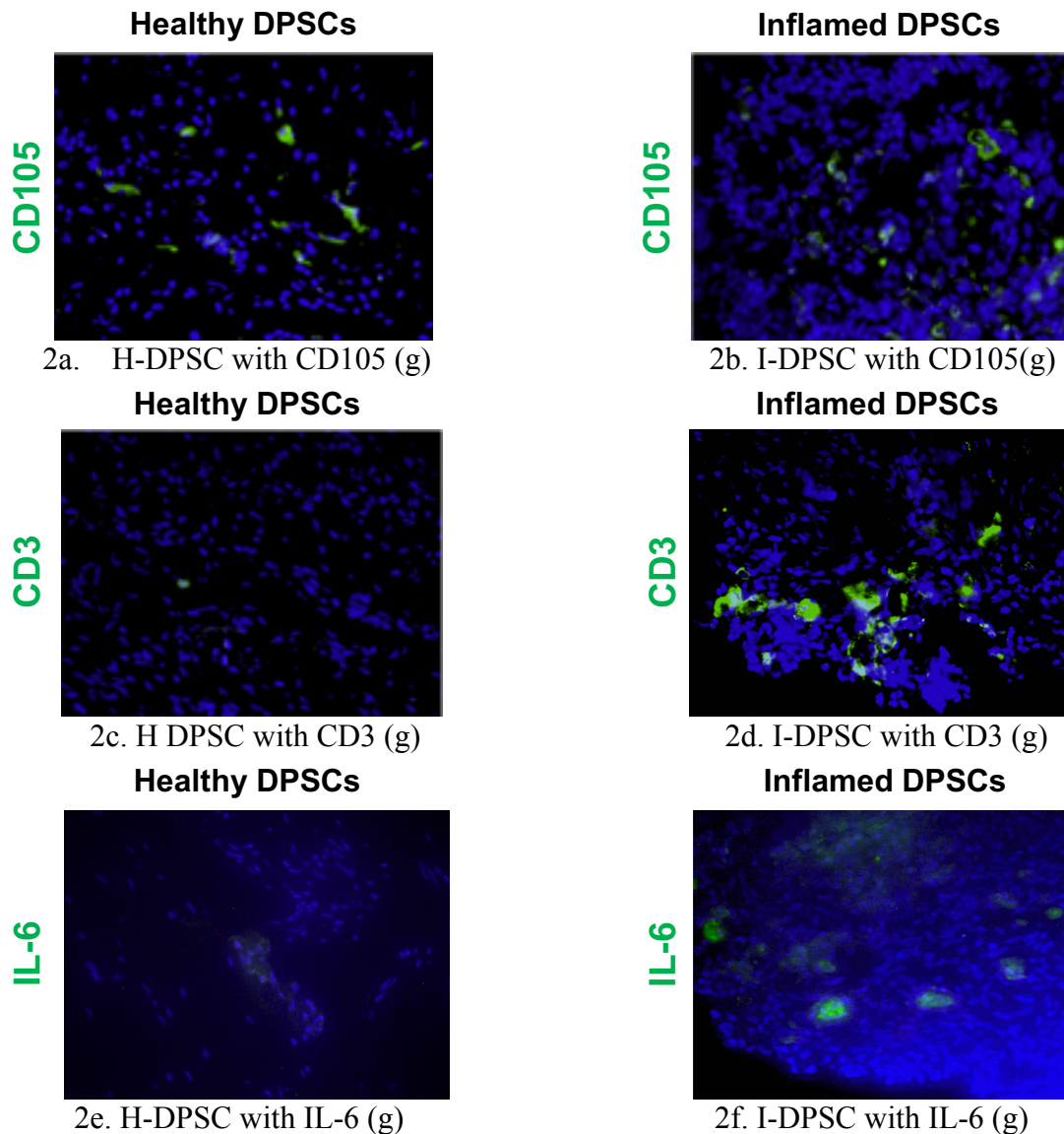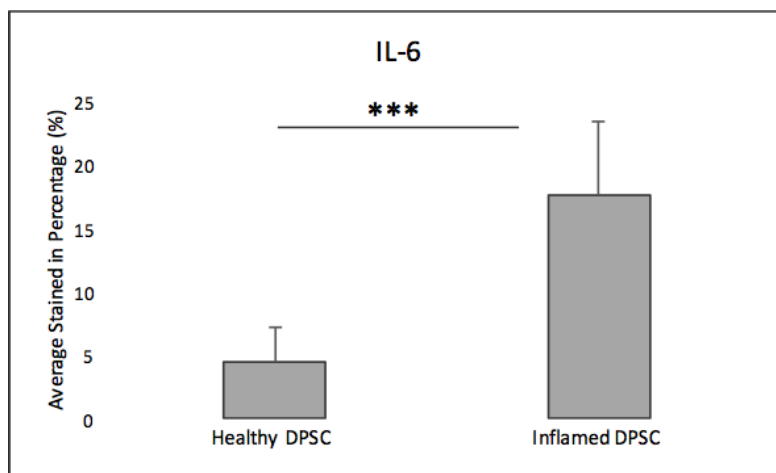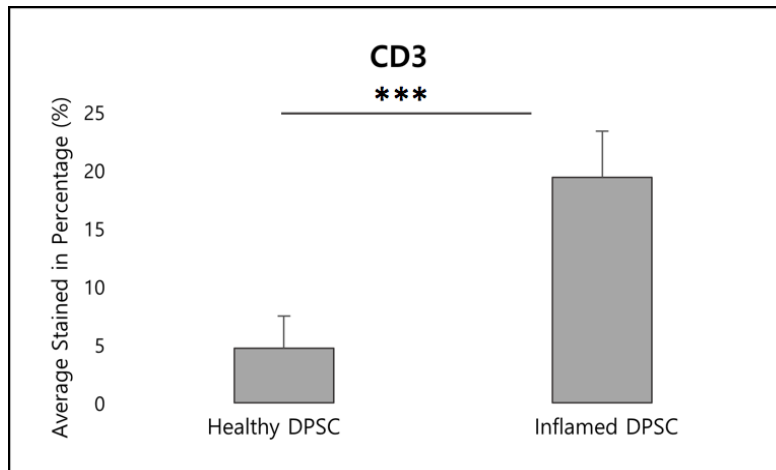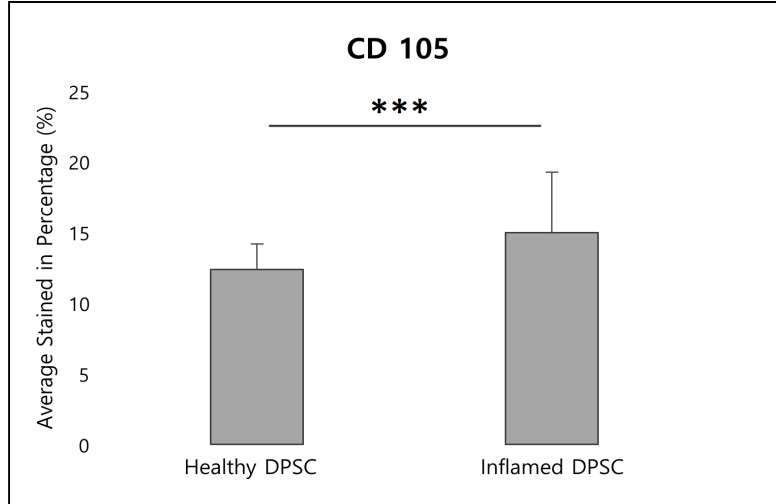
Immunofluorescence staining was performed to assess the difference between the baseline characteristics of healthy and inflamed pulp tissues from frozen sections. CD105 is a common surface marker for DPSCs, while CD3 and IL-6 are common indicators of inflammation. As seen by the quantification analysis, while there was no statistical difference between healthy (12.4% 41± 1.8) and inflamed (15% ± 4.2) pulp tissues for CD 105, there were statistical differences between healthy and inflamed pulp tissues for CD3 and IL-6 (p=0.000028 and p=00032, respectively). The positively stained cells for CD3 were 4.69 % ± 2.7% and 19.3% ± 4% for healthy and inflamed pulp tissues respectively, and for IL-6, they were 4.41 % ± 2.87% and 17.7% ± 5.79 % for healthy and inflamed pulp tissues respectively.

Baseline characteristics of Healthy and Inflamed DPSCs

Figure 3. CFU-F Assay

Healthy DPSCs   **Inflamed DPSCs**

Figure 3a. Healthy DPSC   Figure 3b. Inflamed DPSC
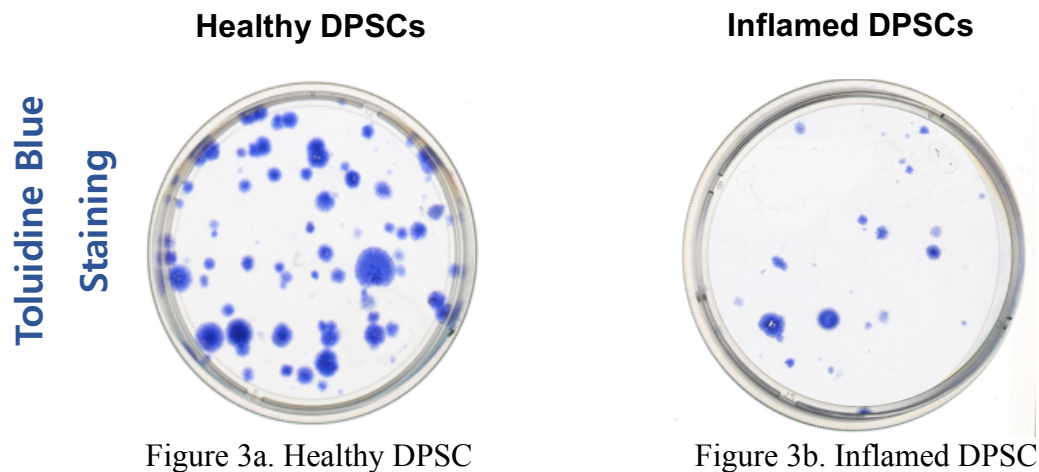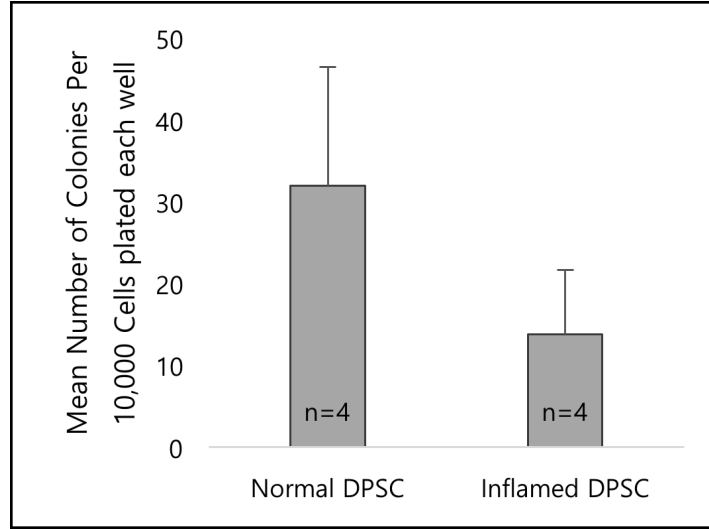
Figure 3c. Mean Number of Colonies Per 10,000 Cells Plated per well

In order to assess the proliferation capacities of H-DPSCs and I-DPSCs, the mean number of colonies per 10,000 cells plated per well for primary culturing was measured. The mean number of colonies for H-DPSCs was 32 with standard deviation of 14.45, while it was 13.75 with standard deviation of 7.89 for I-DPSCs with p value of 0.068. Due to the small sample size, even though there is a substantial difference between the two groups, there was no statistical significance. Each group had 4 samples, and H-DPSCs formed more colonies when compared to I-DPSCs in general as seen in figure 3.

Figure 4. Proliferation assay

Healthy DPSCs   **Inflamed DPSCs**

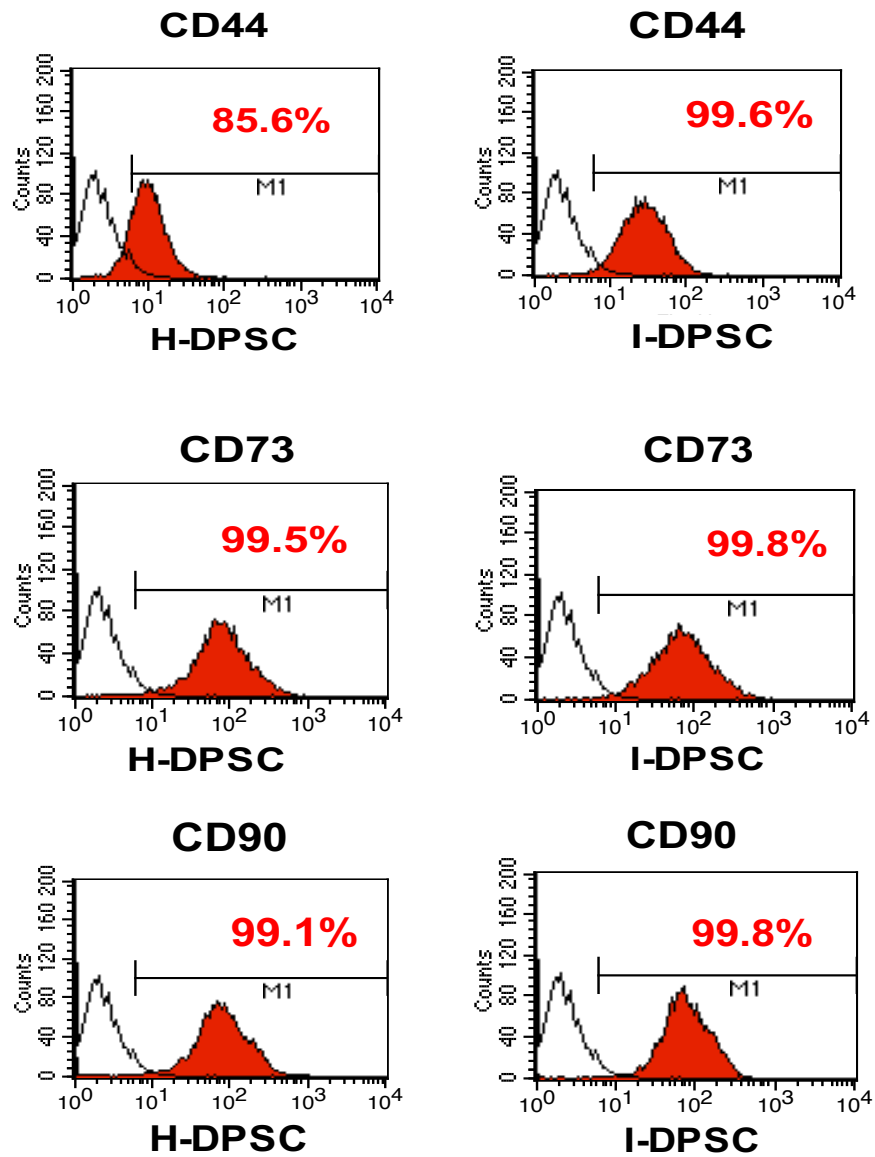Figure 4a. H-DPSC   Figure 4b. I-DPSC
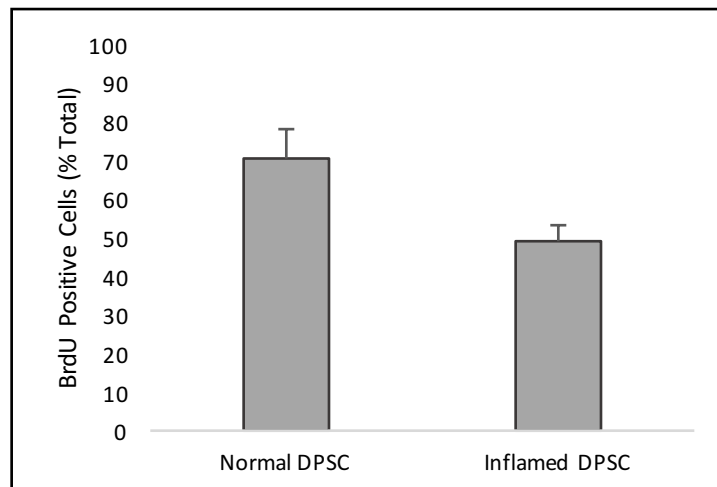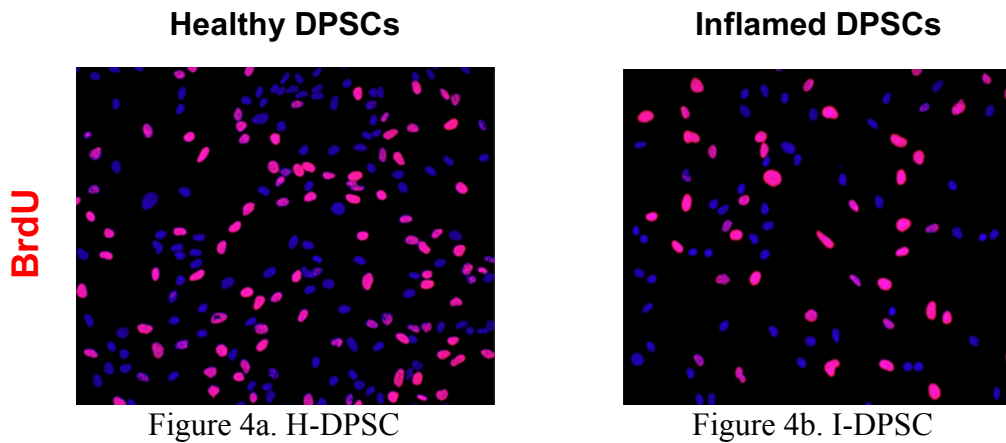
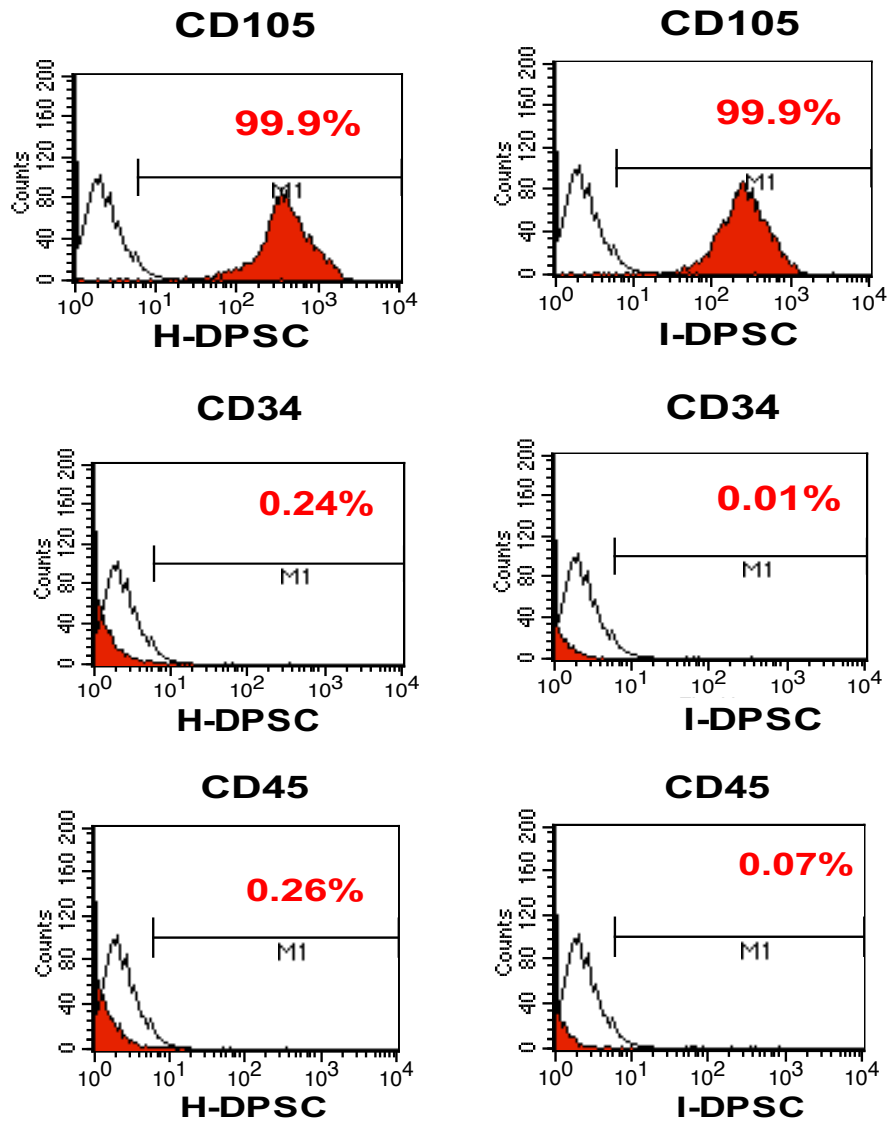

Figure 4c. BrdU positive cells (% Total)

In addition to Toluidine Blue staining, the subconfluent H-DPSCs and I-DPSCs were stained with BrdU for the assessment of proliferation capacities. The results from BrdU staining was in agreement of the results from Toluidine Blue staining. H-DPSCs had an average of 70.3 % ± 7.6 positively stained cells, while I-DPSCs had an average of 49.16 % ± 4.13 positively stained cells. The results indicate that even though DPSCs isolated from both healthy and inflamed pulp tissues have a

high proliferation rate, H-DPSCs had a higher proliferation capacities compared to I-DPSCs.

Figure 5. Flow cytometric analysis

CD105

99.9%

H-DPSC

CD105

99.9%

I-DPSC

CD34

0.24%

H-DPSC

CD34

0.01%

I-DPSC

CD45

0.26%

H-DPSC

CD45

0.07%

I-DPSC

Table 1.

Marker	Normal DPSC (% Gated)	Inflamed DPSC (% Gated)
CD44	85.6	99.6
CD73	99.5	99.8
CD90	99.1	99.8
CD105	99.9	99.9
CD34	0.24	0.01
CD45	0.26	0.07

The flow cytometric analysis was performed on H-DPSCs and I-DPSCs to confirm the purity of stem cell components in the cell culture and to assess the difference between the H-DPSCs and I-DPSCs. CD44, CD73, CD90 and CD105 are considered typical positive stem cell markers while CD34 and CD45 are considered negative markers. As summarized in Table 1, both H-DPSCs and I-DPSCs were positive for CD44, CD73, CD90 and CD105, while both were negative for CD34 and CD45. These results indicate that the cell cultures for both H-DPSCs and I-DPSCs were purely composed of DPSCs, and there was no statistical difference between them with regards to these markers.

Presence of IL-6 in Culture Supernatant from Healthy and Inflamed DPSCs

As mentioned by Barkhordar et al. in 1999, the inflamed pulp tissues contain higher concentration of IL-6 compared to the healthy pulp tissues. ELISA was performed on the culture supernatant from healthy and inflamed DPSCs to investigate if there is a difference in the level of IL-6. On average from our samples, H-DPSCs contained 16pg/mL of IL-6 in their culture medium while I-DPSCs contained 119.5pg/mL of IL-6. These results indicate that I-DPSCs have the capacity to produce a higher concentration of IL-6 with statistical significance (p value < 0.01).

Figure 6. <u>Concentration of IL-6 in culture supernatant from H-DPSCs and I-DPSCs</u>
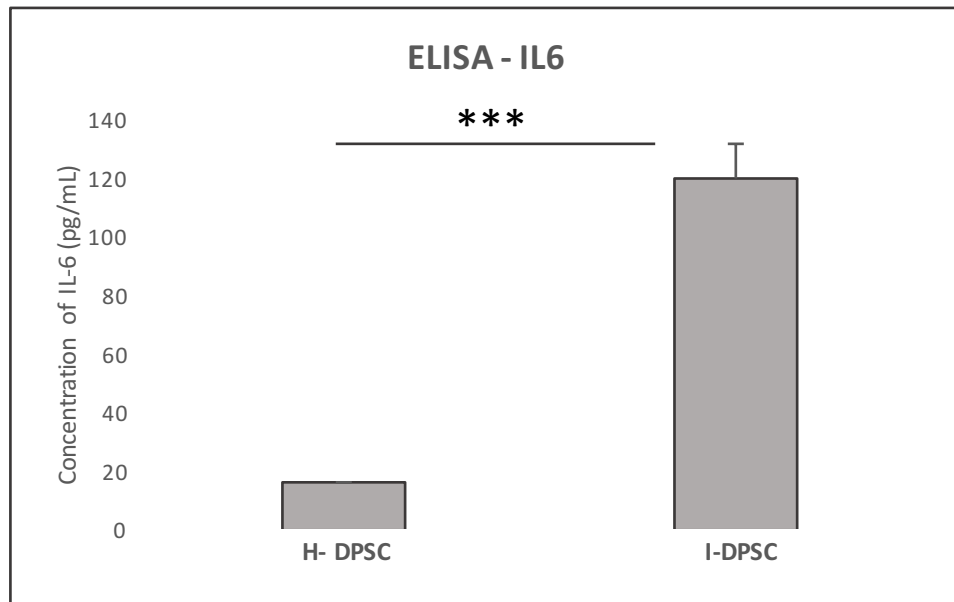
Comparison on Osteogenic Differentiation Potentials between Healthy and Inflamed DPSCs

Figure 7. <u>Osteogenic differentiation potential with Alizarin Red staining and western blotting</u>
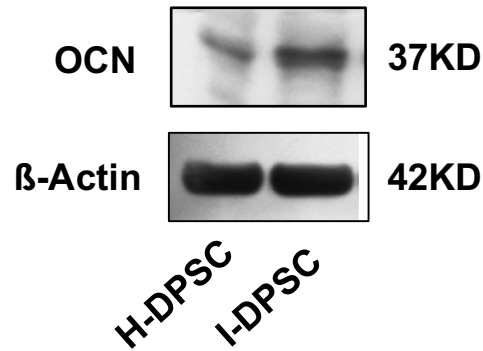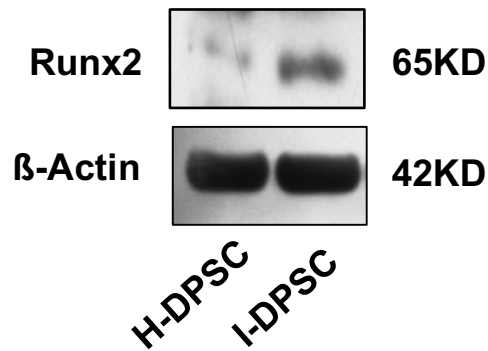

Figure 7a. H-DPSCs
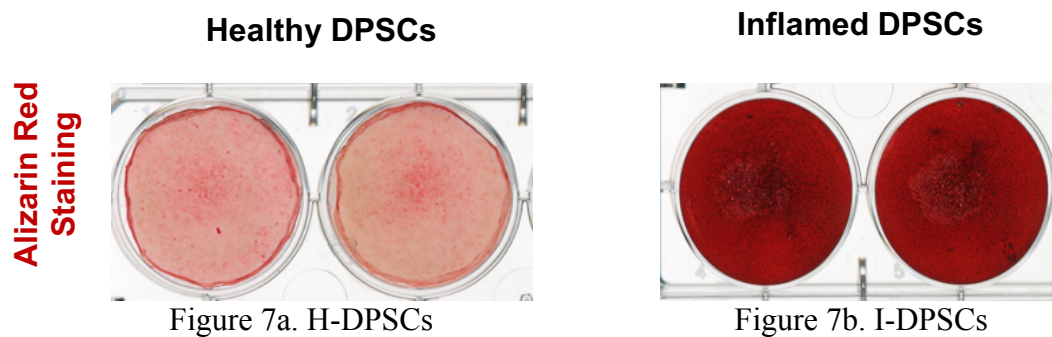
Figure 7b. I-DPSCs

Figure 7c. Western Blotting for OCN

Figure 7d. Western Blotting for Runx2

Although DPSCs are known to have high osteogenic differentiation potentials, there are still conflicting data on the difference between H-DPSCs and I-DPSCs for their capacities for osteogenic differentiation. Therefore, our samples were induced for osteogenesis, and the Alizarin Red staining and the western blotting analysis were performed to assess the difference between H-DPSCs and I-DPSCs. Figure 6 shows both samples have osteogenic differentiation potential. Interestingly, I-DPSCs had stronger Alizarin Red staining and thicker bands for osteogenic markers, Osteocalcin (OCN) and Runx2.

Comparison on Neurogenic Differentiation Potentials between Healthy and Inflamed DPSCs

Figure 8. Neurogenic differentiation potential analysis with immunofluorescent staining and western blotting
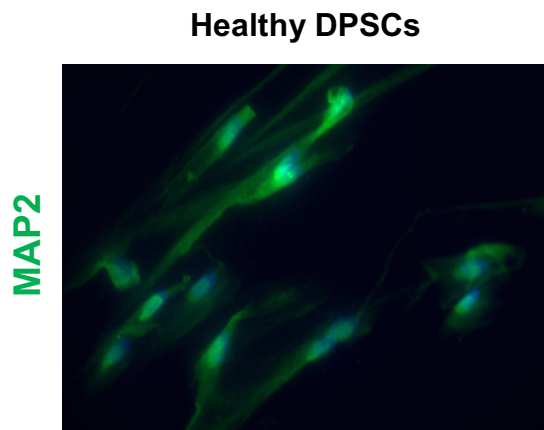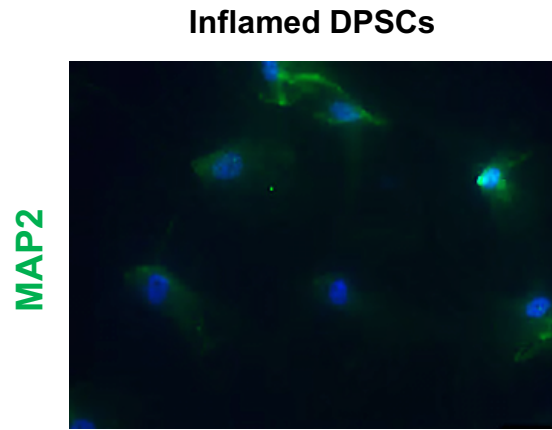
Healthy DPSCs

Inflamed DPSCs

Figure 8a. H-DPSC- MAP2
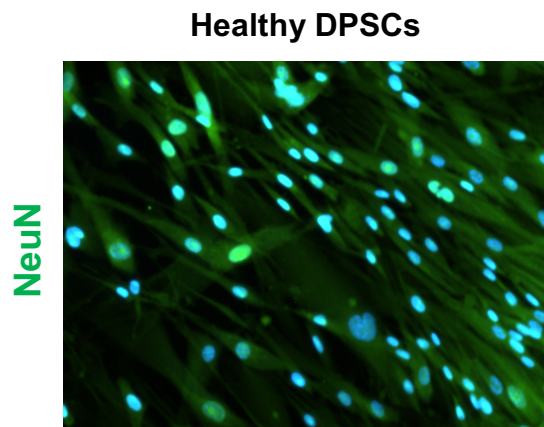

Figure 8b. I-DPSC – MAP2

Healthy DPSCs

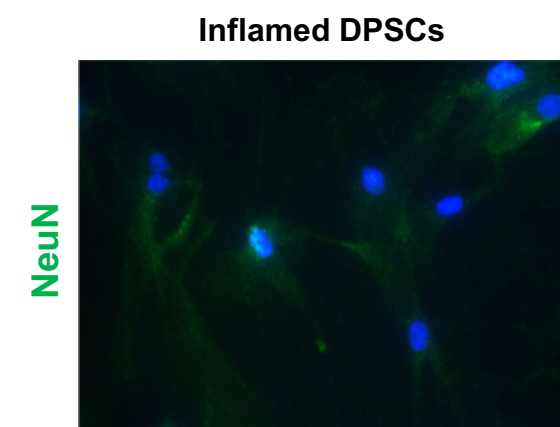**Inflamed DPSCs**

Figure 8c. H-DPSC – NeuN
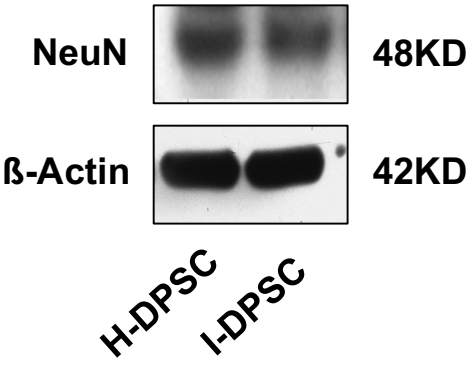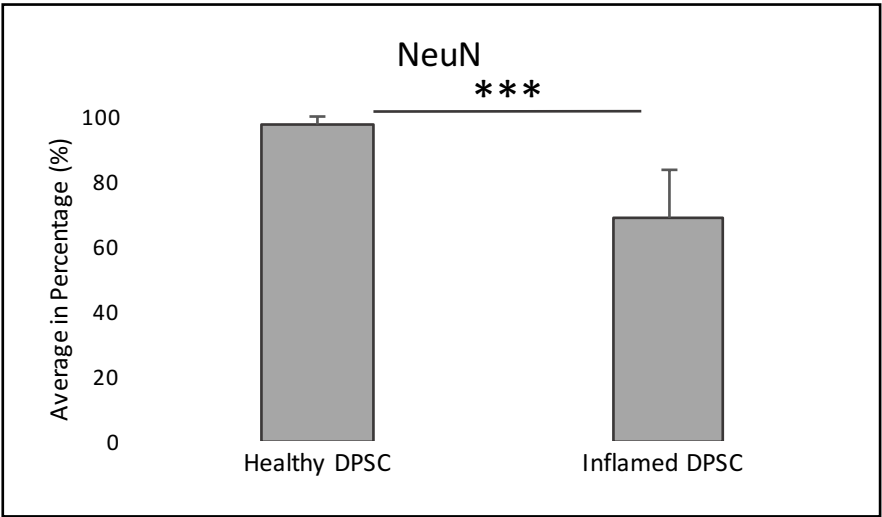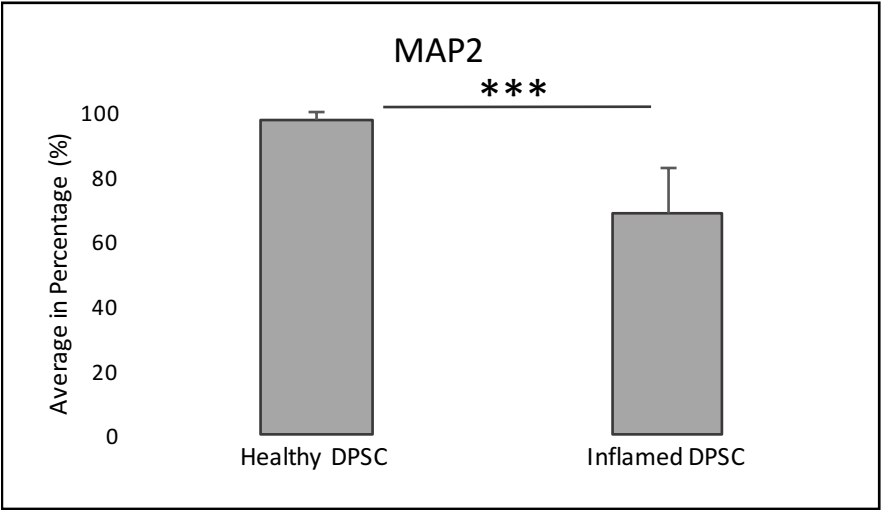

Figure 8d. I-DPSC - NeuN

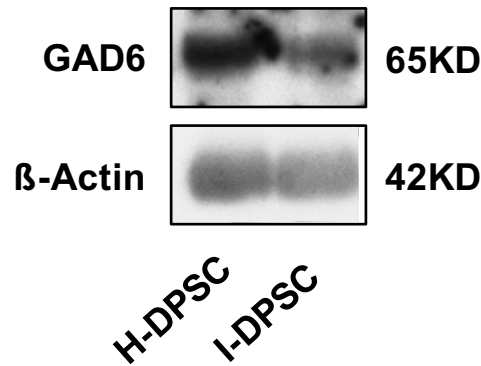Figure 8e. Western Blotting with NeuN

Figure 8f. Western Blotting with GAD6

In addition to osteogenic differentiation, DPSCs are also capable of neurogenesis (Ibarretxe et al., 2012). Our DPSCs samples were induced for neurogenesis, and immunofluorescence and western blotting were performed to investigate if there is any difference between H-DPSCs and I-DPSCs.

Immunofluorescences showed that H-DPSCs are more highly stained with neurogenic markers including MAP2 and NeuN than I-DPSCs. According to the quantification analysis, 97.2 % (± 2.3%) and 98.3 % (± 1.2%) of H-DPSC samples were positively stained for MAP2 and NeuN respectively, while 68.5% (± 13.3%) and 61.7% (±11%) of I-DPSCs samples were positively stained for MAP2 and NeuN respectively. Likewise, the expression of NeuN and GAD6 was more strongly detected in H-DPSCs compared with I-DPSCs.

Effects of IL-6 on Osteogenic and Neurogenic Differentiation Potentials of DPSCs

Figure 9. Osteogenic differentiation potential of IL-6 treated H-DPSC and I-DPSC

H-DPSC

Figure 9a. H-DPSCs

H-DPSC + IL6

Figure 9b. H-DPSCs with additive IL-6

I-DPSC

Figure 9c. I-DPSCs

I-DPSC + Neutralizing IL6
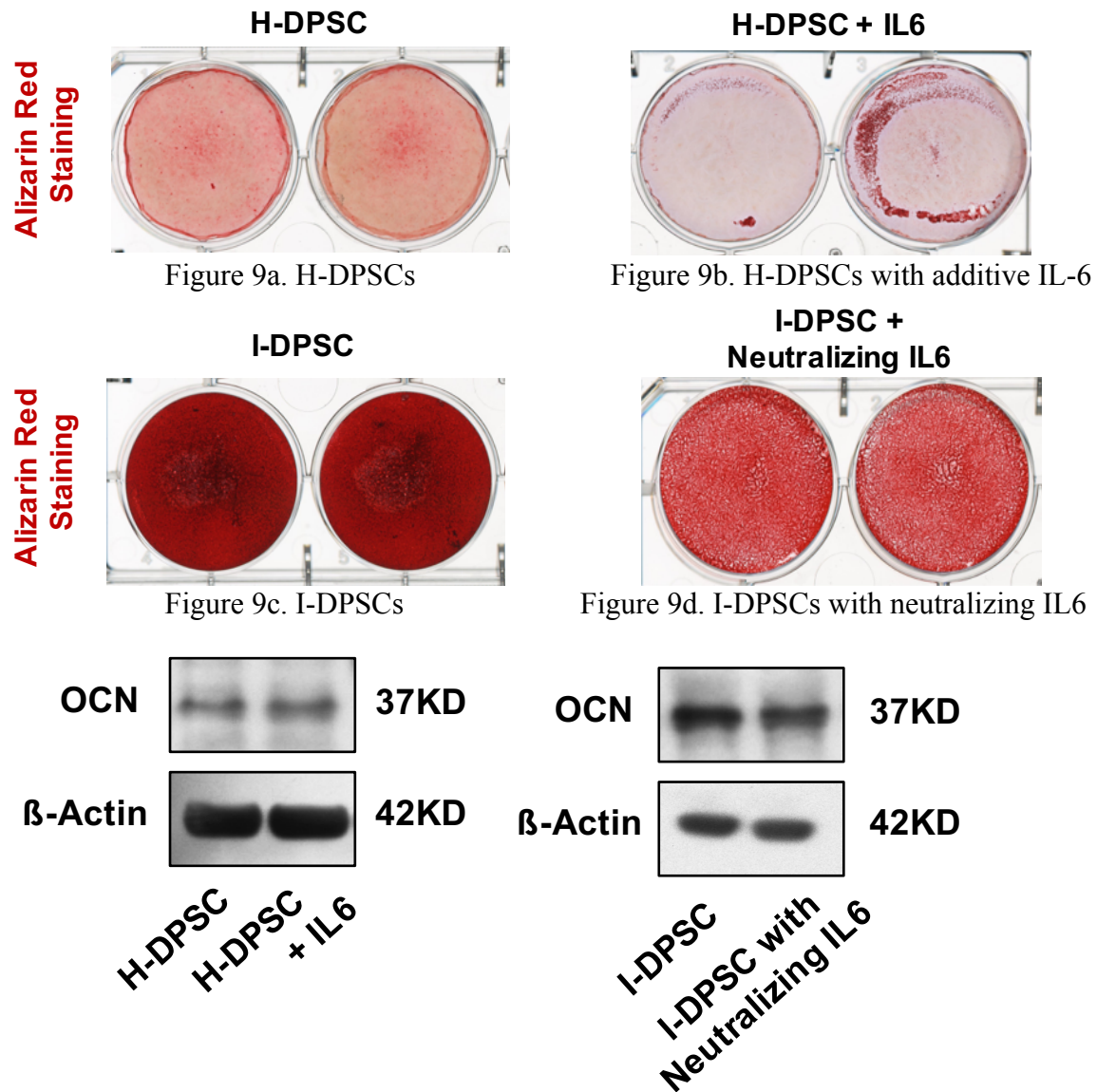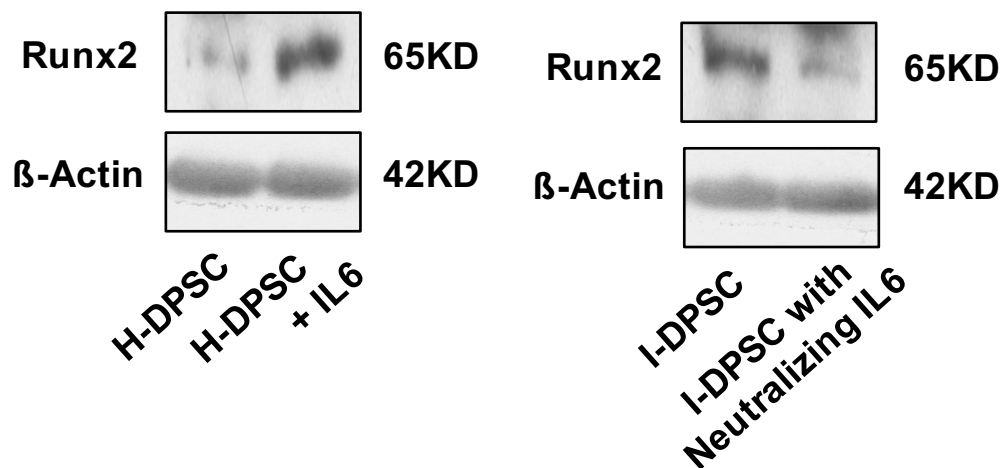

Figure 9d. I-DPSCs with neutralizing IL6

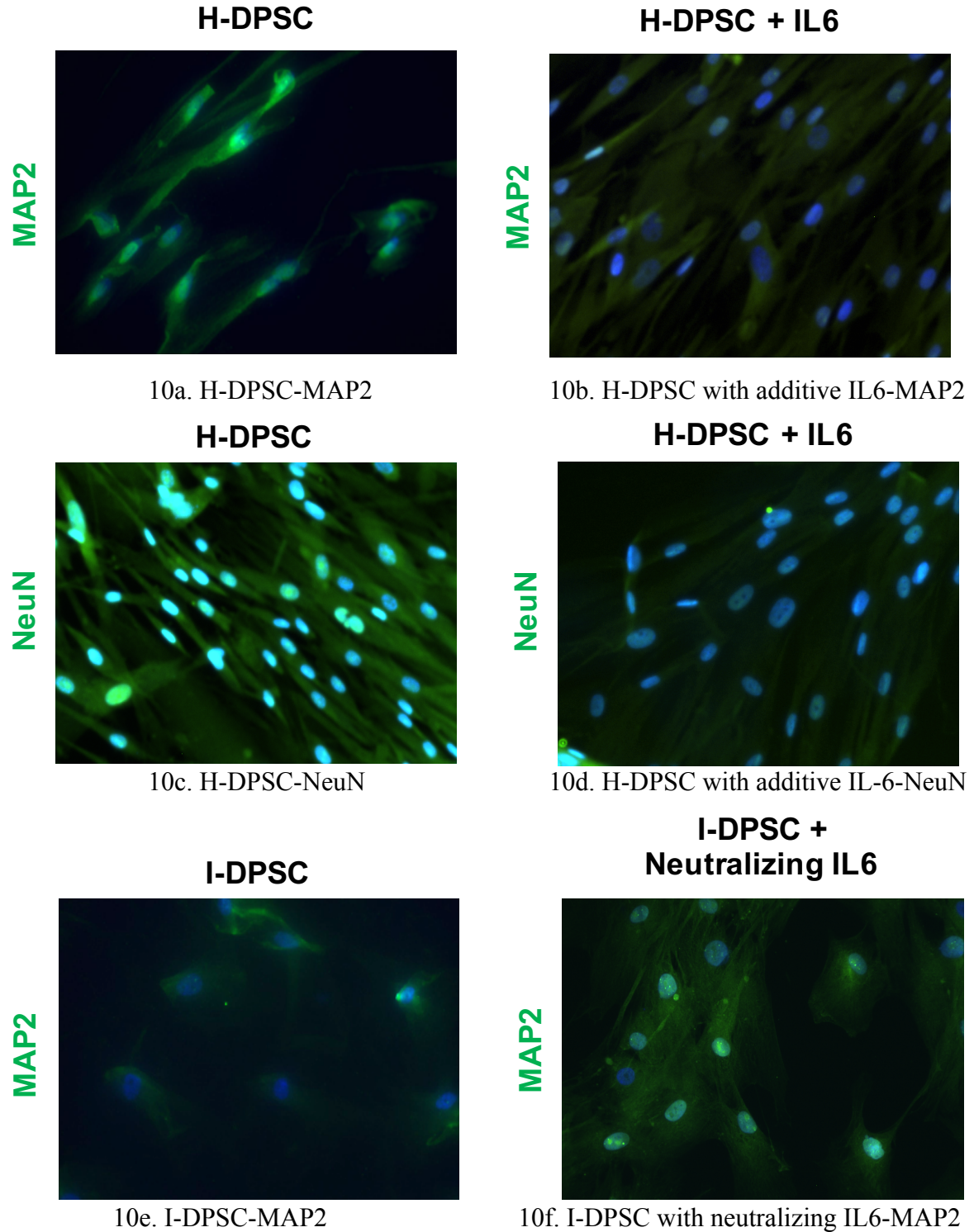

Figure 9e. Western Blotting for OCN
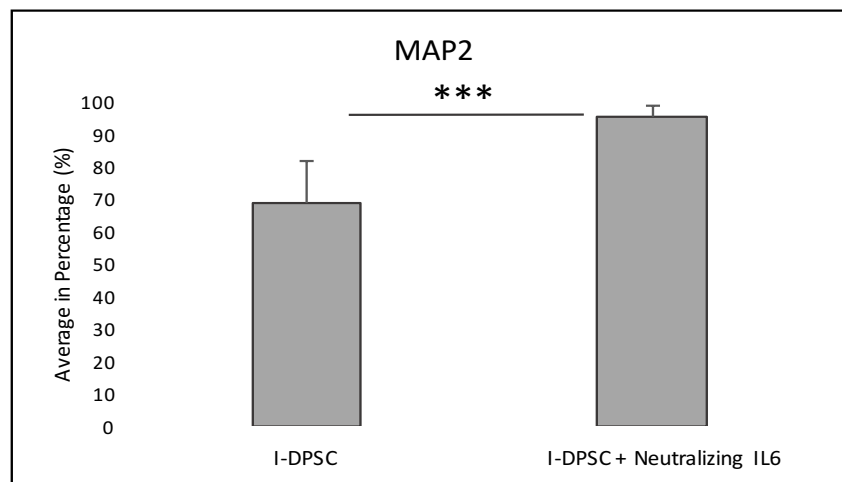
Figure 9f. Western Blotting for Runx2

Since our findings indicate that there are statistical differences between H-DPSCs and I-DPSCs on the secretion of IL-6 during culturing, and osteogenic and neurogenic differentiation, additional experiments were performed to investigate the role of IL-6 on the differentiation potentials of two different types of DPSCs.

Based on Alizarin Red staining and western blotting analysis, IL-6 has an effect on DPSCs for their osteogenic differentiation potentials. Upon stimulation with IL-6, H-DPSCs had higher osteogenic differentiation, as seen by stronger Alizarin Red staining and stronger expression of osteogenic markers including OCN and Runx2 compared to those from non-stimulated H-DPSCs.

In contrast, when neutralizing IL-6 was added to prevent the action of IL-6 for I-DPSCs, a higher degree of osteogenic differentiation was observed when compared to I-DPSCs without neutralizing IL-6 based on weaker Alizarin Red staining and stronger expression of osteogenic markers including OCN and Runx2.

Figure 10. The expression of neurogenic markers in H-DPSC and I-DPSC in immunofluorescence and western blotting

H-DPSC

H-DPSC + IL6

10a. H-DPSC-MAP2

10b. H-DPSC with additive IL6-MAP2

H-DPSC

H-DPSC + IL6

10c. H-DPSC-NeuN

10d. H-DPSC with additive IL-6-NeuN

I-DPSC

I-DPSC + Neutralizing IL6

10e. I-DPSC-MAP2

10f. I-DPSC with neutralizing IL6-MAP2

I-DPSC

**I-DPSC +
Neutralizing IL6**

NeuN

NeuN

10g. I-DPSC-NeuN

10h. I-DPSC with neutralizing IL6-NeuN

MAP2

***

Average in Percentage (%)

100
90
80
70
60
50
40
30
20
10
0

H-DPSC H-DPSC + IL6

MAP2

***

Average in Percentage (%)

100
90
80
70
60
50
40
30
20
10
0

I-DPSC I-DPSC + Neutralizing IL6

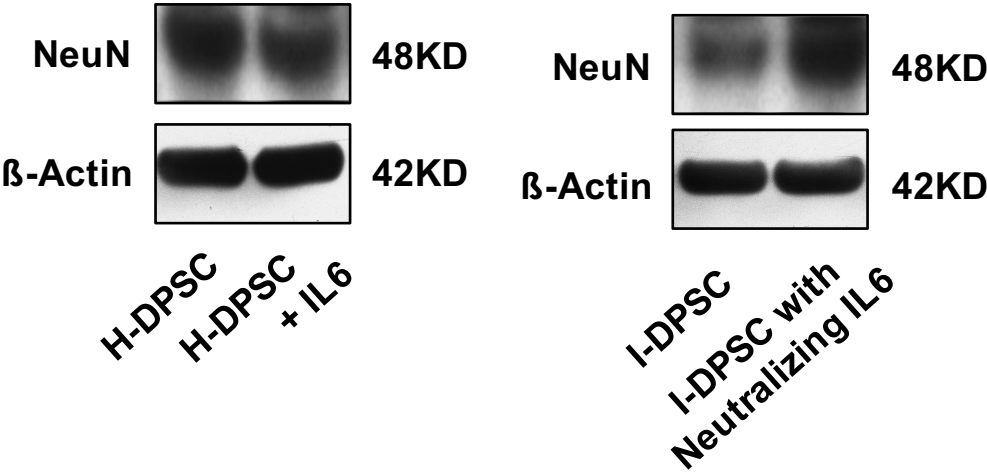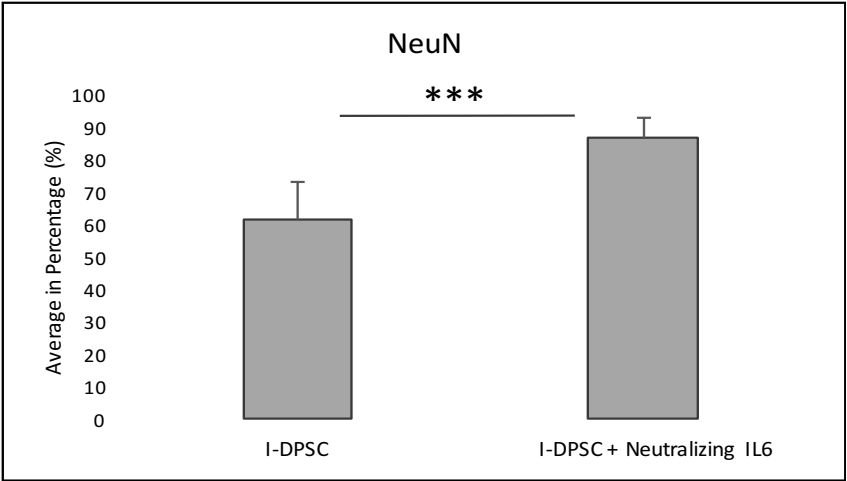Figure 10i. Western Blotting with NeuN
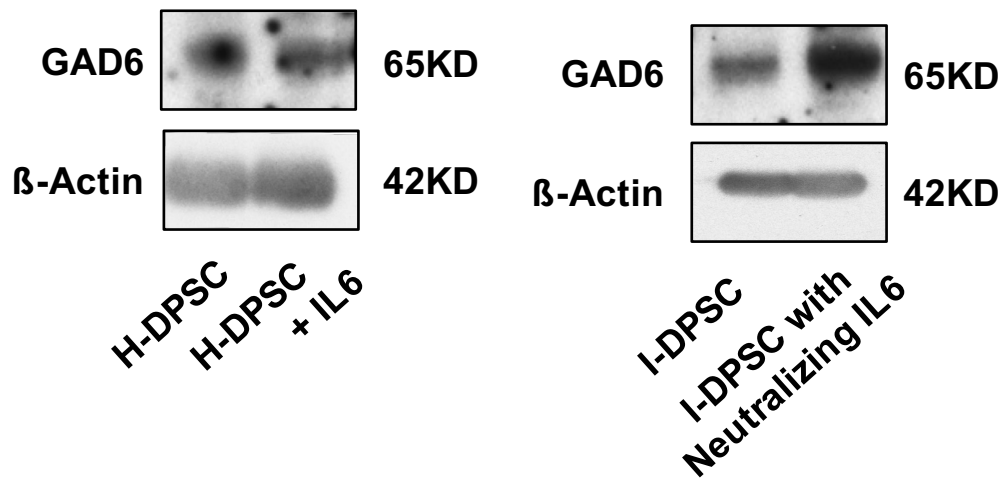
Figure 10j. Western Blotting with GAD6

Immunofluorescence staining and western blotting analysis were performed to assess the effect of IL-6 on neurogenic differentiation potentials of DPSCs. With addition of IL-6, the neurogenic differentiation potentials of H-DPSCs seemed to be decreased based on less immunofluorescence staining and weaker expression of neurogenic markers including NeuN and GAD6. However, based on quantification analysis from immunofluorescences, H-DPSCs without additional IL-6 had 97.2% (±2.3%) for MAP2 and 98.3% (±1.2%) for NeuN for positively stained cells, while H-DPSCs with additional IL-6 had 81.7% (±7.8%) for MAP2 and 88.4% (±3.6%) for NeuN for positively stained cells. For I-DPSCs, there were 68.5% (±13.4%) for MAP2 and 61.8% (±11%) for NeuN for positively stained cells, while I-DPSCs with neutralizing IL-6 had 95.3 % (±3.2%) for MAP2 and 86.7 % (±5.8%) for NeuN for positively stained cells.

Discussion

The differences on baseline characteristics, including expressions for stem cell markers, proliferation rate, and differentiation potentials, for H-DPSCs and I-DPSCs have been thoroughly investigated by many different groups. However, the results from these studies vary from one another, and there is no general consensus yet. For example, Alongi et al. in 2010 showed that H-DPSCs have higher osteogenic differentiation potentials and express lower levels of MSC markers including STRO-1, CD90, CD105 and CD146 when compared to I-DPSCs, while Wang et al. in 2010 showed that H-DPSCs showed similar STRO-1 expression and osteogenic induction potentials when compared to I-DPSCs (Alongi et al. 2010 & Wang et al. 2010). However, Pereira et al. in 2012 showed that there are more significant differences between each patient than there are between H-DPSCs and I-DPSCs. In addition, there are not many literatures that assess the difference between H-DPSCs and I-DPSCs on their neurogenic differentiation potentials. Since there is no general consensus, experiments were performed to investigate the baseline characteristics of H-DPSCs and I-DPSCs for their proliferation rate by counting colony-forming unit and BrdU staining, stem cell markers, and osteogenic and neurogenic differentiation potentials.

In order to investigate the general morphology of healthy and inflamed pulp tissues, H & E staining was performed on two healthy pulp tissues collected from impacted third molars and on two inflamed pulp tissues collected from severely decayed

molars from paraffin sections. As expected, healthy pulp tissues were devoid of immune cells, while inflamed pulp tissues were saturated with them. This result confirms our selection criteria for choosing healthy and inflamed pulp tissues.

Immunofluorescence staining was performed to assess the difference between healthy and inflamed pulp tissues from the paraffin sections from the same samples that were used for H & E staining. CD105 was used as a stem cell marker while CD3 and IL-6 were used as indicators for inflammation. Quantification analysis was performed to assess how many cells are positively stained with secondary antibodies for fluorescence. Five fields were randomly selected at 40 times magnification, and cells stained with DAPI and fluorescence were counted for the analysis. For CD 105, there were 12.4% ($\pm$1.8) and 15% ($\pm$4.2) positively stained cells for healthy and inflamed pulp tissues, respectively. Healthy pulp tissues had 4.69% ($\pm$2.8) and 4.4 %($\pm$2.9) positively stained cells for CD3 and IL6 respectively, while inflamed pulp tissues had 19.3% ($\pm$4.0) and 17.7% ($\pm$5.8) positively stained cells for CD3 and IL-6 respectively. Interestingly, results showed that there is no statistical difference between healthy and inflamed tissues for CD105 (p=0.21) while there were statistical differences for CD3 (p=0.000029) and IL-6 (p=0.00032), indicating that the concentration of DPSCs do not change much, while levels of CD3 and IL-6 are increased during inflammation.

In order to examine the proliferation rate of H-DPSCs and I-DPSCs, the colony forming unit (CFU) and BrdU staining were analyzed. During primary culturing for DPSCs, 10,000 cells for counted for four samples for H-DPSCs and I-DPSCs, and were plated for assessing colony forming unit. For the first passage DPSCs grow in colonies, and the number of these colonies represents the proliferation of each cell line (Gronthos et al., 2000). After two weeks, cells were stained with Toluidine Blue and colonies were defined as cells with more than 50 cells. The mean number of colonies for H-DPSCs was 32 ± 14.4 and for I-DPSCs, it was 13.75 ±7.9. According to our statistical analysis, there was no statistical difference between H-DPSCs and I-DPSCs (p=0.068), even though H-DPSCs tended to have higher number of CFU's than I-DPSCs. This could be due to small sample size.

Additionally, BrdU staining was performed to further assess the proliferation rate of DPSCs. BrdU stands for bromodeoxyuridine which is a synthetic nucleoside, and it can be incorporated into the DNA of replicating cells by replacing thymidine during DNA replication; therefore, it's used for detecting proliferation cells (Lehner et al. 2011). For H-DPSCs, 70.3% (±7.6) cells were stained with BrdU, while 49.16% (±4.1) were stained for I-DPSCs. The statistical analysis indicated that there is a statistical significant difference between H-DPSCs and I-DPSCs (p=0.0006).

In addition, the flow cytometric analysis was performed to assess the purity of cell cultures and to investigate if there is any difference between H-DPSCs and I-

DPSCs. Markers that were evaluated include CD44, CD73, CD90, CD105, CD34 and CD45. CD44 is a cell membrane glycoprotein with lymphocyte activation function, and its expression has been detected on DPSCs (Kawashima, 2012). CD73 is also a positive marker for DPSCs and it is a cell adhesion molecule (Alongi et al., 2012). CD90 is a glycoprotein expressed mainly in leukocytes and is another positive marker for DPSCs (Kawashima, 2012). CD105 is also known as endoglin which is a glycoprotein associated with the human vascular endothelium, and is expressed on DPSCs (Rius et al. 1998). CD34 and CD45 are both hematopoietic markers with CD34 commonly found in hematopoietic stem cells and in endothelium and CD45 being a common leukocyte antigen, and these two markers to be absent on DPSCs (Gronthos et al. 2000). For positive markers including CD44, CD73, CD90, and CD105, both H-DPSCs and I-DPSCs had high positive values, and they were both negative for CD34 and CD45 according to the flow cytometric analysis. These results indicate that cell cultures included purely of DPSCs, and there is no significant difference between H-DPSCs and I-DPSCs on the expression level for stem cell markers.

IL-6 is involved in various steps through inflammatory processes, including induction of synthesis of neutrophils and acute phase proteins. It is also involved in coordination of the activity of the innate and adaptive immunities through stimulation of antibody production and effector T cell development (Tanaka et al., 2014). Although many attempts have been made to investigate the function of IL-6,

little is known about its role in pulpal inflammation. One of the first studies on IL-6 from the dental field was done by Barkhordar et al. in 1999 where they showed that there is an elevated level of IL-6 in inflamed pulp tissues and periapical lesions compared to the healthy pulp tissues. Many studies, including Park in 2002 and Sabir & Sumidartia in 2016, showed similar results where the level of IL-6 is increased in inflamed pulp tissues. Based on these findings, our study conducted an experiment to assess if I-DPSCs can play a role in increasing the level of IL-6. According to the statistical analysis from ELISA, I-DPSCs expressed a higher level of IL-6 compared to H-DPSCs ($p < 0.01$). This result indicates that during inflammation, DPSCs become activated to release IL-6.

Before investigating further into the role of IL-6, the baseline osteogenic and neurogenic differentiation potentials were assessed for H-DPSC and I-DPSC samples. As Pereira et al. in 2012 demonstrated that the differentiation potentials vary among each sample. In this study, all four samples of H-DPSCs and another four samples of I-DPSCs showed osteogenic and neurogenic differentiation potentials, but as seen in figures 7 and 8, there were significant differences. When compared to I-DPSCs, H-DPSCs had lower differentiation potentials for osteogenesis, based on Alizarin Red staining and western blotting analysis for osteogenesis markers including Runx2 and OCN. However, H-DPSCs had higher neurogenic differentiation potentials compared to I-DPSCs based on immunofluorescence staining for neurogenic markers including MAP2 and NeuN,

and western blotting analysis for GAD6 and NeuN. The quantification analysis for immunofluorescence staining was done, and there was statistical difference between H-DPSCs and I-DPSCs for MAP2 (p=0.0038) and NeuN (p=0.00011). This result indicates that H-DPSCs have higher neurogenic differentiation potentials from our samples. The effects of inflammatory processes on MSCs could be a controversial topic. However, in general, when the levels of pro-inflammatory cytokines including TNF-α, IL-1β, and INF-γ are elevated, pulp tissue regeneration and dentinogenic differentiation potentials of DPSCs are decreased along with collagen synthesis, odotogenesis and proliferation rates (Alongi et al., 2010). Although the samples from our study show that H-DPSCs have higher proliferation and neurogenic differentiation potentials with lower osteogenic differentiation potentials compared to I-DPSCs, it does not implicate that inflammation leads to such findings, because there are other factors involved and our sample size was small to make such generalization.

Lastly, the role of IL-6 on the differentiation potentials for DPSCs were evaluated. Based on our result from ELISA, I-DPSCs contained higher level of IL-6 in their cell culture, while H-DPSCs contained minimum amount of IL-6. Therefore, IL-6 was added to the cell culture of H-DPSCs, while neutralizing IL-6 was added to the cell culture of I-DPSCs. The effect of anti-human IL-6 antibodies from the same company on the neutralization has been investigated and proved by several studies, including Zou et al. 1999 and Abrams 2001. The same experiments were

performed to DPSCs treated with IL-6, following osteogenic and neurogenic induction. Based on the Alizarin Red staining and western blotting analysis, IL-6 has a crucial role in osteogenesis for DPSCs. When IL-6 was added to H-DPSCs culture, it led to stronger staining for Alizarin Red and thicker bands for osteogenic markers including OCN and Runx2. When the activity of IL-6 was prevented by adding neutralizing IL-6, osteogenic potentials of I-DPSCs were reduced based on weaker Alizarin Red staining and thinner bands for osteogenic markers. Our results are in agreement with other recent studies including Balto et al. 2001 and Xie et al. 2018, where it showed that IL-6 can lead to higher degree of osteogenesis. The role of IL-6 on the neurogenic differentiation potentials seemed to be the exact opposite of the osteogenic differentiation potentials. When IL-6 was added to H-DPSCs, their neurogenic differentiation potentials seemed to have been reduced based on the lower expression for GAD6 and NeuN from the western blotting analysis and less immunofluorescence staining for NeuN and MAP2. Although both samples were highly positively stained, according to our statistical analysis, there was statistical difference between H-DPSCs and H-DPSCs with additive IL-6 for MAP2 (p=0.0026) and NeuN (p=0.0003). This could be due to weaker effect of IL-6 on neurogenesis. When the neutralizing IL-6 was added to I-DPSCs, the neurogenic potentials were reduced based on less expression on western blotting and immunofluorescences. The quantification analysis showed that there were statistical differences between I-DPSCs and I-DPSCs with neutralizing IL-6 for MAP2 (p=0.0037) and NeuN (p=0.0027).

In recent years, pulpal regeneration with the use of stem cell therapies has been gaining much attention in the field of dental research. In 2017, Nakashima et al. conducted a first human clinical study to regenerate pulp tissue by transplantation of healthy DPSCs from wisdom teeth into pulpectomized teeth with the diagnosis of irreversible pulpitis. In addition, Xuan et al. 2018 showed pulpal regeneration with root maturation on 100% of teeth from the study population via autologous dental pulp stem cell implantation on necrotic immature anterior teeth. From the findings from our study, IL-6 has shown the ability to decrease the neurogenic differentiation potential of DPSCs; therefore, neutralizing IL-6 antibodies can be added on collagen to promote neurogenesis of DPSCs when being implanted into the root canal space for pulpal regeneration which enhances the clinical success.

In addition, many studies are being conducted to investigate the ways to enhance bone healing following endodontic surgeries, commonly known as apicoectomy. In 2011, von Arx and AlSaeed published a review paper on the use of regenerative techniques in apical surgery and showed that many regenerative techniques are being done including guided tissue and bone regeneration with membranes, growth factors, bone fillers or bone substitutes. They mentioned that many studies indicate that the regenerative techniques are needed for better outcome with through-and-through lesions. From our study, it was shown that IL-6 has the capacity to promote osteogenesis; therefore, IL-6 can be further investigated for

the use as regenerative technique in endodontic microsurgery to promote faster bone healing in the diseased area.

Conclusion

DPSCs are being thoroughly studied for their unique characteristics, not only for dentistry but also for medicine nowadays. Our study is one of the very first and few studies where the role of IL-6, another important molecule in our body, on interactions with DPSCs is investigated. Our results indicated that H-DPSCs and I-DPSCs express similar amount of stem cell markers, while H-DPSCs tend to have higher neurogenic differentiation potentials and proliferation rate, and lower osteogenic differentiation potentials when compared to I-DPSCs, although there are variations among each samples. Furthermore, our results showed that the level of IL-6 was elevated in the cell culture of I-DPSCs, which may indicate that I-DPSCs are involved in the production of IL-6. In addition, IL-6 is capable of promoting osteogenesis while inhibiting neurogenesis as seen by our results.

Our study is a pilot study where we showed that IL-6 has an effect on the differentiation potentials of DPSCs. Further studies are required to examine the detailed pathway of IL-6 interaction with DPSCs to develop the idea into promoting healing capacities of DPSCs for pulpal regeneration and periapical bone healing.

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