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Strategies for prevention and treatment of periodontitis are lacking due to difficulty studying this disease in a controlled clinical environment and the lack of suitable animal models. In this study, the Lewis rat was evaluated as a potential model of periodontitis. It was hypothesized that the Lewis rat, when provided a high sucrose/high casein diet (H-SC) long-term (23 weeks), will develop characteristics of periodontitis. This study evaluated: 1) gene expression of local inflammation, disease progression, and bacterial infection in the gingival and alveolar mucosa tissue; 2) gene expression of systemic inflammation in the liver; and 3) metabolic endpoints of body composition, bone densitometric endpoints, and serum leptin and insulin levels. Additionally, age-related differences were evaluated between old control rats and young (6 weeks old) control rats. Interleukin-6 was 2-fold higher in gingival tissue of the old H-SC fed rats when compared to old control rats. No other differences between the old H-SC fed rats and old control rats were found for any other genes in gingival or alveolar mucosa tissue or in the liver. Metabolic endpoints of the old H-SC group were significantly higher in only bone mineral density (5% higher) and liver mass (10% higher) compared to old control rats. Two-fold higher levels of RANKL gene expression and markedly lower metabolic endpoints were evident in young control rats when compared to old control rats, as was expected due to the ~5 month age difference. In conclusion, this animal model developed mild periodontitis. Furthermore, there was no age-related differences between old control rats and young control rats in periodontal endpoints.

EVALUATION OF THE LEWIS RAT AS A MODEL OF PERIODONTITIS: A PRELIMINARY STUDY

by

Stephanie La

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APPROVAL PAGE

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Periodontitis is the inflammation of the periodontium, which includes the gingiva, periodontal ligament, cementum, and/or alveolar bone (Di Benedetto et al 2013). In the development of periodontitis, there is infiltration or overgrowth of toxins and bacterial pathogens, such as *porphyromonas gingivalis*, which causes inflammation within the tissues that surround the teeth. Chronic inflammation, when left untreated, can cause loss of connective tissue and alveolar bone ultimately leading to loss of bone and teeth within the jaw and possibly to the development of osteonecrosis of the jaw (ONJ). Periodontitis is categorized in degree of mild, moderate, and severe. Severe periodontitis is the 6th leading disease in the world (Kassebaum 2014) and is a major health problem. In the United States, mild to severe periodontitis affects 64.7 million adults, which is about half of the adult U.S. population. Of this, severe periodontitis affects 5.5 million people, which is 8.5% of the adult U.S. population (Eke et al 2012). In the 2009-2010 National Health and Nutrition Examination Survey (NHANES), data were evaluated for the prevalence of periodontitis in the United States and it was reported that the prevalence was positively correlated with age, with greatest prevalence in adults and the elderly (Eke et al 2012).

ONJ is characterized as the severe breakdown of tissues in the gingiva of the oral cavity, causing bone exposure and bone loss in the maxillofacial region that does not heal

within 8 weeks after identification by a health care provider (Khosla et al 2007), which differentiates it from severe periodontitis. ONJ has been associated with the use of high-dose nitrogen containing antiresorptives, used for bone disease and cancer treatment, but not with lower doses, used for treatment of osteoporosis (Bilezikian 2006, Marx 2003, Khosla et al 2007, Thumbigere-Math et al 2012). Patients currently taking high doses of antiresorptives or bisphosphonates, who have preexisting periodontitis, may be at greater risk of developing osteonecrosis of the jaw.

Historically, research evaluating periodontal disease has been conducted using a number of animal models, including rabbits and dogs. However, results of these animal models have been hard to generalize to humans because location, development, and appearance of periodontal abnormalities do not closely characterize human periodontitis (Oz and Puleo 2011). Rats, specifically Rice rats, develop spontaneous periodontitis similar to, but more severe than, human disease progression and conditions when fed a high sucrose/high casein (H-SC) diet (Aguirre et al 2012a,b). Rice rats are not available commercially, and therefore must be purposely bred for each experiment. Thus, their general use as an animal model of periodontitis and/or ONJ is limited because of the need to breed the animals, the accompanying technical expertise needed, time involved, and greater costs involved.

As a focus of this study, the Lewis rat was evaluated as a potential model of periodontitis because this strain of rats have a genetically determined defect in their immune system of the central nervous system (Breivik et al 2000). This defect makes the Lewis rat susceptible to experimental induced autoimmune diseases, such as rheumatoid

arthritis (Shi et al 2012), encephalomyelitis (Kurkowska-Jastrzebska et al 2013), and myocarditis (Schmerler et al 2014). This study evaluated the extent that the Lewis rat develops periodontitis under the same experimental conditions used by Aguirre et al in Rice rats (Aguirre et al 2012a,b). As the overall main research question, this preliminary study evaluated if the Lewis rat would be a suitable animal model to study human periodontitis by observing characteristics of periodontal disease production and development when provided the H-SC diet for several months. Inflammation, level of disease and bacterial infection, and metabolic endpoints were evaluated. Since the H-SC diet was used to induce periodontitis, the evaluation of this diet on a number of systemic inflammatory and metabolic outcomes were also examined, as a high sucrose diet has resulted in hyperlipidemia, hyperinsulinemia, hepatic steatosis, and increased inflammatory cytokines in the liver (Oliveira et al 2014). The three aims of this study were to evaluate: 1) local inflammation, disease progression, and bacterial infection in the oral gingival mucosa and oral alveolar mucosa of old H-SC fed rats, compared to old control rats, by evaluating gene expression of cytokines, a proteinase, and bacterial receptors; 2) systemic inflammation of the old H-SC fed rats, compared to old control rats, by evaluating gene expression of several cytokines in the liver; and 3) to evaluate metabolic endpoints of the old H-SC fed rats, compared to old control rats, by evaluating body composition, bone densitometric endpoints, and serum leptin and insulin levels. It was hypothesized that the Lewis rat, when provided the H-SC long-term (23 weeks), will develop characteristics similar to periodontitis under the same experimental conditions as

Rice rats without marked systemic inflammation or alterations in metabolic outcomes, making this a possible new animal model for studying periodontitis

CHAPTER II

REVIEW OF LITERATURE

Periodontitis

Severe periodontitis is the 6th leading disease in the world (Kassebaum 2014) and is a major health problem. In the United States, data evaluated from 2009-2010 NHANES indicate that 47.2% of the adult U.S. population has periodontitis (Eke et al 2012), representing about 64.7 million adults, ages 30 years and older. This study was the first NHANES study to look at a full-mouth examination to assess for mild, moderate, and severe periodontitis. They found that within the adults affected with the disease, 8.7% had mild, 30% had moderate, and 8.5% had severe periodontitis. Periodontitis is the inflammation of the periodontium, including gingival tissue, periodontal ligament, cementum, and/or alveolar bone within the jaw (Di Benedetto et al 2013). Mild periodontitis within the 2009-2010 NHANES study is characterized as ≥ 2 mm of attachment loss surrounding the tooth at the affected site. Moderate periodontitis is ≥ 4 mm of attachment loss at 2 sites surrounding the tooth at the affected sites. Severe periodontitis is ≥ 6 mm of attachment loss at 2 or more sites surrounding the tooth at the affected sites (Eke 2012). ONJ, different from severe periodontitis, is characterized as the severe breakdown of tissues in the gingiva of the oral cavity, causing teeth loss, bone exposure, and major alveolar bone loss in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider (Khosla et al 2007).

Immunocompromised individuals who are also undergoing high doses of bisphosphonate treatment are most likely to develop ONJ (Bilezikian 2006, Marx 2003, Khosla et al 2007, Thumbigere-Math et al 2012).

Little information is known about susceptibility, etiology, and mechanisms of periodontitis progression, as it is challenging to evaluate this disease in humans. Genetic and environmental risk factors may increase individual susceptibility to periodontitis, such as older age (Eke et al 2012), tobacco use (Van Dyke and Sheilesh 2005), poor oral hygiene (Pihlstrom et al 2005), and individual bacterial response (Van Dyke and Sheilesh 2005, Pihlstrom et al 2005), but not much information is known about which individuals are more susceptible to developing periodontitis. In individuals who are susceptible, the bacteria involved induces a severe inflammatory state within the affected areas. As the number of bacteria and toxins increase and develop severe inflammation, especially when left untreated, the bacteria and toxins colonizing the gingiva can travel into the bloodstream and give rise to systemic inflammation that may increase susceptibility to acute and chronic inflammatory reactions as well as to possible systemic diseases (Li et al 2000). For those who are not susceptible, the bacteria tends to never progress into any inflammation or disease state. However, immune-compromised individuals, such as people with rheumatoid arthritis, have a higher susceptibility in developing this disease due to immune dysregulation and autoimmune responses (El-Shinnawi et al 2013).

Animal Models for Studying Periodontitis

Current animal models for studying periodontitis usually involve manipulation of the oral cavity, such as an inoculation of periodontopathogens within the gingiva or a forced tooth movement model (Oz and Puleo 2011). A forced tooth movement model is done by tying a ligature around the molars of a rat along with a collection of the gingival crevicular fluid, the fluid located between the tooth and periodontal gingival tissue, to look at inflammation associated with periodontitis. Inoculations of periodontopathogens, such as *P. gingivalis*, and ligature tying (Oz and Puleo 2011, Meulman et al 2011) can result in the identification of specific localized inflammatory markers of periodontitis in response to a physical insult, but these models do not mimic human periodontal disease which involves the entire gingiva (Oz and Puleo 2011, Meulman et al 2011).

The lack of a commercially available and suitable animal model, that is comparable to human onset, progression, or physiology, hampers the investigation of periodontitis. A suitable, but not widely available, animal model using Rice rats fed a H-SC diet was developed five decades ago (Gupta and Shaw 1956a,b). The use of this animal model was not pursued further, at that time, because researchers thought the progression of disease was too rapid and not comparable to humans. In 2011, re-evaluation of the suitability of the Rice rat model to study periodontal disease was reported by Aguirre et al (2012a). Aguirre et al (2012a) altered experimental conditions, such as providing the diets in pelleted form rather than powder form, and accounting for variations of genes in the rat colonies, which reported as a slower and therefore more suitable animal model for studying disease progression that more closely mimicked

human disease. Additionally, the development of advanced histomorphometry, morphometry, histochemistry, and micro-computed tomography technologies, enhanced the analytical evaluation of samples.

A H-SC diet, used as an inducer of periodontitis, was fed to Rice rats for 18-24 weeks (Aguirre et al 2012a). Disease characteristics and progression were evaluated at intervals throughout the study. Both the maxilla and mandible bones of the jaw from the rats were analyzed for disease lesions, alveolar bone loss, histomorphometry, and histologic characteristics. These investigators demonstrated periodontal lesions that progressed at a rate that more closely mimicked periodontal progression in humans that was reported by Gupta and Shaw (1956a, b). They also observed consistent progressive alveolar bone loss, bone remodeling, and gingival recession starting at 12 weeks, which is a characteristic of periodontitis observed in humans. Interestingly, they reported more periodontal disease-like characteristics in the female rats than in the male rats.

In a subsequent study conducted by Aguirre and coworkers (2012b), the researchers were able to replicate the results of inducing moderate to severe periodontal lesions with the addition of ONJ. Osteonecrosis of the jaw is characterized as the severe breakdown of tissues in the periodontium of the oral cavity, causing bone exposure and bone loss in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider (Khosla et al 2007). Osteonecrosis of the jaw has been associated with the use of high-dose nitrogen containing antiresorptives, used for bone disease and cancer treatment, but not with lower doses, used for treatment of osteoporosis (Bilezikian 2006, Marx 2003, Khosla et al 2007, Thumbigere-Math et al 2012). Results

indicated the H-SC diet and added bisphosphonate treatment resulted in ONJ with severe bone lesions only seen in the rats who developed periodontitis, compared to those in the control group (Aguirre et al 2012b). Results of this study suggest that patients currently taking high doses of bisphosphonates, who have preexisting periodontitis, may be at greater risk of developing osteonecrosis of the jaw. These authors concluded that long term feeding of the H-SC diet, along with high dose bisphosphonates, created an optimal microenvironment that allowed bacteria to proliferate and activate inflammatory responses responsible for loss of bone and soft tissues relating to periodontal disease progression (Aguirre et al 2012a,b). However, disease progression was more rapid than observed in human ONJ and these studies are hampered by the lack of commercially available Rice rats. Use of the Rice rat is limited because of the need to breed the animals for each experiment, and the accompanying technical expertise, time to breed for animals, and greater costs incurred in managing a breeding colony.

The current study, evaluating the Lewis rat as a possible alternative animal model, was undertaken because these rats are commercially available and have a known susceptibility to experimentally induced autoimmune diseases. The Lewis rat has a genetic defect in the central stress response of its central nervous system (Breivik et al 2000). This genetic defect affects the reactivity of the hypothalamic pituitary adrenal axis affecting susceptibility to infectious disorders. Within the genetic defect, stress elicited in the body by either infections or inflammation causes the paraventricular nucleus of the hypothalamus to release corticotropin releasing hormone, which stimulates the anterior pituitary gland to secrete adrenocorticotropin hormone. The adrenocorticotropin hormone

releases glucocorticoids in abnormally higher and longer lasting levels than when compared to species that do not have a genetic defect in the stress response system. The high levels of glucocorticoids can shift the ratio balance between T helper cells Th1 to a more dominate Th2. The dominate Th2 response results in improper and insufficient immune responses that can increase susceptibility to diseases, such as periodontitis (Breivik et al 2000). In other studies using the Lewis rat, the strain has been susceptible to experimental induced autoimmune diseases, such as myocarditis (Schmerler et al 2014) and rheumatoid arthritis (Shi et al 2012).With this genetic defect in this strain of rat and its wide commercial availability, the Lewis rat may be considered a potential animal model to study periodontitis.

Periodontal Inflammation

In animal models, using the forced tooth movement and ligature tying methods, studies have been able to study proinflammatory cytokines and genes associated with inflammation in the gingiva (D'Apuzzo et al 2013, Nishijima et al 2006). As an initial pathological onset of periodontitis, the inflammatory response of interleukin-1 beta (IL-1β) is stimulated and is considered to be the most important cytokine in the gingiva for forced tooth movement-induced periodontitis (D'Apuzzo et al 2013). IL-1β is known to promote osteoclastogenesis by activating the receptor activator of nuclear factor-kβ ligand (RANKL) (Nishijima et al 2006). IL-1β is also an inducer of interleukin-6 (IL-6), which regulates inflammatory responses at inflamed sites. TNF-α is a cytokine that represents acute or chronic inflammation and stimulates bone resorption (D'Apuzzo et al 2013). Ligature tying used as a method, can result in a specific identification of localized

inflammatory markers of periodontitis, however it would not be a suitable method or model to study as it does not mimic human periodontitis which affects the entire periodontium.

A H-SC diet was used in the current study to induce periodontitis in the Lewis rats. The H-SC diet, used previously by Gupta and Shaw (1956a,b) and Aguirre et al (2012a,b), provides an ideal optimal environment for bacteria in the gingiva to grow. Past different combination of diets, such as only high sucrose produced too much inflammation rapidly, and only casein and lard produced a reduction of periodontal lesions, were not ideal diets for inducing periodontitis in an animal model (Gupta and Shaw 1956a,b, Keyes 1954) .

A key bacterial pathogen normally observed in periodontitis is *P. gingivalis* (Sloan et al 2013, Meulman et al 2011, Darveau et al 1997, Li et al 2000). This species of bacteria along with other pathogens and toxins cause inflammatory bone destruction by activating a cascade of inflammatory signaling events. In a study looking at an *ex vivo* culture model of inflammatory bone destruction, Sloan et al (2013) treated cells from mandible bone slices of a CD-1 mice with *P. gingivalis* and examined matrix proteins, osteoclasts, macrophages, and neutrophils. As a result, loss of structure within the bone, increased inflammatory responses from interleukins and tumor necrosis factor alpha (TNF-α), and increased osteoclastogenesis was seen (Sloan et al 2013). These results suggest that inflammatory bone loss is associated with *P. gingivalis* pathogenesis in periodontitis.

Collection of gingival crevicular fluid has also been used to assess and characterize level of bacterial and disease presence of periodontitis in human subjects (Baliban et al 2013, Tsuchida et al 2013, Swaminathan et al 2013). In a study looking at gingival crevicular fluid in periodontal disease subjects and healthy subjects, many different proteins biomarkers of disease quantified by proteomic analysis were found in samples from the diseased subjects when compared to the healthy subjects. Matrix metalloproteinase-9 (MMP-9), stimulated from inflammatory cytokines caused from bacterial infiltration and overgrowth, induces connective tissue degradation (Tsuchida et al 2013) and were primarily focused on by the researchers because elevated levels of MMP-9 were seen in the subjects that suffer from periodontitis than in healthy subjects. They reported a positive correlation between MMP-9 level and disease presence (Baliban et al 2013, Tsuchida et al 2013). In a study evaluating bacterial presence and infection in human subjects with chronic periodontitis, compared to healthy subjects, unstimulated whole saliva was collected and evaluated for the role of salivary epithelial cells on toll-like receptors, which mediate host recognition of bacteria. Results indicated that there was an increase in toll-like receptor 2 and 4 (TLR-2, TLR-4) in the saliva of subjects with chronic periodontitis compared to healthy subjects (Swaminathan et al 2013). Bacteria are recognized by TLR-2 and TLR-4, located on the cell surface, which then activates signaling for cytokine stimulation, inducing periodontal disease pathogenesis. TLR-2 specifically recognizes a variety of pathogen-associated molecular patterns, such as lipoproteins, lipotechoic acids, and peptidoglycans and TLR-4 recognizes bacterial lipopolysaccharides (LPS) (Kawasaki and Kawai 2014).

Periodontal disease has also been shown to produce systemic inflammation. In a study looking at a mouse liver hepatocyte cell line, Hepa-1.6, and a mouse macrophage cell line, RAW 264, treated with periodontal bacteria, *P. gingivalis*, researchers were able to see an increased stimulation level in inflammatory cytokines TNF-α and IL-6 (Takano et al 2012), similar to those seen in periodontitis. In a study looking at systemic inflammatory markers and periodontal disease in an elderly population, researchers found that systemic inflammatory markers of plasma IL-6, C-reactive protein (CRP), and TNF-α were significantly higher in participants with a more severe state of periodontal disease than in others with a more mild state of disease (Bretz et al 2005).

Periodontal disease associated with systemic inflammation was reported in human participants in a nationwide 1999-2004 NHANES study. This study also reported greater prevalence of periodontal disease in individuals with insulin resistance (Demmer et al 2012). In this NHANES study, periodontal examinations looking at probing depth and attachment loss at sites of teeth and fasting blood draws were assessed in non-diabetic adults. Researchers suggested there may be a possible relationship between periodontitis and diabetes in humans, although this association was not directly evaluated. In a Sprague-Dawley rat animal model, evaluating inflammation levels induced by ligature tying in diabetic rats and non-diabetic rats, researchers found that diabetic rats with ligature showed an increased TNF-α, IL-1β, and LPS production in the gingiva when compared to the non-diabetic rats with ligature and the non-diabetic, non-ligature control rats. (Jiang et al 2013). This study found that periodontitis and diabetes can both contribute to a more enhanced inflammatory response than in non-diabetic rats. Overall,

Demmer et al (2012) and Jiang et al (2013) provide evidence of a possible relationship between periodontitis and diabetes.

The H-SC diet, containing 70% of kcals as sucrose and 18% of kcals as casein, may also influence the metabolic profile of the rats. A diet containing 30% sucrose increased body fat mass and the development of leptin and insulin resistance in rats within 18 days (Vasselli et al 2013). The H-SC diet, as replicated from Aguirre et al (Aguirre et al 2012a,b), contains 70% sucrose providing a possible influence on the metabolic profiles of the Lewis rats. The impact on H-SC on bone densitometric endpoints was unknown.

In conclusion, severe periodontitis is the sixth leading disease in the world (Kassebaum 2014) and a major health problem affecting 5.5 million U.S. adults (Eke et al 2012). Genetic and environmental risk factors that may increase a person's susceptibility of developing periodontitis include older age (Eke et al 2012), tobacco use (Van Dyke and Sheilesh 2005), poor oral hygiene (Pihlstrom et al 2005), and response to bacterial presence (Van Dyke and Sheilesh 2005, Pihlstrom et al 2005). Additionally, there is possibly a greater susceptibility in individuals who are immunocompromised, such as those with rheumatoid arthritis (El-Shinnawi et al 2013) and lupus (Kobayashi et al 2003). Furthermore, there is a greater risk of developing ONJ, with high dose bisphosphonate treatment or oral surgery in a person with periodontitis (Bilezikian 2006, Saia et al 2010). There is a lack in knowledge of human periodontal disease and ONJ etiology and mechanisms of disease progression. Research in this area has been

hampered by the complexity of studying the disease in humans and by the lack of a commercially available animal model.

CHAPTER III

RESEARCH QUESTION AND SPECIFIC AIMS

Research Question: Will the Lewis rat, when provided a long term (23 week) H-SC diet, develop characteristics of periodontal disease without developing systemic inflammatory and altered metabolic endpoints compared to age-matched (old) control rats? Since this is the first evaluation of this animal model, measurements of the old control rats will also be compared to those of young control rats, in order to evaluate the extent that the "control" values change, compared to starting baseline values.

Aim 1: To evaluate local inflammation, disease progression, and bacterial infection in the oral gingival mucosa and oral alveolar mucosa of old H-SC rats, compared to old control rats, by evaluating gene expression of cytokines, a proteinase, and bacterial receptors.

> Objective: Gene expression of gingival mucosa (~2mm wide section of tissue of the oral mucosal membrane on the buccal surface in close proximity to the three molars (M1-M3) of the left mandible) and alveolar mucosa (~2mm oral mucosal membrane adjacent to the gingival tissue, but that is more distal from M1-M3) will be evaluated using quantitative real time polymerase chain reaction (qPCR) and analyzed for genes that suggest local inflammation and disease progression (IL-1β, IL-6, RANKL, MMP-9, and TNF-α) and bacterial infection (TLR-2 and TLR-4). Results will be compared between old control and old H-SC rats, and also compared between old control and young control rats.

Aim 2: To evaluate systemic inflammation of the old H-SC rats, compared to old control rats, by evaluating gene expression of several cytokines in the liver.

Objective: Gene expression of liver samples will be quantified by qPCR and analyzed for systemic inflammation by evaluating mRNA of IL-1β, IL-6, and TNF-α. Results will be compared between old H-SC and old control rats, and also compared between old control and young control rats.

Aim 3: To evaluate metabolic endpoints of the old H-SC rats, compared to old control rats, by evaluating body composition, bone densitometric endpoints, and serum leptin and insulin levels.

Objective: Metabolic endpoints will be evaluated, including body composition (percent fat, tissue mass, fat mass, and lean mass), bone densitometric endpoints (bone mineral density (BMD), bone mineral content (BMC), and bone area), and serum leptin and insulin levels. Results will be compared between old H-SC and old control rats, and also between old control and young control rats.

Results from the current study provide an initial characterization of the Lewis rat's response to the H-SC diet. Results of the current study will be evaluated along with concurrent histological evaluation of the maxilla and mandible bones of these animals (conducted by collaborators and is beyond the scope of the thesis project). These results provide important insights into determining whether the Lewis rat has potential to be considered an appropriate animal model in evaluation of human periodontal disease.

CHAPTER IV

EVALUATION OF THE LEWIS RAT AS A MODEL OF PERIODONTITIS: A PRELIMINARY STUDY

Abstract

Strategies for prevention and treatment of periodontitis are lacking due to difficulty studying this disease in a controlled clinical environment and the lack of suitable animal models. In this study, the Lewis rat was evaluated as a potential model of periodontitis. It was hypothesized that the Lewis rat, when provided a high sucrose/high casein diet (H-SC) long-term (23 weeks), will develop characteristics of periodontitis. This study evaluated: 1) gene expression of local inflammation, disease progression, and bacterial infection in the gingival and alveolar mucosa tissue; 2) gene expression of systemic inflammation in the liver; and 3) metabolic endpoints of body composition, bone densitometric endpoints, and serum leptin and insulin levels. Additionally, age-related differences were evaluated between old control rats and young (6 weeks old) control rats. Interleukin-6 was 2-fold higher in gingival tissue of the old H-SC fed rats when compared to old control rats. No other differences between the old H-SC fed rats and old control rats were found for any other genes in gingival or alveolar mucosa tissue or in the liver. Metabolic endpoints of the old H-SC group were significantly higher in only bone mineral density (5% higher) and liver mass (10% higher) compared to old control rats. Two-fold higher levels of RANKL gene expression and markedly lower metabolic endpoints were evident in young control rats when compared to old control

rats, as was expected due to the ~5 month age difference. In conclusion, this animal model developed mild periodontitis. Furthermore, there was no age-related differences between old control rats and young control rats in periodontal endpoints.

Introduction

Periodontitis is the inflammation of the periodontium, including gingiva, periodontal ligament, cementum, and/or alveolar bone (Di Benedetto et al 2013). In the development of periodontitis, there is infiltration or overgrowth of toxins and bacterial pathogens, which causes inflammation within the tissues that surround the teeth. If left untreated, the development of severe inflammation can lead to loss of connective tissue and alveolar bone ultimately leading to loss of bone and teeth within the jaw and possibly to the development of osteonecrosis of the jaw (ONJ). Periodontitis is categorized in degree of mild, moderate, and severe. Severe periodontitis is the 6th leading disease in the world (Kassebaum 2014) and is a major health problem. In the United States, mild to severe periodontitis is affects 64.7 million adults. Importantly, severe periodontitis affects 8.5% (5.5 million) in the U.S. (Eke et al 2012).

ONJ is characterized as the severe breakdown of tissues in the gingiva of the oral cavity, causing bone exposure and bone loss, which differentiates it from severe periodontitis. ONJ has been associated with the use of high-dose nitrogen containing antiresorptives, used for bone disease and cancer treatment, but not with lower doses, used for treatment of osteoporosis (Bilezikian 2006, Marx 2003, Khosla et al 2007, Thumbigere-Math et al 2012). Patients currently taking high doses of antiresorptives or

bisphosphonates, who have preexisting periodontitis, may be at greater risk of developing osteonecrosis of the jaw.

Genetic and environmental risk factors may increase individual susceptibility to periodontitis and ONJ, but limited information is known about susceptibility, etiology, and mechanisms of disease progression, as it is challenging to evaluate this disease in humans. In susceptible individuals, the bacteria colonizing the gingiva induces a severe inflammatory state within the affected areas and can increase susceptibility to acute and chronic inflammatory reactions and diseases (Li et al 2000). In non-susceptible individuals, inflammation does not occur.

Strategies for prevention and treatment of periodontitis are lacking due to a low abundance of clinical research and difficulty studying this disease in a controlled clinical environment, as well as a lack of suitable experimental animal models. Current animal models for studying periodontitis usually involve manipulation of the oral cavity, such as an inoculation of periodontopathogens within the gingiva (Meulman et al 2011) or a forced tooth movement of an individual tooth (D'Apuzzo et al 2013, Nishijima et al 2006). The forced tooth manipulation model can result in the identification of specific localized inflammatory markers of periodontitis in response to the physical insult, but these models do not closely mimic human periodontal disease and progression, which involves the entire gingiva (Meulman et al 2011). The Rice rat is the only animal model currently available, where severe periodontitis and ONJ can be induced in the entire gingiva, and most closely resembles human disease progression and conditions. This spontaneous severe periodontitis occurs when fed a high sucrose/high casein (H-SC) diet;

ONJ is then induced when these rats have developed the severe periodontitis and then are given high dose bisphosphonates (Aguirre et al 2012a,b). However, Rice rats are not available commercially, and therefore must be purposely bred for each experiment. Additionally, the H-SC induction of severe periodontitis occurs rapidly in the Rice rat. Thus it would be useful to have access to a commercially available rat model, with the well described immunological abnormalities and for which the required technology and known genome are available.

Therefore, in this study, the Lewis rat was evaluated as a potential model of periodontitis as it is commercially available and has a genetically determined defect in its immune system of the central nervous system. This genetic defect effects the reactivity of the hypothalamic pituitary adrenal axis which in turn causes an abnormally high and long lasting release of glucocorticoids levels than when compared to species that do not have a genetic defect. The abnormal glucocorticoid level causes an increase in susceptibility to infectious disorders and diseases, such as periodontitis (Breivik et al 2000). This defect makes this rat strain susceptible to experimental induced autoimmune diseases, such as rheumatoid arthritis (Shi et al 2012), encephalomyelitis (Kurkowska-Jastrzebska et al 2013), and myocarditis (Schmerler et al 2014).

This study evaluated the extent that the Lewis rat develops periodontitis under the same experimental conditions used by Aguirre et al in Rice rats (Aguirre et al 2012a,b). The specific aims of this study were to evaluate: 1) local inflammation, disease progression, and bacterial infection in the oral gingival mucosa and oral alveolar mucosa of old H-SC rats, compared to old control rats, by evaluating gene expression of

cytokines, a proteinase, and bacterial receptors; 2) systemic inflammation of the old H-SC rats, compared to old control rats, by evaluating gene expression of several cytokines in the liver; and 3) metabolic endpoints of the old H-SC rats, compared to old control rats, by evaluating body composition, bone densitometric endpoints, and serum leptin and insulin levels. As a secondary analysis, age-related differences were evaluated between old control rats and young control rats. The evaluation of the H-SC diet on a number of systemic inflammatory and metabolic outcomes were examined, as a high sucrose diet has resulted in hyperlipidemia, hyperinsulinemia, hepatic steatosis, and increased inflammatory cytokines in the liver (Oliveira et al 2014). It was hypothesized that the Lewis rat, when provided the H-SC long-term (23 weeks), will develop characteristics similar to periodontitis under the same experimental conditions as Rice rats without marked systemic inflammation or alterations in metabolic outcomes, making this a possible new animal model for studying periodontitis.

Study Design and Methodology

Animals and Diet

Five week old female Lewis rats (LEW/SsNHsd, Harlan Laboratories, Indianapolis, IN) weighing on average of 125g ± 7 (5 week old) upon arrival were used for this experiment. Rats were weighed biweekly. The control diet was the Teklad Global 18% protein diet (control diet; Teklad Diets, Madison, WI). The H-SC diet, a modification of the AIN93G (Purina TestDiet, St. Louis, MO), contained 70% kcals from sucrose, 18% from casein, and 12% from fat (Aguirre et al 2012a,b). This study was

approved by The University of North Carolina at Greensboro Institutional Animal Care and Use Committee.

Study Design

Rats were assigned to one of three groups: 1) young control rats, used as a control baseline measurement (n=8), were provided a pelleted control diet and water ad libitum for 1 week; 2) old control rats, used as a control group (n=12), were provided a pelleted control diet and water ad libitum throughout the long-term feeding period; 3) old H-SC rats, used as an experimental group (n=20), were provided a pelleted H-SC diet and water ad libitum throughout the long-term feeding period. Old rats were provided the H-SC or control diet for 23 weeks (June 2013 - December 2013) after 1 week acclimation to the diet. These "old" rats were 29 weeks old at the end of the feeding period. Young control rats arrived 1 week prior to euthanasia and were used to provide baseline data to compare age related differences with old control rats. Animals were housed two per cage with a controlled room temperature, humidity, and 12 hour light/dark cycle. Five days prior to the end of the study, animals were anesthetized for body composition and bone density measurements. At the end of the study, rats were fasted for 12 hours prior to necropsy. All rats were euthanized with CO_2 and tissue, blood, and bone samples were collected as described below.

Total Body Composition and Bone Densitometry

Five days prior to sample collection, body composition and bone densitometric endpoints were assessed with a Dual-energy X-ray absorptiometry (DXA) machine

(Prodigy, GE Healthcare Lunar). Rats were anesthesized with 2.5 - 4.5% isoflurane and scanned for analysis of bone mineral density (BMD), bone mineral content (BMC), bone area, percent fat, tissue mass, fat mass, and lean mass. Percent body fat was calculated using the following formula: fat(g)/(fat(g) + lean(g) + BMC)*100.

Tissue Collection

Gingival tissue, located ~2 mm adjacent to M1 and M2 molars on the buccal surface, from the left mandibles of the rats were extracted and collected. Alveolar mucosa, located ~2mm adjacent to the gingival tissue, were also collected for use as an internal control for each animal. Gingival and mucosa tissues were put in 150 µL of RNAlater Stabilization Reagent in microfuge tubes for immediate stabilization of RNA in the tissues. Whole liver was removed and weighed. Sections of liver tissue were placed in 650 µL of RNAlater in microfuge tubes.

Blood Collection

Blood was collected in unheparinized syringes, and placed in conical tubes at room temperature to coagulate. Serum was obtained by centrifuging tubes at 3,000 rpm for 15 minutes and stored in a -20 refrigerator. Serum was used to evaluate insulin levels (Rat/Mouse Insulin ELISA; Millipore, Billerica, MA) and leptin levels (Rat Leptin ELISA; Millipore, Billerica, MA).

Bone Collection

The right mandibles and right maxillas of the rats were removed and placed in 20 mL vials filled with 10% formalin at a 9:1 ratio for 48 hours, then rinsed, and replaced with 70% ethanol. The left mandible and left maxillas after removal were stored similar

to the right mandibles and maxillas. A micro-computed tomography scan and histological analysis was completed by collaborators, Dr. Aguirre and Dr. Yarrow, on the mandibles and maxillas to detect any differences in treatment groups or with age (beyond the scope of this thesis project).

mRNA Analysis

Total RNA from tissue samples was isolated and extracted for gene analysis by qPCR. The gingival and mucosa tissues were analyzed for genes that suggest inflammation and bacterial infection. Genes reflecting the inflammatory response of periodontitis were tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), nuclear factor-kβ ligand (RANKL), and matrix metalloproteinase-9 (MMP-9). The genes reflecting bacterial infection in periodontitis are toll-like receptor 2 (TLR-2), and toll-like receptor 4 (TLR-4). Liver tissue samples were evaluated for indications of systemic inflammation. The genes that were analyzed to evaluate systemic inflammation were TNF-α, IL-1β, and IL-6.

Statistical Analysis

Data are presented as mean ± SEM. Student's t-tests were conducted to evaluate the significance of differences between old H-SC and old control groups (primary focus of research) and between the old control and young control groups (secondary focus of research). Statistics were performed using SPSS statistics (version 17; Chicago, IL). $P < 0.05$ was considered significant.

Results

Gingival Tissue and Alveolar Mucosa Tissue Gene Expression

Mandibular gingival tissue and alveolar mucosal tissue were evaluated for genes reflective of the inflammatory response (Figure 1). All results were normalized to β-actin. Gingival tissue gene expression of IL-6 (Figure 1A) was ~2-fold higher in the old H-SC group than the old control group ($p < 0.05$). Gingival tissue gene expression of IL-1β (Figure 1B), TNF-α (Figure 1C), and RANKL (Figure 1D) were similar between old control and old H-SC groups ($p > 0.05$). Alveolar mucosa tissue gene expression of IL-6 (Figure 1E) and IL-1β (Figure 1F) were similar between old control and old H-SC groups ($p > 0.05$). Gene expression of TNF-α and RANKL were undetectable in the alveolar mucosa tissue. A significant ~2-fold age-related difference between the young control group and old control group was evident only for gingival tissue RANKL (Figure 1D). There were no significant differences in expression of any other inflammatory genes for either gingival tissue or alveolar mucosa tissue between young and old control groups ($p > 0.05$).

Figure 1

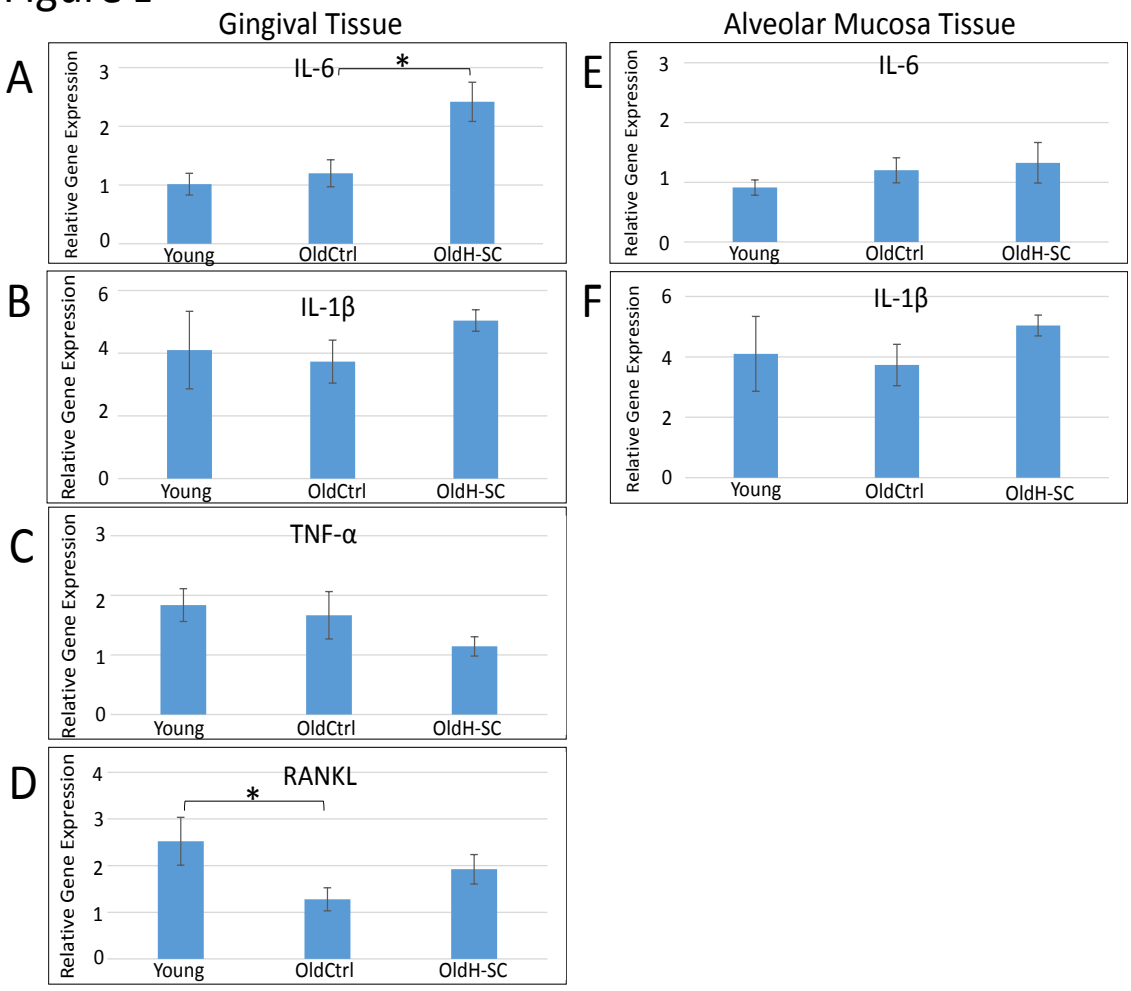

Figure 1. Expression of Genes Reflective of the Inflammatory Response in Mandibular Gingival Tissue and Alveolar Mucosa Tissue. All samples were normalized to β-actin. (A) Gingival IL-6 (B) Gingival IL-1β (C) Gingival TNF-α (D) Gingival RANKL (E) Mucosa IL-6 (F) Mucosa IL-1β. Mucosa TNF-α and RANKL gene expression were undetectable. Mean +/- SEM. * Significance differences ($p < 0.05$) between groups were determined by Student's *t*-test. Young, n = 4-6; old control n = 8-9; H-SC, n = 12-16. Young = young control group; old ctrl = old control group; old H-SC = old high sucrose/high casein group.

Mandibular gingival tissue and alveolar mucosa tissue were evaluated for genes reflective of disease progression and bacterial infection (Figure 2). All results were normalized to β-actin. There were no significant differences between old control and old H-SC group or between young control and old control group ($p > 0.05$), for gene

expression of gingival tissue MMP-9 (Figure 2A), gingival tissue TLR-2 (Figure 2B), and gingival tissue TLR-4 (Figure 2C). Additionally, there were no significant differences between old control and old H-SC group or between young control and old control group ($p > 0.05$), for gene expression of alveolar mucosa tissue MMP-9 (Figure 2D), alveolar mucosa tissue TLR-2 (Figure 2E), an alveolar mucosa tissue TLR-4 (Figure 2F).

Figure 2

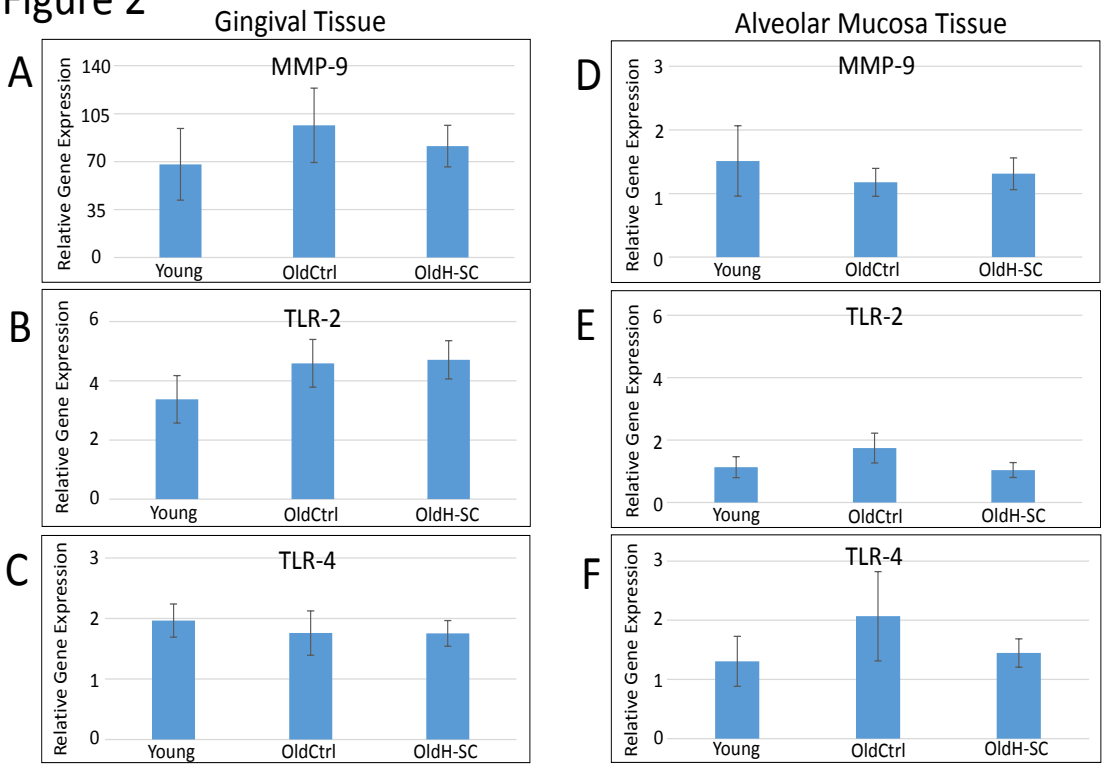

Figure 2. Expression of Genes Reflective of Disease Progression and Bacterial Infection in Mandibular Gingival Tissue and Alveolar Mucosa Tissue. All samples were normalized to β-actin. (A) Gingival MMP-9 (B) Gingival TLR-2 (C) Gingival TLR-4 (D) Mucosa MMP-9 (E) Mucosa TLR-2 (F) Mucosa TLR-4. Mean +/- SEM. Young, n = 4-6; old control n = 9-10; H-SC, n = 14-16. Young = young control group; old ctrl = old control group; old H-SC = old high sucrose/high casein group. $p > 0.05$ between groups.

Liver Gene Expression

Liver samples were evaluated for genes reflective of systemic inflammation (Figure 3). All results were normalized to 18S rRNA. There was no significant differences in TNF-α (Figure 3A) and IL-1β (Figure 3B) gene expression between old H-SC and old control groups or between young control and old control groups ($p > 0.05$). IL-6 gene expression was undetectable in the liver samples.

Figure 3

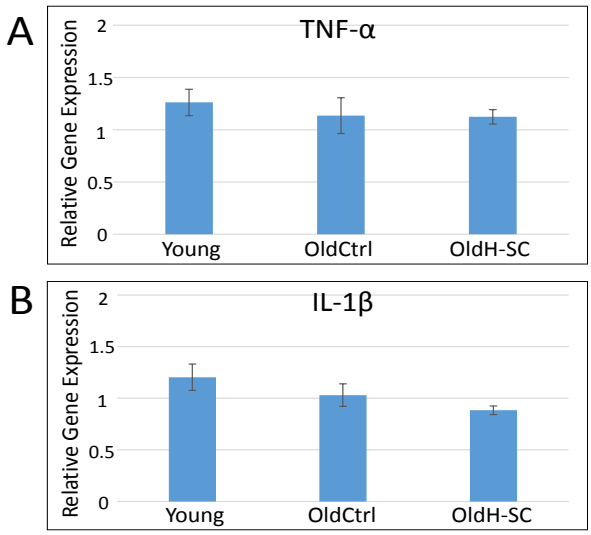

Figure 3. Gene Expression of TNF-α (A) and IL-1β (B) of Liver Samples. All samples were normalized to 18S rRNA. IL-6 gene expression was undetectable. Mean +/- SEM, Young, n=8; OldCtrl, n=11; and old H-SC, n=20. $p > 0.05$.

Metabolic Endpoints

Metabolic endpoints were assessed through evaluating bone densitometry endpoints, body composition, and serum leptin and insulin levels (Table I). Related to the effect of the H-SC diet on metabolic endpoints, body weight between the old control group and the old H-SC treatment group were similar ($p > 0.05$). BMD between the old control group and old H-SC group were significantly different ($p < .001$) with the old H-SC rats having a 5% higher total body bone density than old control rats. BMC, area, fat mass, lean mass, percent fat, leptin and insulin were similar between the old control and old H-SC groups ($p > 0.05$). However, liver mass was 10% higher ($p < 0.05$) for the old H-SC group than for the old control group.

Related to the question of age related differences in metabolic endpoints, significant differences were evident for all endpoints, between young control and old control groups (Table 1). Body weight in the young control group was ~53% less than in the old control group ($p < 0.001$). BMD was ~60% less ($p < 0.001$), BMC was ~34% less ($p < 0.001$), and bone area was ~57% less in the young control rats than in the old control group. Body composition measurements were also markedly lower in the young control group than in the old control group. Fat mass was ~24% less ($p < 0.001$), lean mass was 64% less ($p < 0.001$), and percent fat was 50% less ($p < 0.001$) in the young control group than in the old control group. Liver mass in the young control group was 88% lower in the old control group ($p < 0.05$). Serum insulin levels in the young control group were 58% less than in the old control group ($p < 0.001$). Serum leptin levels in the young control samples were undetectable.

Table 1. Total Body Bone Densitometry, Body Composition, and Serum Leptin and Insulin Levels

	Young Control (n=8)	Old Control (n=12)	Old H-SC (n=20)
Final Body Weight (g)	123 ± 3**	234 ± 21	238 ± 15
Bone Densitometry			
BMD (mg/cm^2)	109 ± 3**	183 ± 5	193 ± 6*
BMC (mg)	2819 ± 107**	8329 ± 651	8776 ± 1932
Area (cm^2)	26 ± 2**	46 ± 3	48 ± 4
Body Composition			
Fat Mass (g)	20 ± 1**	82 ± 16	88 ± 15
Lean Mass (g)	94 ± 3**	148 ± 7	149 ± 8
Percent Fat (%)	17 ± 1**	34 ± 4	36 ± 5
Liver Mass (g)	4.37 ± 0.32 *	4.99 ± 0.67	5.51 ± 0.59*
Leptin (ng/mL)	ND	6.08 ± 2.23	6.64 ± 3.30
Insulin (ng/mL)	0.69 ± 0.05**	1.19 ± 0.24	1.08 ± 0.27

$\bar{X} \pm$ SD; ND = Not detected; Leptin, young control n=8; old control n=12; old H-SC n=13. Insulin, young control n=8; old control n=9; old H-SC n= 14. Student's t-test between young control and old control; and between old control and old H-SC. * $p < 0.05$ compared to old control ** $p < 0.001$ compared to old control.

Discussion

Results of the current study suggest that mild periodontal inflammation, but not systemic or metabolic alterations, are induced in the Lewis rat animal model fed the H-SC diet for 23 weeks. This finding, coupled with the observation of worse periodontal disease score and alveolar bone loss in the H-SC animals compared to old control animals (Aguirre et al 2014 [abstract]), suggests that H-SC diet-related mild periodontitis occurs, but develops more slowly than in Rice rats. Results of the current study also indicate that there were no age related differences in the periodontal indices measured. However,

marked differences in RANKL gene expression and metabolic endpoints of body weight, bone densitometry, and body composition were evident, due to difference in age of the young control and old control rats, as expected of greater bone turnover at young age (Misawa et al 2007) and normal growth and development of the Lewis rat (Altun et al 2007, Montanya et al 2000).

As demonstrated by the 2-fold higher IL-6 gene expression in gingival tissue, a mild gingival inflammation occurred in the old H-SC rats. There was no inflammation present in the alveolar mucosal tissue, which was expected as this tissue is usually free of disease, making this tissue an appropriate internal control for each animal. Alveolar mucosa tissue may be non-diseased due to the location and distance away from the diseased gingival tissue and affected area surrounding the tooth, where periodontal bacteria infiltration and overgrowth usually reside. The IL-6 gene is a good indicator of inflammation as others have found that IL-6 regulates inflammatory responses at inflamed sites (D'Apuzzo et al 2013). In a ligature-induced periodontal disease animal model using Wistar rats, gingival tissue was extracted from around the ligated and non-ligated sites and gene expression was assessed. These investigators reported a significant increase in IL-6 gene expression in the tissues from the ligated sites when compared to the non-ligated sites (Peruzzo et al 2008). In a human study looking at inflammatory markers in gingival tissue biopsies of chronic periodontitis patients, and as a result, the investigators reported that IL-6 gene expression was more highly expressed in the gingival tissue of chronic periodontitis patients when compared to gingival tissue of non-

diseased patients. In addition, protein levels of IL-6 were also elevated in the biopsy samples (Venza et al 2010).

Gene expression of other inflammatory markers of IL-1β, TNF-α, and RANKL was similar between old H-SC and old control groups in both gingival tissue and alveolar mucosa tissue at the time point evaluated (23 weeks on the experimental diet). There was no evaluation of these genes at earlier time points in the study, however inflammatory markers may have been upregulated earlier and transiently. This earlier upregulation of inflammation markers is likely because measurements of alveolar bone height and periodontal disease scores in mandibles and maxillas of these same animals, completed by our collaborators (Aguirre et al 2014 [abstract]) reflect the presence (or prior presence) of inflammation. Other researchers have reported that with the initial onset of periodontitis in humans, IL-1β is stimulated. IL-1β is also considered to be a major cytokine in the gingiva for forced tooth movement-induced periodontitis in animal models (D'Apuzzo et al 2013). IL-1β is known to promote osteoclastogenesis by activating RANKL (Nishijima et al 2006). TNF-α is also induced with acute or chronic inflammation, which can also stimulate bone resorption (D'Apuzzo et al 2013). In a study looking at a ligature-induced periodontitis model using rhesus monkeys, researchers reported a significant overexpression of IL-1β and RANKL, within 2 weeks to 1 month of ligature application. The IL-1β gene expression returned to baseline levels 2 months after ligature removal (Ebersole et al 2014). In the biopsy samples of periodontitis patients, researchers reported TNF-α gene and protein levels were significantly higher in the gingival tissue of chronic periodontitis patients when compared to patients without

periodontal disease (Venza et al 2010). Thus, analysis of inflammation at earlier time points would confirm early onset inflammation.

There were no significant differences in gene expression of TLR-2 and TLR-4 with the old H-SC group compared to old control group, for either gingival tissue or alveolar mucosa tissue. These genes are upregulated in the presence of periodontal bacteria, such as *P. gingivalis,* which is a key bacterial pathogen prominently observed in periodontitis in both humans and animal models (Sloan et al 2013, Meulman et al 2011, Darveau et al 1997, Li et al 2000). In a study evaluating bacterial presence and infection in chronic periodontitis of human subjects, toll-like receptors in salivary epithelial cells were evaluated as an indicator of host recognition of bacteria. As a result, it was found that there was an increase stimulation in TLR-2, TLR-4 in the saliva of subjects with chronic periodontitis compared to saliva of healthy subjects (Swaminathan et al 2013). In the biopsy samples of periodontal diseased and non-diseased patients of either smokers or non-smokers, researchers reported that regardless of smoking status, gene expression of TLR-2 and TLR-4 were significantly greater in the gingival tissue of periodontal patients when compared to the gingival tissue of periodontal free patients (Fatemi et al 2013).

There were no significant differences in gene expression of MMP-9 with the old H-SC group compared to old control group, for either gingival tissue or alveolar mucosa tissue. Periodontal disease progression, as reflective by MMP-9, is a major part of severe periodontitis due to the induction of connective tissue degradation. MMP-9 is stimulated from inflammatory cytokines as a result of initial periodontal bacterial infiltration and overgrowth (Tsuchida et al 2013), and reflects increased tissue degradation with

increased periodontal disease progression (Baliban et al 2013, Tsuchida et al 2013). In a study looking at gingival crevicular fluid in periodontal disease subjects and healthy subjects, researchers reported elevated levels of MMP-9 in the periodontal subjects than in healthy subjects. Researchers reported a positive correlation between MMP-9 level and disease presence (Baliban et al 2013, Tsuchida et al 2013). In a study looking at a ligature-induced periodontitis model using rhesus monkeys, researchers reported that gene expression of MMP-9 levels were significantly elevated throughout a 5 month evaluation (Ebersole et al 2014).

There were no significant differences in gene expression of TNF-α, IL-1β, and IL-6 in the liver of the old H-SC group compared to old control group, suggesting systemic inflammation did not occur under the current experimental conditions. However, researchers have reported that periodontitis can produce systemic inflammation in humans. In a nationwide 1999-2004 NHANES study on human participants, periodontal examinations looking at probing depth and attachment loss at sites of teeth and fasting blood draws were assessed in non-diabetic adults. Results indicated positive correlations between periodontal disease and systemic inflammation, and also periodontal disease and insulin (Demmer et al 2012). In a study looking at a mouse liver hepatocyte cell line, cells were treated with a periodontal bacteria, *P. gingivalis*, and researchers reported an increased stimulation level in inflammatory cytokines TNF-α and IL-6 (Takano et al 2012). In liver samples of a sepsis rats, researchers reported that IL-6 expression was significantly increased in the liver. Researchers evaluated IL-6 measurements through gene expression levels and plasma protein levels and as a result, the expression of IL-6 in

the plasma protein levels resulted in greater levels, but both gene expression and protein levels had significant results (Ding et al 2014). If severe periodontitis was present in this study, then an increase in systemic inflammation may have been detectable by upregulation of these genes in the liver.

Metabolic endpoints were evaluated to characterize the effects of the H-SC diet and aging. Bone densitometric endpoints, body composition, and serum leptin and insulin levels were assessed. Significant differences were evident in only for BMD (5% higher) and liver mass (10% higher) in the old H-SC group compared to the old control group. A high protein diet resulted in higher BMD in humans compared to a low protein diet (Dawson-Hughes 2003). The higher liver mass in the old H-SC group may indicate increased triglyceride storage, resulting in a larger liver. A similar observation has been reported with experimental diets containing 30% sucrose in animals by other investigators (Oliveira et al 2014, Apolzan and Harris et al 2012). Furthermore, our results indicate that a 70% sucrose diet did not appear to produce major metabolic effects. However, other researchers have reported that diets containing 30% - 70% sucrose produced metabolic differences of increased body fat mass and the development of leptin and insulin resistance in adult rats within 18 days (Vasselli et al 2013).

Age-related differences were found in the young control and old control groups in all metabolic endpoints evaluated, as was expected. This age-related difference reflects the normal growth and development of the Lewis rat as has been reported by others (Lewis et al 1942, Altun et al 2007, Montanya et al 2000, Wolden-Hanson 2010). In an aging study looking at changes in tissue levels of Vitamin A, using Lewis rats, body

weight differences between young rats (6-8 weeks old) and old rats (8 months old) were reported (Zolfaghari and Ross 1995). These age-related body weight differences in Lewis rats are consistent with the results of this current study. Age-related increases in body weight, body composition, fat mass, and plasma insulin levels have also been reported in Sprague-Dawley rats. For example, younger rats (2 months old) had lower body weight, fat mass, and circulating insulin levels than older rats (4 months old) (Barzilai and Rossetti 1996). Serum leptin levels were undetectable in the young control rats in the current study, attributed to the lower body adiposity at 6 weeks of age. Aging positively correlates with increased adipose tissue and leptin concentrations in rats, so younger rats have lower amounts of adipose tissue and leptin concentrations compared to older rats (Mooradian et al 2000).

In conclusion, this animal model developed mild periodontitis with an observed slower periodontal disease progression, than in Rice rats. Localized inflammation was present in the gingival tissue, but not in alveolar mucosal tissue or liver. This gingival inflammation, along with the higher periodontal disease score and alveolar bone loss in mandibles and maxillas of the H-SC groups (results completed by our collaborators) suggest this animal model displays mild periodontitis. Further evaluation is needed to evaluate the effects of a longer feeding time frame on inducing a moderate to severe periodontitis progression and possibly evaluation of co-treatment with high doses of bisphosphonates.

CHAPTER V

EPILOGUE

Mild periodontal inflammation, but not systemic or metabolic alterations are induced in the Lewis rat animal model fed a H-SC diet for 23 weeks. This finding, coupled with our collaborator's data of observed worse periodontal disease score and alveolar bone loss in the old H-SC animals, indicate that H-SC diet-related periodontitis occurs. This study provides valuable information on the initial evaluation and characteristic of the Lewis rat as an animal model to study periodontitis, however further evaluation is needed to evaluate a moderate to severe periodontitis disease progression similar to that of humans.

This study was only able to produce mild periodontal inflammation under the experimental conditions conducted within this study. Rice rats, starting at week 18, were able to produce moderate periodontal lesion when fed a H-SC diet (Aguirre et al 2012a, b). This indicates that Lewis rats develop periodontitis more slowly than in Rice rats. In order for Lewis rats to develop moderate or severe periodontitis, a longer feeding time frame may need to be evaluated. This study only provided a 23 week feeding study, as this was the first evaluation of the Lewis rat as a model for periodontitis and variations in this strain or effects of the H-SC diet on this strain was unknown. A 23 week feeding study was established as a best estimate for when periodontal like characteristics may possibly be present, as Rice rats, under the same experimental conditions, had shown

moderate periodontitis by 18 weeks of feeding. A longer feeding time frame past 23 weeks would be needed to determine if Lewis rats develop a more moderate to severe periodontitis. Additionally, an evaluation of ONJ by co-treatment of bisphosphonates is needed to further study periodontitis and ONJ development.

Gene expression of IL-6 presented mild inflammation within the gingival tissue in the old H-SC group, however no significant changes were seen in the other inflammation related genes evaluated. To evaluate inflammation markers in periodontal disease, studies have also assessed the protein levels in gingival tissue, in gingival crevicular fluid, and systemically in blood. Studies have confirmed that evaluating protein levels directly does result in higher detected levels than gene expression levels, but if protein levels were found significant, then an effect would have also been seen in gene expression levels (Ding et al 2014, Venza et al 2010). Evaluating protein levels of inflammatory markers in gingival tissue and blood would have been desirable, but these analyses were too costly for a preliminary study. Further evaluation of IL-6 gene and protein expression, as well as of the other genes and proteins, including evaluation at several time intervals, would be informative.

There are many limitations for a preliminary animal model study, such as a preliminary study has unknown variations, effects, and a budget restraint and that an animal cannot be generalizable to the human population. Despite these limitations, this study provides valuable information on the evaluation and characteristic of the Lewis rat as an animal model to study periodontitis and future evaluations are needed to further study and understand human periodontitis

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