Upregulation of Adipogenic Lineage

Differentiation and Markers in Inflamed-Dental Pulp

BY

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THESIS

Submitted as partial fulfillment of requirements
For the degree of Master of Science in Oral Sciences
In the Graduate College of the
University of Illinois at Chicago, 2016

Chicago, Illinois

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To my parents, Helen and James Czapla, my sister, Anna Czarples, and my husband, Mark Czaperacker. Without you, none of this could be possible.
ACKNOWLEDGMENTS

Thank you to my thesis committee—(Drs. Alapati, Johnson, and Colvard)—for their support and assistance. I would also like to thank the Edgar D. Coolidge Endodontic Study Club of Chicago, IL, the American Association of Endodontists Foundation, and the National Institute of Health (DE019514-SBA NIDCR Grant) whose donations and grant helped make my research possible.

A special thanks to all members of the Alapati Lab who were helpful with developing a research protocol, data collection, and having some fun along the way.
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# LIST OF ABBREVIATIONS

<table>
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<th>Abbreviation</th>
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<tr>
<td>AAE</td>
<td>American Association of Endodontists</td>
</tr>
<tr>
<td>AAPD</td>
<td>American Academy of Pediatric Dentistry</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Ca(OH)$_2$</td>
<td>Calcium hydroxide</td>
</tr>
<tr>
<td>CEJ</td>
<td>Cementoenamel junction</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
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<tr>
<td>DM</td>
<td>Differentiation medium</td>
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<tr>
<td>DPSC</td>
<td>Dental pulp stem cell</td>
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<td>DSPP</td>
<td>Dentin sialophosphoprotein</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>IL-2</td>
<td>Interleukin-2</td>
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<tr>
<td>IM</td>
<td>Induction medium</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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<td>Lep-R</td>
<td>Leptin receptor</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>MTA</td>
<td>Mineral trioxide aggregate</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Sodium hypochlorite</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PDGFR-α</td>
<td>Platelet-derived growth factor receptor-α</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PPAR-γ</td>
<td>Proliferator-activated receptor-gamma</td>
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<tr>
<td>SILAC</td>
<td>Stable Isotope Labeling by Amino Acids in Cell Culture</td>
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<tr>
<td>Th1</td>
<td>T-helper lymphocyte type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper lymphocyte type 2</td>
</tr>
<tr>
<td>UIC</td>
<td>University of Illinois at Chicago</td>
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<td>VPT</td>
<td>Vital pulp therapy</td>
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Vital pulp therapy (VPT) and pulp regeneration or revascularization are procedures currently utilized in endodontics. Even though these therapies are commonly employed, the biomolecular pathways by which they are working are not completely understood.

One such aspect that remains to be fully understood is how the microenvironment of inflammation due to pulpitis or pulpal necrosis effects the stem cells employed in VPT and pulp revascularization. Preliminary reports have illustrated that on the cellular level, stem cells challenged by inflammatory stresses, such as hypoxia, have a significant up-regulation of leptin and leptin receptors.

Leptin is a hormone that regulates many processes including energy intake and expenditure, hematopoiesis, reproduction, bone formation and turn over, and immune modulation. In the context of this study, leptin’s contribution to immune modulation is of particular interest.

The aim of this study is to investigate the role of inflammation and leptin on VPT and pulp revascularization. The hypothesis is that pulp tissue inflammation alters the differentiation potential of the dental pulp stem cells (DPSCs) towards an adipogenic lineage, resulting in an increase in leptin production and upregulation of leptin receptors.

In the first aim of the current study, DPSCs were isolated from seven human extracted teeth. Half of the DPSCs were cultured in hypoxia (5% oxygen) and the remaining half were cultured in normoxia (21% oxygen) for 72 hours at 37°C in 5% CO₂. DPSCs were then cultured in adipogenic differentiation medium for 14 days in order to induce adipogenic differentiation. Cells were then evaluated for lipid accumulation by staining with oil red O stain (Sigma–
Aldrich, MO, USA). This aim of the study demonstrated that DPSCs exposed to hypoxia had a higher concentration of cells differentiate into adipocytes compared to those cells exposed to normoxia.

In the second aim of the study, dental pulp was collected from six extracted virgin human premolars with normal pulp and six extracted human teeth with irreversible pulpitis without periapical radiolucency. The collected pulp tissue was subjected to a BioPlex Luminex® Assay (BioRad, CA, USA) in order to quantify protein analytes specific for leptin and adipogenesis. Statistical analysis was preformed using two-tailed Student’s t-test with p-value ≤ 0.05. This portion of the study demonstrated that leptin and leptin receptors were significantly up-regulated in the inflamed pulp compared to the healthy pulp; and furthermore, adipogenic protein markers were also significantly up-regulated.

This study suggests that inflammation drives dental pulp stem cells towards an adipogenic lineage with an ensuing upregulation of leptin production. With this finding, the biomolecular pathways of pulp repair and regeneration are better understood. Perhaps in the future, this information will be useful in employing leptin as a therapeutic molecule in order to regenerate tissue that more closely resembles the dentin-pulpal complex more quickly and safely.
I. INTRODUCTION

A. Background

Dental pulp stem cells (DPSCs) are essential for the success of vital pulp therapies, such as indirect and direct pulp caps and pulpotomies, in teeth with reversible pulpitis, and for pulpal regeneration procedures in immature, necrotic teeth (Zhang, 2010). DPSCs vitality and differentiation potential for these procedures are directly influenced by the pulpal environment (Wagers, 2012). Currently, the knowledge of the local influence of inflammation in this context is still limited, but is fundamental to understanding the signaling of DPSCs. Such knowledge of the multifaceted interplay in molecular signaling of inflammation and DPSCs will be instrumental to clinical translation of vital pulp therapies and pulpal regeneration procedures (Smith et al., 2016).

Preliminary studies have demonstrated that in a cell model with a simulated inflammatory stressor of hypoxia, DPSCs are driven to up-regulate leptin and leptin receptor (LEP-R) (Byun et al., 2015).

Leptin has been observed to protect dental pulp cells from bacterial and chemical insults via pro-inflammatory responses. Its signaling plays an essential role in cell survival, growth, differentiation, and mineralization capacity of DPSCs (Martin-Gonzales et al., 2013).

This research examines a diseased tissue model, whether up-regulation of leptin is evident in inflamed dental pulp and if the increase in leptin expression is associated with an increased differentiation of DPSCs into adipogenic lineage.
B. **Significance of the Study**

This study brings novel insight into the current understanding of DPSCs in an inflamed microenvironment. With the knowledge gained from this study, the driving mechanisms of pulpal repair and regeneration will be better understood. Additionally, these findings could serve as an inspiration for a potential therapeutic target to promote faster and higher quality pulpal intervention.
C. **Specific Aims**

1. To address whether an up-regulation of leptin in hypoxia-exposed DPSCs, result in adipogenic lineage differentiation, *in vitro*.

2. To investigate the differential expression profiling of adipogenic lineage markers in inflamed dental pulp tissue *ex vivo* when compared to healthy pulp tissue.
D. **Hypothesis**

Inflammation or hypoxia alters the differentiation potential of dental pulp tissue towards an adipogenic lineage, resulting in an increase in leptin production and upregulation of leptin-receptor.
II. REVIEW OF THE LITERATURE

A. Pulp Biology and Embryology

Dental pulp is composed of highly vascular and innervated loose connective tissue. The pulp is responsible for supplying the tooth with immune surveillance and defense, nutrition, proprioception, and formation of dentin during development and in response to pathologic stimuli (Yu and Abbott, 2007).

The dental pulp is embryonically formed by the dental papilla. The dental papilla and thus the pulp are derived from cells that migrated from the neural crest (ectomesenchymal cells) and mingled with cells of local mesenchymal origin. The dental papilla is officially termed the dental pulp once its outermost layer of cells differentiate into odontoblasts and lay down dentin (Torabinejad and Walton, 2008).

The pulp is composed of four zones including the odontoblast layer, cell-free zone, cell-rich zone, and pulp core (Berman and Hargreaves, 2016).

The outermost layer is the odontoblast layer which contains odontoblasts, nerve fibers, capillaries, and dendritic cells. The cell-free zone, also known as the zone of Weil, is innervated with nerve networks and is rich with capillaries. The cell-rich zone contains a greater density of fibroblasts and undifferentiated mesenchymal cells. The pulp core is located in the center of the pulp chamber and encompasses nerve fibers, blood vessels, fibroblasts, undifferentiated mesenchymal stem cells, immune cells, ground substance, and collagen (Berman and Hargreaves, 2016).
B. Pulpal Response to Caries

Pulpal inflammation occurs when caries advances to within 1.5mm of the pulp. At this critical distance, bacterial antigens and metabolites can diffuse through the dentinal tubules and directly affect the pulp (Bergenholtz, 1977).

If the bacterial challenge continues, the pulp mounts an immune response which is initially characterized by increased inflammation, edema, and pulpal pain (Hahn and Liewehr, 2007). Inflammation is a protective biologic response intending to remove damaging etiology produced by pathogens and reinstate pulpal equilibrium. However, in the process of mounting these inflammatory mechanisms, host factors may contribute to the injury of the pulp and escalate the ultimate demise of the pulp tissue. Prolonged inflammation of the pulp is of particular concern because the pulp, unlike other connective tissues, is enclosed in a low-compliance environment with minimal collateral circulation which can hasten or intensify the harmful side effects of host inflammatory mediators eventually leading to pulp disintegration and apical pathosis (Cohenca et al., 2013). Prompt clinical intervention, through the removal of decay and bacterial antigens before irreversible pulpitis commences, can resolve pulpal inflammation and promote recovery (Berman and Hargreaves, 2016).

C. Vital Pulp Therapy

Vital pulp therapy (VPT) is defined as a treatment that aims to preserve and maintain the pulp in teeth that have been exposed to caries or trauma. The treatment can be completed in permanent teeth that show normal pulp or reversible injury to the pulp.
VPT is a broad term that encompasses a number of procedures including indirect pulp capping, direct pulp capping, and pulpotomy (Berman and Hargreaves, 2016).

Indirect pulp capping is a VPT indicated for teeth with large carious lesions that approximate the pulp. During an indirect pulp cap procedure, caries are excavated in a step-wise manner so as to slow or arrest caries progression, promote formation of tertiary dentin to protect the pulp, and avoid carious pulpal exposure. Indirect pulp capping usually takes two appointments. At the first visit, the outermost infected dentin is removed—leaving the innermost affected dentin over the pulp—and a sedative restoration, like zinc oxide-eugenol, is placed. In three to six months, the patient presents for the second visit, where the sedative restoration is removed, the remaining caries are excavated, and final restoration is placed (AAPD, 2014).

![Figure 1](image)

**Figure 1 – Indirect Pulp Cap:** (a) Pre-operative radiograph of tooth #19 disto-occlusal decay approximating the pulp; (b) Post-operative radiograph of indirect pulp cap first visit tooth #19, incomplete caries removal with sedative restoration; (c) Post-operative radiograph of indirect pulp cap second visit tooth #19, complete caries removal with final restoration (Fagundes et al., 2009)
Direct pulp capping is a VPT indicated for small pulp exposures incurred as a result of caries removal, trauma, or tooth preparation. The goal of direct pulp capping is to preserve pulp vitality. Direct pulp capping is a single appointment procedure. With rubber dam isolation in place, acutely inflamed pulp tissue is removed, hemostasis is achieved with a moist cotton pellet, and a medicament, like mineral trioxide aggregate (MTA) or calcium hydroxide \((\text{Ca(OH)}_2)\), and final restoration are placed (AAPD, 2014).

![Figure 2 – Direct Pulp Cap](image)

Pulpotomy is a more invasive VPT procedure where the coronal portion of the pulp is removed in order to maintain the vitality of the radicular portion. Pulpotomy is indicated in teeth where caries or trauma exposed a large portion of the pulp. Much like direct pulp capping, pulpotomy is a single appointment procedure where the acutely inflamed coronal pulp tissue is removed, hemostasis is easily achieved with a moist cotton pellet, and a medicament, MTA or \(\text{Ca(OH)}_2\), and final restoration are placed (Berman and Hargreaves, 2016).
Figure 3 – Pulpotomy:  (a) Pre-operative radiograph of tooth #30 occlusal decay to the pulp;  
(b) Post-operative radiograph of pulpotomy tooth #30, complete caries removal with MTA in 
pulp chamber and final restoration

D. Pulpal Regeneration or Revascularization

Pulp regeneration or revascularization is a procedure used to treat immature 
permanent teeth with a necrotic pulp. The primary goal of revascularization is to 
eliminate symptoms and to prevent or resolve apical periodontitis. The secondary goal of 
revascularization is to increase root wall thickness and root length of the immature tooth. 
The tertiary goal is to regain a positive response to pulp sensibility testing which would 
reflect successful regeneration of an organized pulp-like tissue (AAE, 2016).

Revascularization is currently outlined as a two-appointment procedure by the 
American Association of Endodontics. At the first appointment, endodontic access is 
created under local anesthetic and rubber dam isolation. The canal is copiously irrigated 
with 20mL 1.5% sodium hypochlorite (NaOCl) followed by 20mL 17% 
ethylenediaminetetraacetic acid (EDTA) at a rate of 4mL per minute. The canal is then 
dried with paper points. Calcium hydroxide is placed via syringe to the cemento-enamel 
junction (CEJ). And the access is sealed with 2-4mm of temporary restorative material. 
The patient is to return in one to four weeks for the second visit (AAE, 2016).
At the second visit, the patient is anesthetized using local anesthetic without vasoconstrictor. Under rubber dam isolation, the temporary restoration is removed and the canal is irrigated with 20mL of 17% EDTA at a rate of 4mL per minute. The canal is then dried with paper points. Next, bleeding into the canal is achieved by over-instrumenting until the blood reaches the level of the CEJ. A resorbable matrix such as Collacote® (Zimmer Dental, IN, USA) or CollaPlug® (Zimmer Dental, IN, USA) is placed over the blood and capped with a bioceramic material such as mineral trioxide aggregate (MTA). Finally, the access is restored with a 3-4mm layer of light-cured glass ionomer (AAE, 2016).

![Figure 4](image)

**Figure 4 – Revascularization:** (a) Pre-operative radiograph of tooth #18 occlusal decay to the pulp with periapical radiolucency; (b) Post-operative radiograph of revascularization tooth #18, complete caries removal with MTA and glass ionomer restoration

**E. Tissue Engineering Concepts**

VPT and pulp revascularization as described above is clinical application of tissue engineering (Nakashima and Akamine, 2005). The term “tissue engineering” was officially defined in 1988 at the National Science Foundation workshop. The term was coined to describe “the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in
normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function,” (O’Brien, 2011).

Tissue engineering concepts have been compartmentalized into three parts and are collectively referred to the “tissue engineering triad” or the “dogma of tissue engineering” (Scheller et al., 2009). The triad is based on the three main components of tissues—cells (stem cells), their extracellular matrix (scaffolds), and a signaling system (growth factors) (Scheller et al., 2009).

The challenge of tissue engineering is being able to identify and successfully employ the three components of the triad. The stem cells, for example, should be undifferentiated and capable of proliferation and differentiation into the desired cell type. Stem cells may be embryonic or adult; autogenous or allogenic; unaltered or genetically modified (Scheller et al., 2009). The scaffold should be designed in order to provide structure and substrate for tissue growth and development (Murphy et al., 2013). The most important characteristics of a scaffold are biocompatibility, ability to biodegrade, and mechanical properties consistent with the intended anatomical site (O’Brien, 2011). And finally, growth factors or biophysical stimuli should be selected in order to provide appropriate growth and differentiation signals to the cells (Murphy et al., 2013).

F. **Dental Stem Cells**

Stem cells have been isolated from a number of dental tissues including exfoliated deciduous teeth, periodontal ligament, dental papilla, dental follicle of developing third molar teeth, and dental pulp (Aly, 2015). The main advantage of dental stem cells is that they are derived from post-embryonic or adult tissues. Therefore, there are fewer ethical
dilemmas in using them for therapeutic purposes. Another advantage of dental stem cells is that they are able to differentiate into multiple cell types as they are multipotent in nature (Watt and Hogan, 2000).

G. DPSCs

Dental pulp stem cells were first isolated and partially characterized in 2000 by Gronthos et al. The most remarkable characteristic of DPSCs, according to Gronthos et al. (2000), was their ability to differentiate into a mineralized matrix containing odontoblast-like cells, fibrous tissue, and blood vessels in a configuration akin to the dentin-pulp complex found in healthy human teeth.

DPSCs originate from ectodermal-derived neural crest cells. They possess mesenchymal stem cell properties, such as fibroblast-like appearance, ability to adhere to a surface, colony forming potential when cultured in vitro, and multi-lineage differentiation capabilities (Nuti et al., 2016). DPSCs have been shown to differentiate into chondrocytes, adipocytes, odontoblasts, and neural-like cells (Fawzy et al., 2012).

H. Fate of DPSCs in Repair and Revascularization

Stem cell behavior and fate is regulated by intrinsic factors, like transcription factors, as well as extrinsic factors, like systemic and local microenvironmental cues (Lunyak & Rosenfield, 2008). According to Wagers (2012), of these factors, microenvironmental cues have the most influential role.

The microenvironment regulates how stem cells participate in repair and regeneration. The DPSC microenvironment or niche is comprised of numerous elements
including but not limited to connective tissue cells, immune cells, growth factors, cytokines, adhesion molecules, other bioactive molecules, extracellular matrix, and nerve fibers (Lin, 2014). If there is even a slight modification to the niche, the biological behavior and ultimate fate of the DPSCs could be altered (Scadden, 2006).

One such modification is inflammation. Nonspecific mediators of pulp inflammation include neuropeptides, fibrinolytic peptides, kinins, complement fragments, vasoactive amines, lysosomal enzymes, arachidonic acid metabolites, and various cytokines (Torabinejad, 1994).

Since the inflammatory niche affects the biologic behavior of DPSCs, interactions between DPSCs and the inflammatory microenvironment are worth investigating (Zhou, 2015). In 2013, Martin-Gonzalez et al. investigated DPSCs from teeth induced with pulpal inflammation via mechanical exposure of the pulp and found that a hormone, leptin, was up-regulated.

I. **Leptin**

Leptin is a 16-kDa protein hormone comprised of 146 amino acids that is primarily synthesized and secreted by adipocytes. Initially, leptin was described as playing an integral role in metabolism and homeostasis, regulating body weight through the central control of energy intake and expenditure (Martin-Gonzales et al., 2013). However, through more studies, it is evident that leptin is a cytokine-like hormone with multiple actions including vascular, reproductive, mineralization, and immune modulation functions (Lago et al., 2007).
Figure 5 – Peripheral Targets of Leptin: Schematic representation of the peripheral targets of leptin. Abbreviations: IL, interleukin; NO, nitric oxide; TH1, T-helper lymphocyte type 1; TH2, T-helper lymphocyte type 2 (Lago et al., 2007).

In their review, Fantuzzi and Faggioni discuss leptin’s role in inflammation and immunity (2000). The authors discuss leptin as a pro-inflammatory mediator regulating phagocytosis and production of cytokines (Fantuzzi and Faggioni, 2000). Leptin has been show to affect both innate and adaptive immunity by activation of phagocytosis and cytokine production by monocytes and macrophage, chemotaxis and oxidative species productions by polymorphonuclear leukocytes (PMNs), development and maintenance of natural killer (NK) cells, and shifting T-cell response towards T-helper cell (Th1)
cytokine type (interleukin-2 (IL-2) and interferon-γ (INF-γ)) (Karthikeyan and Pradeep, 2007).

Leptin receptor (Lep-R) is expressed in six isoforms which are divided into three classes—long, short, and secretory. Lep-R has been identified in oral tissues including gingiva, crevicular fluid, pulp fibroblasts, and periapical lesions (Martin-Gonzalez et al., 2013).

J. Adipocytes

As mentioned earlier, leptin is synthesized and secreted by adipocytes. Adipocytes are also known as fat cells and are the cells that compose adipose tissue (Rosen and Spiegelman, 2011). Identification of adipocytes in cell culture can be achieved directly through staining with oil red O stain and indirectly through identification of adipogenesis-related proteins.

Oil red O is a fat soluble dye used for identifying triyglycerides and lipids. Positive oil red O stain indicates presence of adipocytes (Mehlem et al., 2013).

Adipogenesis-related proteins used for identification of adipocytes include CD140a, proliferator-activated receptor-gamma (PPAR-γ), AdipoQ, alkaline phosphatase (ALP), apolipoproteins, and lipoprotein lipase (LPL).

CD140a, also known as platelet-derived growth factor receptor-α (or PDGFR-α), is a cell surface tyrosine kinase protein expressed by adipose-derived stem cells (Mohsen-Kanson, 2013). CD140a functions in wound healing and acts as a chemoattractant for fibroblasts, smooth muscle cells, glial cells, monocytes, and neutrophils (BioLegend, 2012).
PPAR-γ is a ligand-dependent nuclear receptor essential for adipocyte terminal
differentiation (Berry et al., 2013). PPAR-γ also has been shown to inhibit the cytokine
production and inflammation (Chawla, 2001).

AdipoQ is an adipose cDNA protein or gene that is highly specific to adipose tissue.
The expression of adipoQ is highly regulated during the adipose differentiation process
and is expressed predominately in adipose tissue (Hu, 1996).

ALP is a hydrolytic enzyme involved in lipid metabolism and gene expression as well
as secretion of adipokines in adipocytes (Hernandez-Mosqueria, 2015). Apolipoproteins
are proteins that form lipoproteins by binding lipids in order for transportation through
the lymphatic and circulatory systems (Saito, 2004). LPL is an enzyme that plays a
critical role in breaking down fat in the form of triglycerides in order for lipid transport to
occur (Dallinga-Thie, 2016).
III. MATERIALS & METHODS

A. Study Design

The aim of the study was two-fold. First, using an in vitro, or simulated stressor, model to investigate why leptin is up-regulated in DPSCs exposed to hypoxic conditions. And second, using an ex vivo, or disease, model to investigate if leptin is up-regulated in the irreversibly inflamed dental pulp, and if so why.

B. Sources of Extracted Teeth

Extracted teeth were obtained according to the protocol approved by the University of Illinois at Chicago (UIC) Institutional Review Board (IRB), Protocol-2011-0129. According to this protocol, written informed consent to donate pulp tissue for research was obtained from either the patient or his/her legal guardian. All teeth were extracted for orthodontic or therapeutic reasons by UIC’s Postgraduate Oral Surgery Department.

C. Procurement of Pulp from Extracted Teeth

Freshly extracted teeth were first washed with 5% sodium hypochlorite to eliminate the remains of periodontal ligament tissue. Teeth were then mechanically fractured using a sterile surgical chisel and mallet. Dental pulp tissue was obtained using sterile tissue forceps under 4.5x loupes magnification.
D. \textit{In Vitro (Simulated Stressor) Model}

1. \textbf{Cell Culture}

Seven (n=7) prophylactically extracted human third molar teeth were used for this portion of the study. All teeth were clinically and radiographically normal, and thus considered healthy. The dental pulp tissue was procured as described above (Sections III.B and III.C).

The dental pulp tissue was enzymatically dissociated in order to obtain DPSCs as follows. First, pulp tissues were transferred into an enzyme solution for 1 hour at 37\(^\circ\)C and vortexed for 30 minutes. Afterwards, large cell aggregates were removed and single-cell suspensions by passing the cells through a 70µm strainer. Cells were maintained and refreshed in basal medium, which contains 10% fetal bovine serum (FBS) in \(\alpha\)-MEM (Life Technologies, NY, USA), until confluency was achieved.

DPSC viability and characterization was performed using flow cytometry analysis.

2. \textbf{Hypoxia Treatment}

Half of the DPSCs were cultured in room air, or normoxia, (21\% oxygen) for 72 hours at 37\(^\circ\)C in 5\% CO\(_2\) in a tri-gas incubator (Thermo Fisher Scientific, MA, USA). The remaining half of the DPSCs were cultured in hypoxia (5\% oxygen) for 72 hours at 37\(^\circ\)C in 5\% CO\(_2\) in a tri-gas incubator (Thermo Fisher Scientific, MA, USA).

Five percent oxygen was selected for the hypoxia treatment in this study because it was determined to be low enough to elicit stressed conditions, but also high enough so as to allow cell survival (Werle et al., 2016).
3. **Adipogenic Differentiation**

For the induction of adipogenic differentiation, DPSCs were seeded in 6 well plates at a density of $2 \times 10^4$/cm$^2$. After the cells reached 80% confluence, they were either cultured in an adipogenic differentiation medium (DM), 0.5 mM methylisobutylxanthine, 0.5 mM hydrocortisone, and 60 mM indomethacin to the α-MEM (10% FBS) (Sigma–Aldrich, MO, USA), or were further cultured in nonspecific IM as a negative control.

The cells were cultured in their respective medium for a total of 14 days and were evaluated for intracellular lipid accumulation by staining with oil red O (Sigma–Aldrich, MO, USA).

E. **Ex Vivo (Disease) Model**

1. **Healthy and Diseased/Inflamed Dental Pulp Collection**

   All dental pulps for this portion of the study were procured as described above (Sections III.B and III.C).

   *Healthy Pulp:* Dental pulp was collected from six (n=6) extracted human premolars with normal pulp, without caries or restorations. All healthy teeth were extracted prophylactically for orthodontic treatment.

   *Diseased/Inflamed Pulp:* Dental pulp was collected from six (n=6) extracted human premolars and molars with a clinical diagnosis of irreversible pulpitis. All diseased teeth were carious without radiographic periapical radiolucency.
2. **Quantification of Leptin, Lep-R, and Adipogenesis-Related Protein Analytes**

Leptin, Lep-R, and adipogenesis-related protein analytes were quantified using BioPlex Luminex® Assay (BioRad, CA, USA). Dental pulp tissue was first treated with antibody-coated bead suspension. The protein analytes were quantified following 2-hour incubation in darkness at room temperature using the Luminex® Assay (analysis of 100 microspheres per region) (BioRad, CA, USA). Protein analytes were compared between healthy and diseased pulp tissues. Fold change was documented. All assays were made in triplicate.
IV. STATISTICAL ANALYSIS

Experimental values were reported as mean ± standard error. Statistical comparison among groups was calculated using the two-tailed Student’s t-test (SPSS for Windows, Version 19, SPSS Inc., NY, USA). A p-value ≤ 0.05 was considered statistically significant.

Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) ratios are also reported. The SILAC ratio reflects the relative protein abundance between groups. According to the manufacturer, BioPlex Luminex® Assay (BioRad, CA, USA), a SILAC ratio above 2 is considered significant.
V. RESULTS

A. *In Vitro (Simulated Stressor) Model*

Dental pulp stem cells exposed to hypoxic conditions had a higher concentration of cells differentiate into adipocytes compared to those DPSCs exposed to normoxic conditions.

![Figure 6 – Adipogenic Differentiation:](image)

*Figure 6 – Adipogenic Differentiation:* This figure contains light microscopy photographs of DPSCs stained with oil red staining. The left column depicts DPSCs treated in adipogenic DM for 14 days in normoxic (21% O₂) conditions. The right column depicts DPSCs treated in adipogenic DM for 14 days in hypoxic (5% O₂) conditions. There is a marked increase in differentiation of DPSCs to adipocytes in hypoxic, or stressed conditions, when cultured in adipogenic DM.

B. *Ex Vivo (Disease) Model*

Leptin and leptin receptor (Lep-R) are significantly (p≤0.05) up-regulated in the diseased pulp compared to healthy pulp.
Figure 7 – Leptin and Leptin-R Receptors in Diseased vs. Healthy Pulp: An upregulation of Leptin and its receptor (Lep-R) in diseased pulp tissue compared to healthy pulp tissue. (* Denotes significant difference, p-value $\leq 0.05$.)

Concurrently, there is a significant ($p \leq 0.05$) increase in adipogenesis-related protein markers including CD140a, peroxisome proliferator-activated receptor-gamma (PPAR-$\gamma$), adipoQ, alkaline phosphatase (ALP), apolipoprotein, and lipoprotein lipase (LDL) proteins.
Figure 8 – Protein Markers for Adipocytes in Diseased vs. Healthy Pulp: An upregulation of CD140a, PPAR-γ, and AdipoQ proteins in diseased pulp tissue compared to healthy pulp tissue. (*) Denotes significant difference, p-value ≤ 0.05.

Figure 9 – Protein Markers for Adipocytes in Diseased vs. Healthy Pulp: An upregulation of alkaline phosphatase, apolipoprotein, and LPL proteins in diseased pulp tissue compared to healthy pulp tissue.
VI. DISCUSSION

The aims of this study were to first investigate on a cellular level how a stressor, hypoxia, effects DPSC differentiation and leptin production. And similarly, on a tissue level, how caries-driven irreversible pulpitis impacts levels of leptin and adipogenesis-related proteins in pulp tissue. With these findings, the molecular mechanisms of pulp repair and regeneration can be better understood and perhaps manipulated in the future to promote faster and higher quality repair and regeneration in vital pulp therapies and revascularization techniques.

Hypoxia was selected as the stressor in the *in vitro* portion of this study for several reasons. First, hypoxia is one of many components of an inflammatory microenvironment. As Yu and Abbott (2007) described in their literature review, the end result of pulpal inflammation is tissue hypoxia.

Second, there is a well-established link between hypoxia and leptin in the literature. In 2001, Grosfeld et al. aimed to investigate the relationship between preeclampsia-induced-placental-hypoxia and leptin. Through their study, Grosfeld et al. was able to demonstrate that decreased oxygen tension is a positive regulatory factor of leptin gene expression. Similarly in 2008, Wang et al. demonstrated that leptin synthesis and release could be readily induced in human subcutaneous preadipocytes and adipocytes by hypoxic (1% O₂ and 5% CO₂) conditions.

And lastly, oxygen concentrations during cell culture could easily be manipulated and controlled in a tri-gas incubator as described earlier. The tri-gas incubator uses nitrogen gas to suppress oxygen (Thermo Fisher Scientific, 2013).

The *in vitro* portion, or simulated stressor model, of this study demonstrates that the stress of hypoxic conditions causes an increase in differentiation of DPSCs to adipocytes.
compared to unstressed, normoxic cells. When considering findings from a preliminary study (Byun et al., 2015) which demonstrated upregulation of leptin and Lep-R receptor with the findings from the current study, the upregulation in leptin and its receptor can be attributed to a concurrent increase in DPSC differentiation into adipocytes.

Similar findings were also demonstrated in the ex vivo, or disease model, portion of this study at the tissue level. Dental pulps with irreversible pulpitis due to caries have a significantly higher concentration of leptin and its receptor as well as adipocyte-related proteins including CD140a, PPARγ, adipoQ, ALP, apolipoprotein, and LPL. Further illustrating that DPSCs and dental pulp tissue are driven to an adipogenic lineage with increased leptin production when stressed by inflammatory conditions.

Leptin has been reported in a number of dental structures including gingiva (Johnson & Serio, 2001), gingival crevicular fluid (Bozkurt et al., 2006; Dilszia et al., 2010), periapical lesions (Haghighi et al., 2010), and in dental pulp (Martin-Gonzalez, 2012).

Martin-Gonzalez et al. (2013) discussed the likelihood of leptin’s role in defensive and reparative responses of dental pulp against deep carious lesions involving an unknown feedback mechanism. The significantly higher relative amount of leptin and Lep-R in dental pulp with irreversible pulpitis found in the present study supports Martin-Gonzalez’s concept that leptin plays a role in dental pulp inflammatory process.

Um et al. (2011), presented evidence that leptin acts as an important modulator of DPSC differentiation. According to their study, treating DPSCs with exogenous leptin for two weeks enhanced cementogenic/odontogenic activity and also inhibited adipogenesis (Um et al., 2011).
In addition to immune modulation and DPSC differentiation, leptin has also been associated with promotion of wound healing in the oral mucosa. Leptin is known to accelerate epithelial cell migration as well as enhance angiogenesis of a wounded area (Umeki et al., 2014).

In their 2015 study, Martin-Gonzalez investigated leptin’s effect on mineralization by evaluating dentin sialophosphoprotein (DSPP) expression in dental pulp. The authors found a dose-dependent relationship between leptin and DSPP. DSPP is a well-known mediator in dentinogenesis and the dental pulp reparative process (Martin-Gonzalez et al., 2015).

Synthesizing the information gained from previous leptin-DPSC studies as well as the results from the present study suggest that DPSCs are driven to an adipogenic lineage with increased leptin production when stressed by inflammatory conditions. In these conditions, leptin aids in inflammation, promotes cementogenic/odontogenic activity, inhibits adipogensis by a negative feedback mechanism, aids in epithelial migration and angiogenesis, and increases the mineralization potential of DPSCs.
VII. CLINICAL RELEVANCE AND LIMITATIONS

With this research, it is evident that leptin plays a valuable role in pulpal inflammation, repair, and regeneration. Perhaps in the future, leptin can be administered exogenously or incorporated as a scaffold, much like growth factors, to increase pro-inflammatory events (Martin-Gonzalez et al., 2013), epithelial migration and angiogenesis (Umeki et al., 2014), and mineralization (Martin-Gonzalez et al., 2015) as well as inhibit adipogenesis (Um et al., 2011) during vital pulp therapies and regeneration procedures.

There are, of course, limitations to this study. First, leptin is not acting independently during inflammation, repair, or regeneration. There are other biomolecules and molecular pathways involved concurrently. In order to elicit accurate and meaningful results, only the leptin pathway was investigated in this study.

Second, medical histories of the tissue donors were unknown. Perhaps obesity, anorexia, diabetes, or other metabolic disorders could have skewed the results of the study. In the future, teeth from such donors should be excluded.

Third, in the \textit{ex vivo} portion of the study, the experimental group of teeth with irreversible pulpitis, were not matched by tooth type (i.e. anterior/premolar/molar), age, gender, or jaw (maxillary/mandibular) to the control group of teeth with normal pulps. These extraneous variables may have influence on the findings.

And finally, the \textit{ex vivo} section of this study used a dichotomous comparison of disease (i.e. irreversible pulpitis) vs health (i.e. normal pulp). However, a dichotomous comparison may be too simplistic as inflammation of the pulp is more detailed than present or absent—inflammation exists in varying degrees of severity as a disease spectrum. A recent systematic
review concluded that there is insufficient evidence to determine whether the presence, nature, and duration of clinical symptoms offer accurate information about the extent of pulp inflammation or pulp disease (Mejare et al., 2012). Therefore, in the future it may be judicious to investigate the effects of varying degrees of inflammation on DPSCs.
VIII. FUTURE RESEARCH

The future for reparative and regenerative endodontics offers promise. Studies like the present study, which investigate biomolecules necessary for dentin-pulp complex repair and regeneration, will provide better understanding and ultimately new therapeutic pathways for exploration.

Some prospects for future research include determining if there is a correlation between dental pulp leptin concentrations and presence or absence of pulpal pathology. And if an association exists, another area for future research is to investigate a chairside method to efficiently ascertain pulpal leptin concentrations so as to provide meaningful information for diagnosis and aid in treatment planning.

Lastly, as alluded to earlier, another opportunity for research is to investigate the administration of exogenous leptin to DPSCs as a therapeutic biomolecule to promote pulpal repair and regeneration. Exploring leptin manipulation within DPSCs will offer key insight into the mechanism of action of leptin, and how leptin interplays with other biomolecules and pathways.
IX. CONCLUSIONS

This study demonstrates that the stress of hypoxia and caries-driven irreversible pulpitis drive differentiation of DPSCs towards an adipogenic lineage with increased leptin production. In the future, leptin may be employed as a therapeutic molecule in bioengineering models to facilitate regenerative endodontics.
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