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The Effect of Vitamin D Deficiency on Periodontal Inflammation

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The Effect of Vitamin D Deficiency on Periodontal Inflammation

A dissertation submitted in partial satisfaction of the requirements for the degree Master of Science in Oral Biology

by

Sherif Warda

2015

ABSTRACT OF THE THESIS

The Effect of Vitamin D Deficiency on Periodontal Inflammation

by

Sherif Warda

Master of Science in Oral Biology

University of California, Los Angeles, 2015

Professor Sanjay M. Mallya, Co-chair

Professor Flavia Queiroz de Mo Pirih, Co-chair

Background: Periodontitis is a multifactorial inflammatory disease resulting in the destruction of the supporting structures of the teeth. It is currently well established that the host response to bacterial pathogens is the major cause in the pathogenesis and progression of the disease. Moreover, the seminal role of vitamin D in calcium-phosphate homeostasis and bone metabolism has been well established. More recent studies suggested extraosseous effects of vitamin D,

showing association of vitamin D deficiency with risk of multiple diseases including cancer, osteoarthritis, cardiovascular disease, autoimmune diseases, infections and more.
The immunomodulating effects of vitamin D may explain the reported epidemiological associations between vitamin D status and a large number of autoimmune and inflammatory diseases.

Purpose: To analyze the relationship between vitamin D deficiency and its effect on LPS-induced periodontal bone loss in mice.

Methods and Materials: Using the *P. gingivalis* LPS injection model to induce periodontal bone loss, we utilized 32 one-month-old male mice (C57BL/6J). The mice were divided into four groups. Group 1: Vitamin D adequate diet, No injection; Group 2: Vitamin D adequate diet and LPS injections; Group 3: Vitamin D deficient diet, No injections; Group 4: Vitamin D deficient diet and LPS injections. Test groups received 2 µl (20µg) of P. gingivalis–LPS injections in between the first and second maxillary molars on both sides of the maxilla, two times a week for 6 weeks. Animals were sacrificed one day after last injection,and maxillae were dissected and harvested. MicroCT imaging was used to evaluate periodontal bone loss measured using linear analysis (Dolphin imaging) and volumetric analysis (CTAn Software).

Results: Linear analysis showed statistically significant bone loss when comparing both LPS groups to control groups. However, the extent of bone loss did not differ between vitamin D deficient and adequate LPS-injected groups. Volumetric bone analysis similarly showed no statistical difference when comparing vitamin D adequate and deficient LPS-injected groups.

Conclusion: In the present study, vitamin D deficiency did not influence LPS-induced bone loss in our mouse model, however, further investigation is needed to confirm these findings.

The dissertation of Sherif Warda is approved.

Perry Klokkevold

Diana Messadi

Sanjay M. Mallya, Committee Co-chair

Flavia Queiroz de Mo Pirih, Committee Co-chair

University of California, Los Angeles

2015

DEDICATION

This thesis is dedicated to my loving parents, Hussein, Lina and Estela who have always supported me in my academic endeavors.

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ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my committee directors Dr. Flavia Pirih and Dr. Sanjay Mallya, as well as, the members of my committee Dr. Diana Messadi and my program director Dr. Perry Klokkevold for their invaluable guidance.

I would also like to thank my co-residents for their support and encouragement.

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INTRODUCTION

Periodontitis is one of the most common oral diseases. The manifestation of the disease can range from mild cases of marginal gingival inflammation with slight attachment and bone loss to severe cases of attachment and bone loss where teeth are retained only by soft tissue. It is estimated that the prevalence of periodontitis in the US adult population ages 30 years and older is 47% (65 million people) and 65 years and older is 70%. [1]

Evidence show that periodontitis has plagued the human species for thousands of years, Chinese physicians were probably first to describe signs of periodontal diseases in the earliest known textbook, Nei Ching (approximately 2700–2600 BC). The etiology of periodontitis has evolved throughout history, but it wasn't until the 19th century and the advances in microbiology that two German physicians, Robert Ficinus (1809–1852) and Adolph Witzel (1847–1906), described the association of bacteria with periodontal tooth loss. From Europe to the Untied states, pyorrhea alveolaris became the accepted term for any gum disease, eliminating the term Riggs' disease in association to John Riggs (1810-1885) who was considered the father of periodontics because of his attempts to eliminate the disease by thorough calculus removal, curettage of soft tissues and oral hygiene. [2]

Indeed, in the mid-1960's , believed as the start of the plaque era, the classic studies of Loe et al. convincingly demonstrated that plaque accumulation directly preceded and initiated gingivitis. [3]

Many investigators believed that gingivitis was harmful and led to the eventual destruction of the periodontal tissues, probably by host-mediated events. Yet, certain discrepancies continued to baffle clinicians and research workers alike. If all types of plaque were more or less alike and

induced a particular systemic response in the host, why did some individuals with little detectable plaque or clinical inflammation develop rapid periodontal destruction? If inflammation was the main mediator of tissue destruction, why were so many teeth retained in the presence of continual gingivitis? One explanation may have been inconsistencies in the host response.[4]

Current consensus is that the etiopathogeny of periodontitis entails a multifaceted dynamic interaction of periopathogenic bacteria and viruses, innate and adaptive immune responses, adverse environmental events, and genetic susceptibility factors. [5]

Emerging evidence supports the major role of host responses modulated through genetics, immunological and inflammatory responses, stress, smoking, diet, social determinants, and general health as being the major determinants of the outcomes of the classic chronic inflammatory condition we know as periodontitis.[6]

Socransky and Haffajee remind us that the initiation or progression, is a resultant of the interplay between a large number of factors. The presence of bacterial pathogens are necessary, but not sufficient for disease to take place. The host must be susceptible to the pathogen. [7]

One of the well-studied and well established periodontal pathogen is Porphyromonas gingivalis (*P.gingivalis*). *P.gingivalis* has been strongly present in lesions of periodontitis, and unusal in health or gingivitis subjects, and its elimination or suppression resulted in successful therapy. Furthermore, the host response shows elevated serum antibody and local antibody response to the virulent factors like collagenase, trypsin like activity, fibrinolysin, gingipains, endotoxins (LPS) and more.[8]

Alveolar bone destruction in periodontitis as a result of a shift in the RANK-RANKL-OPG axis uncoupling the normally tightly coupled process of bone resorption and formation.

Cytokines like prostaglandin E-2, IL1-alpha, IL-6 and tumor necrosis factor released in response to LPS invasion of the tissue appear to mediate bone resorption in periodontitis.

Circulating factors, including the steroid hormones, parathyroid hormone, calcitonin, and vitamin D regulate the overall bone remodeling process. [9]

Historically, vitamin D was discovered as an essential nutrient for the prevention of rickets, required for optimal absorption of dietary calcium and phosphate. This ability to produce sufficient amounts of vitamin D with adequate sunlight exposure indicates that vitamin D is actually not a vitamin.[10]

Vitamin D is a secosteroid hormone produced chiefly in the skin upon exposure to ultraviolet radiation. Vitamin D can also be supplied by the diet or by supplements. The crucial role for vitamin D in calcium-phosphate homeostasis and bone metabolism is well established.

In addition to regulating calcium homeostasis, vitamin D has many other metabolic effects, which were identified more recently when researchers investigated the immunomodulating properties, regulating the proliferation, differentiation and function of immune cells both directly and indirectly.

Focusing on recent developments in our understanding of the vitamin D endocrine system, including, but not limited to, the discovery that most tissues and cells in the body have a vitamin D receptor and that several possess the enzymatic machinery to convert the primary circulating form of vitamin D, 25-hydroxyvitamin D, to the active form, 1,25-dihydroxyvitamin D, has provided new insights into the function of this vitamin. Of great interest is the role it can play in

decreasing the risk of many chronic illnesses, including common cancers, autoimmune diseases, infectious diseases, and cardiovascular disease. [11]

Although there is no consensus on optimal levels of 25-hydroxyvitamin D as measured in serum, vitamin D deficiency is defined by most experts as a 25-hydroxyvitamin D level of less than 20 ng per milliliter (50 nmol per liter).Vitamin D intoxication is observed when serum levels of 25-hydroxyvitamin D are greater than 150 ngper milliliter (374 nmol per liter). With the use of such definitions, it has been estimated that 1 billion people worldwide have vitamin D deficiency or insufficiency.

According to several studies, 40+ % of the U.S population are deficient in vitamin D. More than 50% of postmenopausal women taking medication for osteoporosis had suboptimal levels of 25-hydroxyvitamin D — below 30 ng per milliliter, 42% of 15- to 49-year-old black girls and women throughout the United States had 25-hydroxyvitamin D levels below 20 ng per milliliter, and 32% of healthy students, physicians, and residents at a Boston hospital were found to be vitamin D–deficient [12]

The realization of general health as a determinant factor of the host response to periodontal inflammation and breakdown, as well as the recent findings of the importance of vitamin D as an immune modulator and its role in chronic illnesses, and finally the high prevalence of vitamin D deficiency in our population brings us to our current interest in the relationship between vitamin D deficiency and Periodontitis.

NHANES III data was analyzed in a cross sectional study in 2007, finding an inverse association between dairy intake (considered at the time 84% of the source of calcium intake)

and periodontitis prevalence. Subjects with higher dairy intake had 20% lower odds of having periodontitis. [13]

Dietrich et al. used the NHANES III data to examine the association of serum vitamin D levels with CAL. They found an inverse association in men and women 50 y or older. Compared with persons in the highest 25-hydroxyvitamin D3 quintile, those in the lowest quintile had mean CALs of 0.39 mm (95% CI 0.17–0.60) and 0.26 mm (95% CI 0.09–0.43) higher for men and women, respectively. The association between lower serum vitamin D levels and higher CAL scores was independent of factors such as ethnicity, social context, smoking habit, and diabetes mellitus. This study suggested that the inverse association might be attributed to the anti-inflammatory effects of vitamin D. The limitations of this study were the cross-sectional design and the fact that vitamin D serum levels were determined only once. [14]

A 2013 study from Harvard School of Public health shows the longest prospective observational study to evaluate the association between predictors of 25(OH)D and incidence of tooth loss and periodontitis, following 51,000+ male health professionals beginning from 1986-2006. In multivariable analyses, adjusted for key confounders and risk factors, the risk of tooth loss and periodontitis was significantly lower with higher levels of the predicted 25(OH)D score. These results suggest that vitamin D itself and/or components associated with vitamin D status including UV-B may be associated with lower risk of tooth loss and periodontitis. [15]

None of the reviewed articles had an RCT design, all studies were cross-sectional studies, particularly on the same data (NHANES III). In general, cross-sectional studies provide a lower level of evidence supporting a causal effect than RCTs and cohort or case–control studies. These results warrant further investigation by epidemiological and clinical studies.[14,15]

SPECIFIC AIM AND HYPOTHESIS

To analyze the relationship between vitamin D deficiency and its effect on LPS induced periodontitis in mice.

Working Hypothesis: Vitamin D deficiency is associated with increased periodontal breakdown in LPS-induced bone loss in mice.

MATERIALS AND METHODS

Timeline: (Figure 1)

Thirty-two six-week-old male mice (C57BL/6J) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in a temperature-and light-controlled environment at UCLA.. All mice were handled according to protocols approved by the UCLA Office for Animal Research Oversight. Sixteen mice were placed on a standard diet and sixteen on a vitamin D deficient diet for four weeks before the start of the study.

Diet (Bio-Serve, Flemington, NJ, USA) was the sole source of vitamin D for the mice. The standard diet contains 1 IU/G cholecalciferol, while the deficient diet is 0.05 IU/G of cholecalciferol (5% of the recommended daily intake). (Table 1)

Blood was drawn for serum level quantification by ELISA before the start of the diet. Below 12 ng/ML was considered deficient, between 12 to 20 ng/ML was considered insufficient, and above 20 ng/ML was considered optimal as defined by the institution of medicine.

Mice were grouped into 4 groups (n=8 mice/group):

1. Vitamin D adequate diet, no injections
2. Vitamin D adequate diet with LPS injections
3. Vitamin D deficient diet, no injections
4. Vitamin D deficient diet with LPS injections

Inflammatory bone loss model:

Mice were anesthetized with 3% isoflurane, administered through a nose cone. Under the microscope (Leica Microsystems, Buffalo Grove, IL, USA), 16 mice (groups 2 and 4) received 2 µl (20µg) of P. gingivalis–LPS (InvivoGen, SanDiego, CA, USA) injections in between the

first and second maxillary molars on both sides of the maxilla, two times a week for 6 weeks. The control animals (Groups 1 and 3) did not receive injections. A 10 µL Hamilton syringe with a 33 gauge needle was used (Hamilton Company, Reno, NV, USA). This regimen was similar to previously published studies [16] No overt signs of tissue inflammation or soft tissue damage were observed during the course of injections (data not shown). All subjects were weighed weekly.

Animals were euthanized 6 weeks after the first injection. Maxillae was dissected and immersed in 10% buffered formalin for 24-48 h. Specimens were then washed with distilled water, then placed in 70% ethanol solution.

Micro-computed tomography (micro-CT) analysis of the maxillae was scanned using a microcomputed tomography scanner with a voxel size of 10 µm (isotropic voxel) and X-ray energy of 55 KVp and 181 mA. Each scan was conducted over a period of 21 min, with steps of 0.4°. Ten frames were averaged and a 0.5 mm aluminum filter was utilized. Virtual image slices were reconstructed using the cone-beam reconstruction software version 1.5 based on the Feldkamp algorithm. [17] Analysis was done in two stages, linear and volumetric bone analysis was performed.

LINEAR BONE ANALYSIS

Volumetric data was converted to DICOM format and imported to Dolphin software (Dolphin Imaging, Chatsworth, CA, USA) and analyzed. To quantify the amount of bone loss, the imaged volume was oriented in the coronal (green) and transverse (blue) planes such that the sagittal plane (red) was parallel to the maxillary midline, identified by the intermaxillary suture and

the coronal plane intersected the proximal area between the first and second maxillary molars. (Figure 2)

Then, at the sagittal plane crossing the interproximal contact point of the first and second molar crowns, the distance between the cemento-enamel junction and the alveolar crest were measured for the distal surface of the first molar and the mesial surface of the second molar just below the contact point and 0.2 mm palatal to the contact point. (Figure 2)

Quantifying the amount of bone loss by measuring the distance between the cemento-enamel junction and the alveolar crest for the distal surface of the first molar and the mesial surface of the second molar just below the contact point and 0.2 mm palatal to the contact point.

To quantify the amount of bone loss, the average distance of all groups were calculated and compared with each other. This regimen was similar to previously published studies. [17]

STATISTICAL ANALYSIS

Groups were compared using the Student's t-test.

Significance level: ($p<0.01$)

VOLUMETRIC BONE ANALYSIS

Volumetric data was converted to DICOM format and imported to DataViewer software, where the samples are oriented so the axial slices are parallel to the horizontal axis using the maxillary suture as a reference. The sagittal slices were oriented so the CEJs are touching the horizontal red line. (Figure 3). The coronal slices are oriented for the palate to be parallel to the horizontal line axis (Figure 4).

Once orientation is completed, the file is saved and re-opened in CtAn software for the measurement of Bone Volume/ Tissue (BV/TV) Volume in between the first and second molar area on the right and left sides of each sample.

The reference point is the CEJ, and by taking the average of the highest bone point from the CEJ to the alveolar crest in our linear analysis, we determine the highest slice. And by taking the average of the lowest point from the CEJ to the alveolar crest in our linear analysis, we determine the lowest slice. We use these slices which comprise of 20 slices as the area of interest to determine the BV/TV.

Once this is determined, on each subject, we measure from the CEJ till the highest point, and start mapping the bone volume in the area each 3 slices. (Figure 5) shows mostly red which illustrates tissue volume, and (Figure 6) shows mostly blue which illustrates bone volume.

The average volumes of each group are measure and compared with each other for differences.

STATISTICAL ANALYSIS

Groups were compared using the Student's t-test.

Significance level: ($p<0.01$)

RESULTS

WEIGHT

Normal increase in the weight of the mice were noted consistent with expected biological growth. (Figure 7) The weight of each mouse was measured at a weekly basis and the average of each group was measured. (Table 2)

CLINICAL PICTURES

Clinical images for all groups are taken. One sample from each group was chosen to represent each group. (Figure 8)

Group 1: Sample 2

Group 2: sample 9

Group 3: sample 23

Group 4: sample 27

Clinical signs of inflammation are mild, slight edema is seen interproximally between the first and second molars in the LPS groups (Group 2 and 4).

RADIOGRAPHIC IMAGES

Radiographic slices showing the average bone loss on the sagittal view for all groups.

Two samples from each group representing the average for each group. (Figure 9)

Group 1: samples 1 and 6

Group 2: samples 14 and 15

Group 3: samples 21 and 23

Group 4: samples 30 and 31

LINEAR BONE ANALYSIS

Tables 3,4,5,6 show the linear measurements of bone loss in mm. The average for group 1 showed a linear bone loss of 0.1530mm with a standard error of the mean (SEM) of 0.00377, group 2 showed an average loss of 0.2719mm with an SEM of 0.00854, group3 showed an average loss of 0.1634mm with SEM of 0.00395 and group 4 with an average loss of 0.2784mm with SEM of 0.00652. The graph shows the differences in mm between all groups. (Figure 10)

The linear measurements of the micro-CT analysis revealed statistical significant alveolar bone loss at the interproximal space between the first and second maxillary molars on both the right and left sides at the groups 2 and 4 (LPS-injected sites) compared to the groups 1 and 3(non-injected sites) ($p<0.01$). No statistical significance was noted in the amount of bone loss comparing the vitamin D deficient (Group 4) and vitamin D adequate (Group 2) LPS-injected groups ($p>0.05$). Similarly, no statistical significance was found when comparing both non-injected groups (Groups 1 and 3) (p.0.05).

VOLUMETRIC BONE ANALYSIS

(Table 7) shows the percentage averages of Bone Volume/Tissue Volume between of all groups. Group 1 showed an average of 73.06% bone to tissue volume in the area of interest, group 2 showed an average of 29.36%, group 3 showed an average of 74.77% and group 4 showed an average of 36.93%.

The graph shows the differences in percentages among all groups. (Figure 11)

Volumetric analysis revealed statistical significance of higher BV/TV at the analyzed sites between the first and second molars on both the right and left sides with groups 1 and 3 (Non-injected groups) as opposed to groups 2 and 4 (LPS injected groups) ($p<0.01$).

No statistical significance could be found when comparing the BV/TV for both LPS-injected groups (Groups 2 and 4) ($p>0.05$), similarly, no statistical significance was found when comparing the non-injected groups (Groups 1 and 3) ($p>0.05$).

DISCUSSION

With the development of the inflammatory periodontal diseases many pathognomonic qualitative and quantitative changes occur in the molecular composition of the periodontal connective tissues. Almost as soon as gingival plaque accumulates adjacent to the gingival margin, an inflammatory infiltrate becomes apparent within the connective tissue. [18]

Murine models have been used in a wide variety of hypotheses related to periodontal pathogenesis, ranging from the role of the host response to virulence traits of suspected periodontal pathogens and the interconnection of those factors with systemic parameters. [19]

In the study of periodontitis, mice have shown similar inflammatory response mechanisms as humans. A study was conducted to observe the host's response to inflammatory components in different strains of inbred mice including C57BL/6J strain. *P. gingivalis*-derived LPS was injected subgingivally into the maxillary molar regions. At the end of six weeks, the maxilla was harvested and scanned with microCT. C57/B6J showed the most severe bone loss. [20-21]

Significant controversy has emerged over the last decade concerning the effects of vitamin D on skeletal and non-skeletal tissues. The demonstration that the vitamin D receptor is expressed in virtually all cells of the body and the growing body of observational data supporting a relationship of serum 25-hydroxyvitamin D to chronic metabolic, cardiovascular, and neoplastic

diseases have led to widespread interest in vitamin D as a therapeutic modality for the prevention of chronic diseases. [22]

Studies on vitamin D and its effects on periodontal breakdown have been limited to cross sectional observational studies, or longitudinal observational studies such as the 2013 Harvard School of Public Health Study which followed health professionals for a 20+year span measuring the association with predictive measures of serum level vitamin D and not blood tests on all the subjects. The studies warranted further investigation by epidemiological and clinical studies.[12-14]

Our study's aim was to examine the influence of vitamin D deficiency on *P.gingivalis* LPS-induced periodontal bone. We examined bone loss by linear measurements on microCT images of maxillae, to detect any association between vitamin D deficiency and LPS-induced bone loss. We also measured the amount of bone loss by volumetric measurements using CTAn software which allowed us to map a specific area of interest (in our case the space between the 1st and 2nd molars) and measure the amount of Bone/Tissue volume. Our controls were 2 groups, one vitamin D adequate and one vitamin D deficient that did not receive any injections.

There was a statistical significance ($p<0.01$) between the control and injected groups, the groups with LPS injections showed more bone loss than those with no injections. These results are consistent with previous studies from Dr Pirih's laboratory.[17]

Our findings showed that there was no statistical significance between the vitamin D deficient group with LPS injections over the vitamin D adequate group with LPS injections. ($p>0.05$).

Our results of a lack of an association between vitamin D deficiency and LPS-induced bone loss in mice does not completely rule out the possibility of this association. The fact that periodontitis is a multifactorial disease suggest that its progression can be a resultant of the interplay between a large number of factors and not just one factor. Alternatively, our results may have been influenced by the timing of our studies, for example, our study design did not evaluate the immediate/rapid differences in bone loss between the two dietary groups. Likewise, our study did not examine the effects of long-standing chronic vitamin D deficiency on bone loss progression.

Recent study evaluating the therapeutic effects of systemic vitamin D3 on gingival inflammation and alveolar bone in rats with experimentally induced periodontitis by measuring the distance between the alveolar bone crest and the cemento-enamel junction (CEJ). Their study was a short 10 day study comparing conventional periodontal therapy alone (scaling and root planing) or in combination with the administration of vitamin D3, vitamin K2 or both together and their effects on the CEJ-Alveolar bone crest distance. The differences in alveolar bone loss seen histologically between groups were not significant. Their findings did not support the notion that vitamin D has additional positive effects on alveolar bone. [23]

Along with the limitations of sample size and the time factor, there may be a possibility that vitamin D does not have an effect on bone levels in murine models with induced periodontal disease.

CONCLUSION

The importance of vitamin D in modulating our host response is unquestionable. Vitamin D deficiency may be part of a more complex host immune response towards perio-pathogenic micro-organisms and their virulence factors.

Within the limitations of the study, vitamin D deficiency did not show an effect on periodontal bone destruction when compared to the vitamin D adequate group in LPS induced periodontitis in mice.

Further studies and investigations are needed to further evaluate the contribution of vitamin D deficiency on establishment and progression of periodontal disease.

FIGURES

Figure.1 Flow Chart.

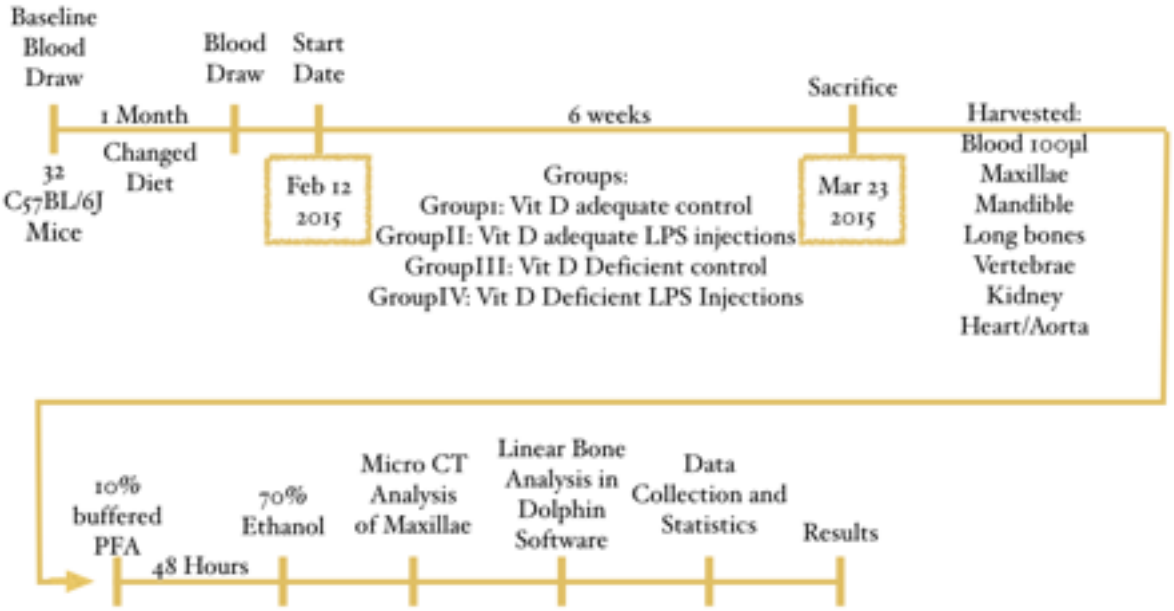


Figure 2. Coronal, axial and sagittal planes on Dolphin Imaging software used for orientation of maxillae for linear measurement analysis.

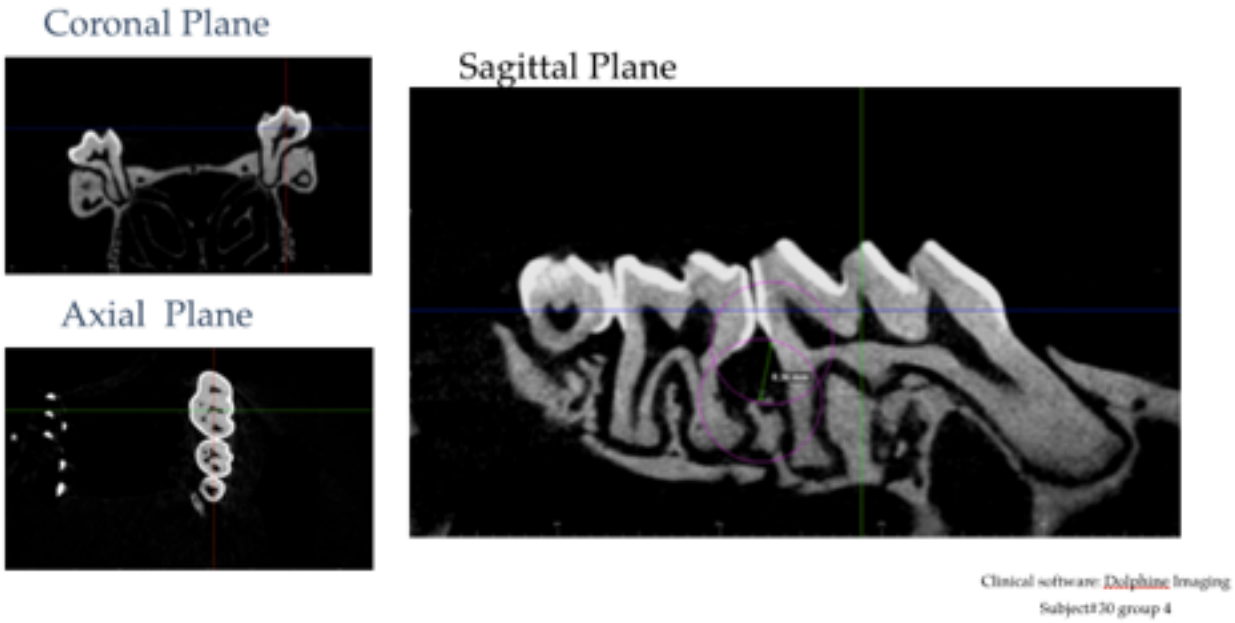


Figure 3. Sagittal view on DataViewer used for orientation of maxillae for volumetric measurement analysis.

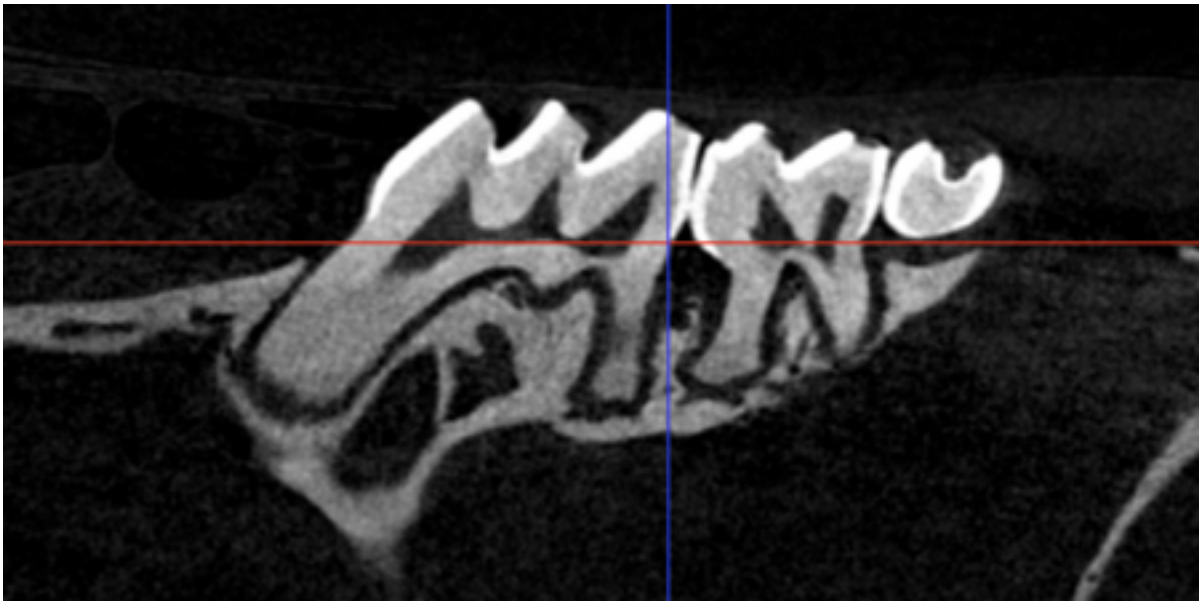


Figure 4. Coronal view on DataViewer used for orientation of maxillae for volumetric measurement analysis.

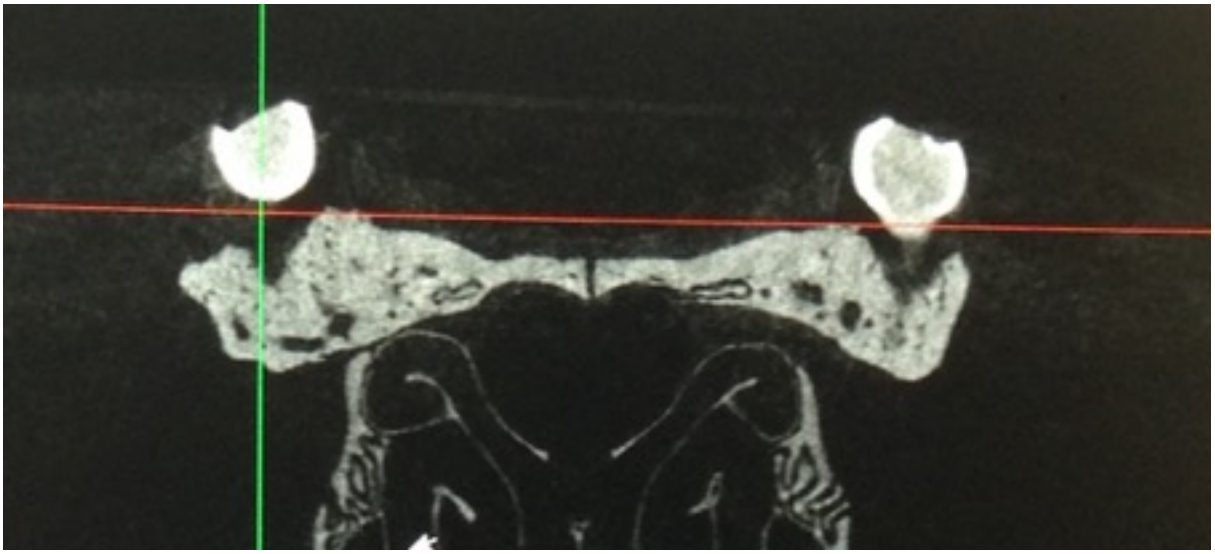


Figure 5. Transverse view on CtAn software for orientation of maxillae and outlining the area of interest for volumetric BV/TV measurements (Group 4), Blue (Bone)- Red (Tissue).

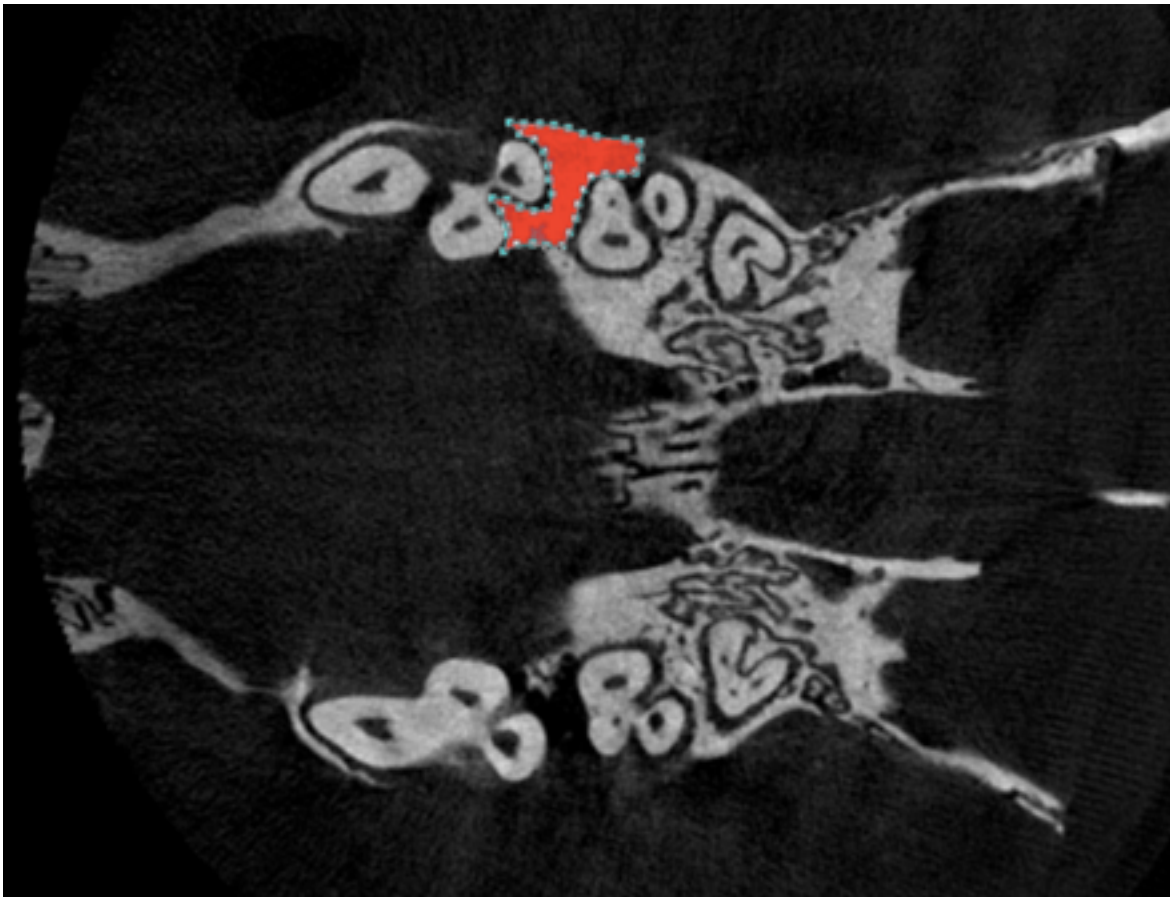


Figure 6. Transverse view on CtAn software for orientation of maxillae and outlining the area of interest for volumetric BV/TV measurements (Group 1), Blue (Bone)- Red (Tissue).

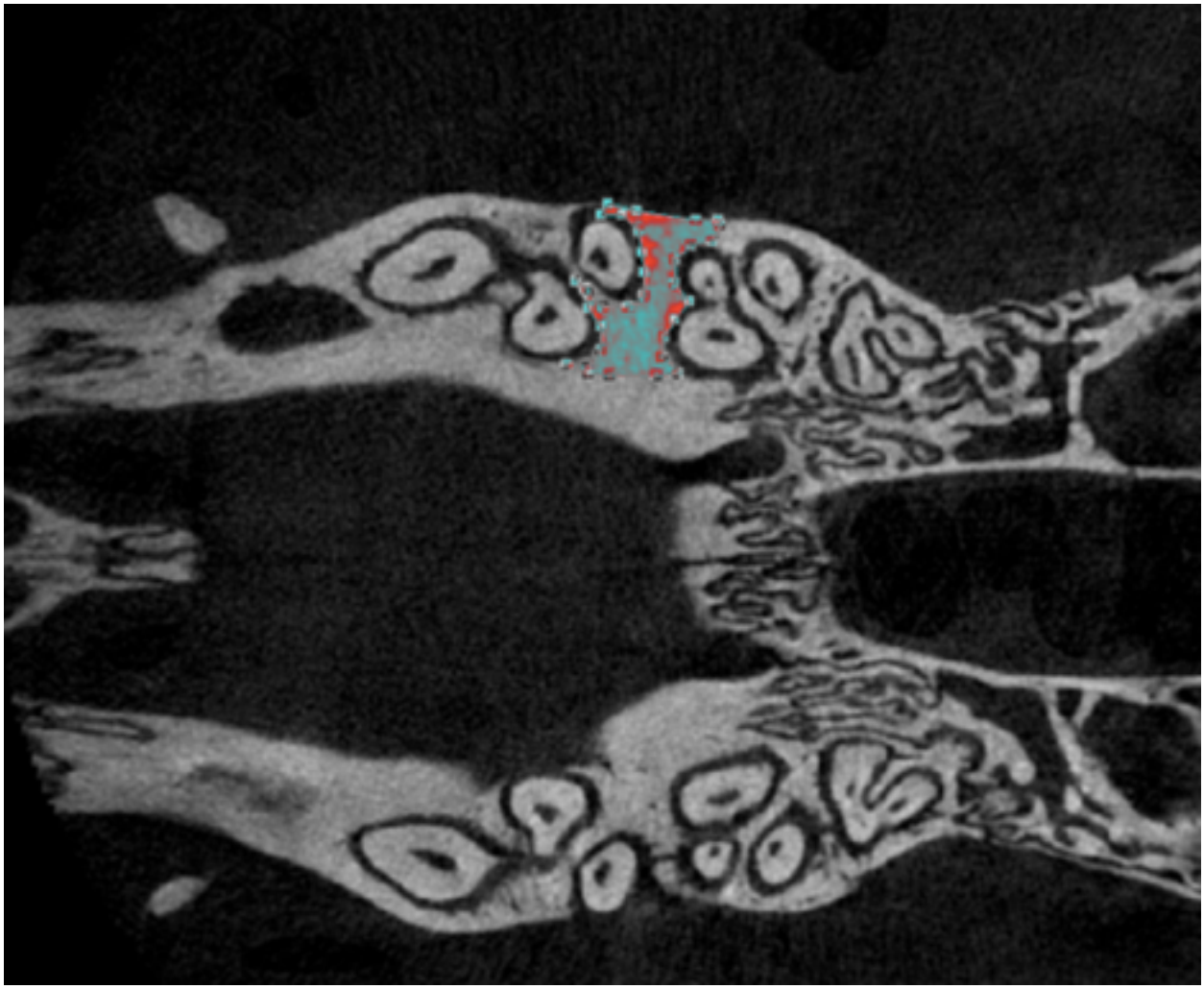


Figure 7. Average weight in grams for all groups from start to end of study measured weekly, showing consistent growth pattern.

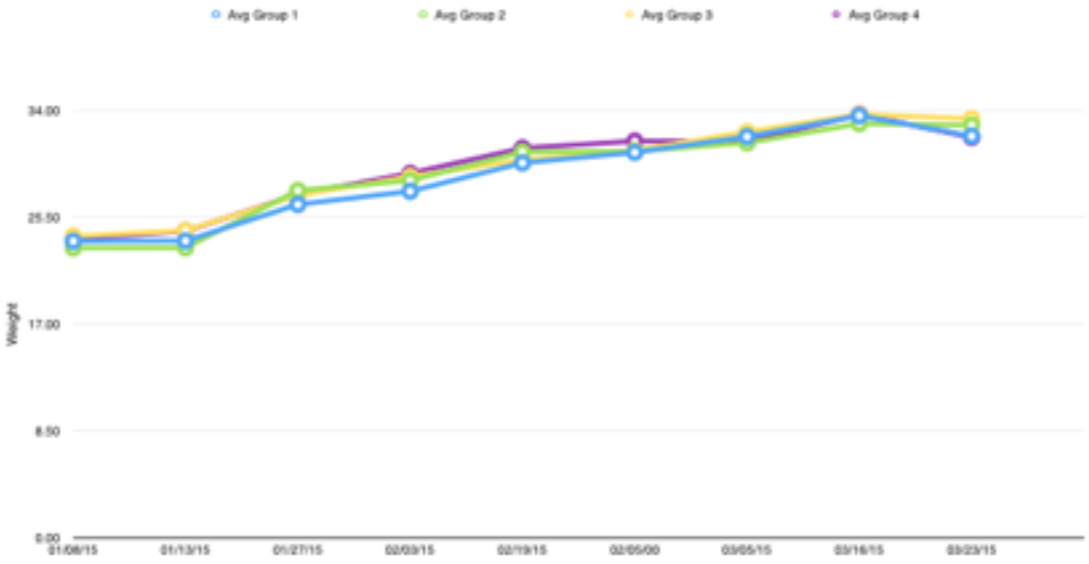


Figure 8. Clinical pictures for all groups.

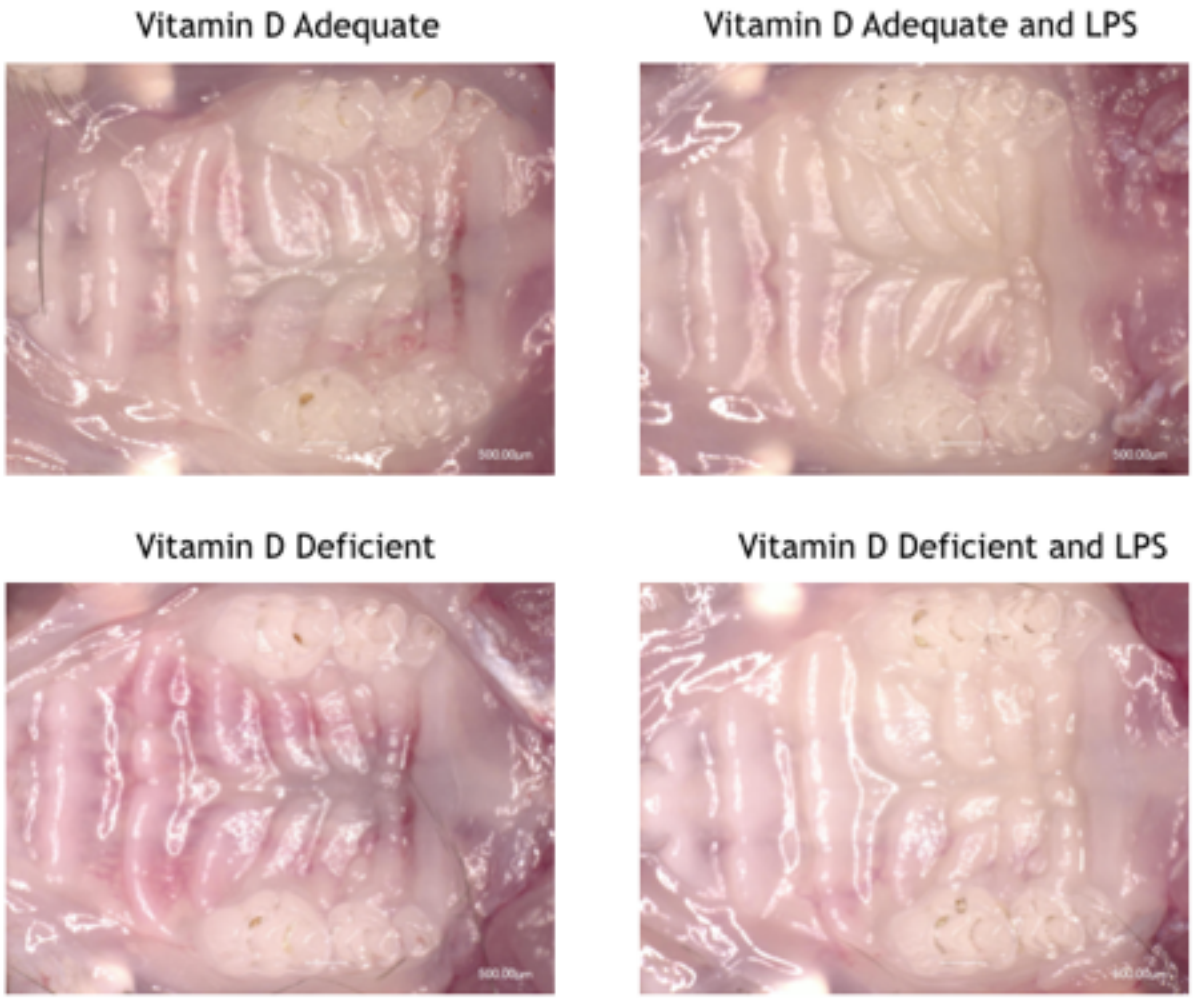


Figure 9. Average bone loss for all groups (2 samples each group).

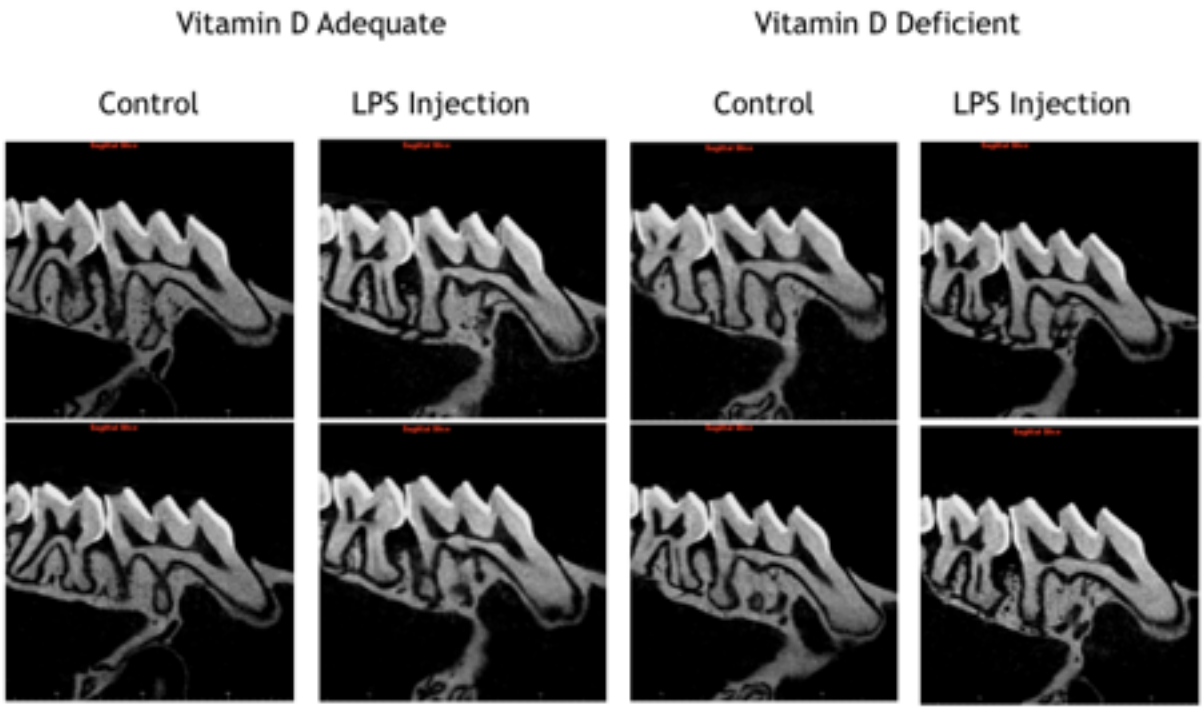
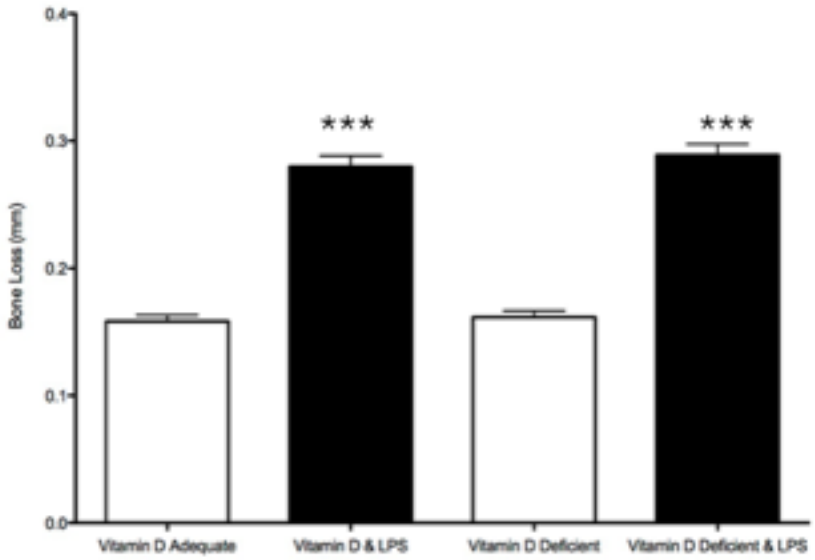
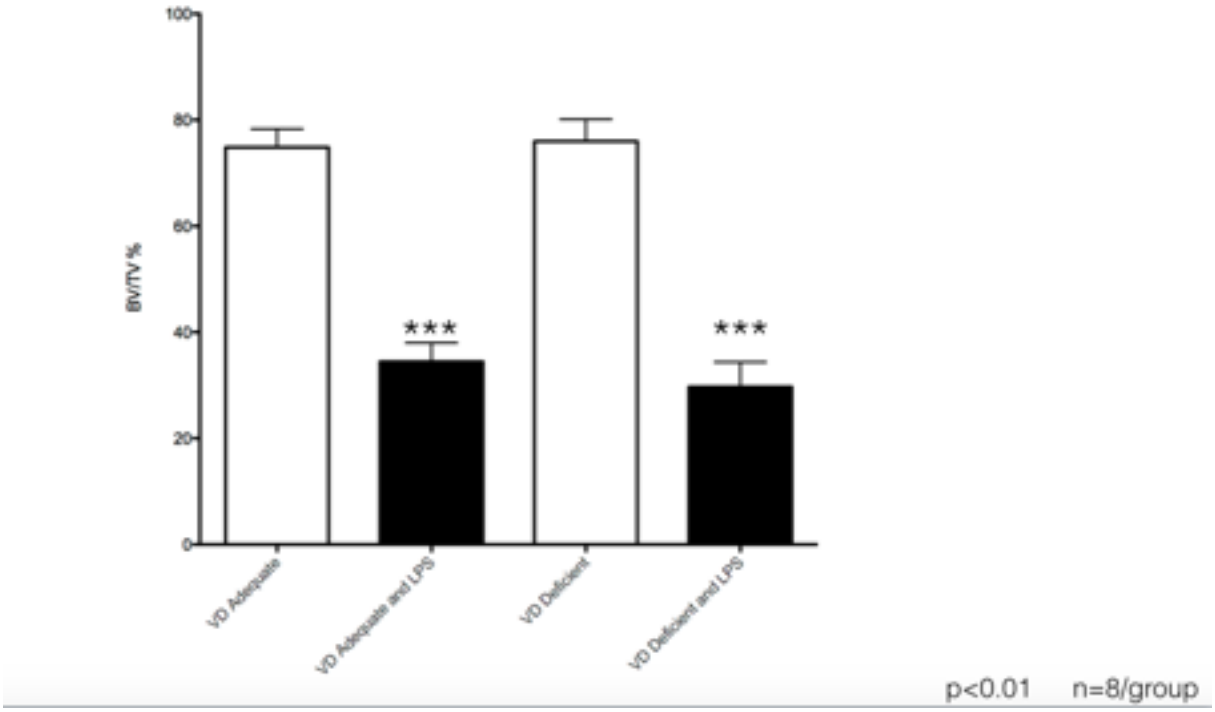


Figure 10. Graph showing the average bone loss in mm for all groups for the linear bone analysis.



p<0.01 n=8/group

Figure 11. Graph showing the average bone volume/ tissue volume in percentage for all groups for the volumetric bone analysis



TABLES

Table 1. Diet (Bio-Serve, Flemington, NJ, USA)

Bio-Serv®
Delivering Solutions™...
◆ Nutritional ◆ Enrichment ◆ Medicated ◆ Special Needs

Nutritional Profile

Bacon Softies™, 1/2" Pellets, Certified (Contaminant Screened)
Packed: 1 kg/bag - Product# F3580-1 and 5 kg/Box - Product# F3580 **Sterile:** 1 kg/bag - Product# S3580-1

Proximate Profile

Protein	%	22.4
Fat	%	12.0
Fiber	%	6.2
Ash	%	4.2
Moisture	%	<10
Carbohydrate	%	45.8

Caloric Profile

Protein	kcal/gm	0.90
Fat	kcal/gm	1.10
Carbohydrate	kcal/gm	1.83
Total	**kcal/gm**	**3.83**

Amino Acids

Alanine	gm/kg	8.9
Arginine	gm/kg	14.0
Aspartic Acid	gm/kg	22.8
Cystine	gm/kg	2.7
Glutamic Acid	gm/kg	43.1
Glycine	gm/kg	8.7
Histidine	gm/kg	6.0
Isoleucine	gm/kg	11.0
Leucine	gm/kg	17.9
Lysine	gm/kg	14.9
Methionine	gm/kg	7.3
Phenylalanine	gm/kg	10.8
Proline	gm/kg	16.0
Serine	gm/kg	12.5
Threonine	gm/kg	9.5
Tryptophan	gm/kg	3.0
Tyrosine	gm/kg	9.6
Valine	gm/kg	12.0

Carbohydrates

Monosaccharides	gm/kg	53.0
Disaccharides	gm/kg	225
Polysaccharides	gm/kg	160

Fatty Acids

C18:2 Linoleic	gm/kg	56.6
C18:3 Linolenic	gm/kg	8.0
Total Saturated	gm/kg	22.0
Total Monounsaturated	gm/kg	27.7
Total Polyunsaturated	gm/kg	64.1

Minerals

Calcium	gm/kg	5.9
Chloride	gm/kg	1.6
Copper	mg/kg	18.9
Chromium	mg/kg	1.0
Fluoride	mg/kg	1.0
Iodine	mg/kg	0.21
Iron	mg/kg	71.5
Magnesium	gm/kg	1.8
Manganese	mg/kg	26.4
Phosphorus	gm/kg	4.6
Potassium	gm/kg	12.4
Selenium	mg/kg	0.20
Sodium	mg/kg	1100
Sulfur	mg/kg	301
Zinc	mg/kg	45.0

Vitamins

Ascorbic Acid	mg/kg	1.8
Biotin	mg/kg	0.20
Choline	mg/kg	1112
Folic Acid	mg/kg	4.4
Niacin	mg/kg	46.1
Pantothenic Acid	mg/kg	22.1
Pyridoxine	mg/kg	8.6
Riboflavin	mg/kg	7.1
Thiamin	mg/kg	7.7
Vitamin A	IU/kg	4243
Vitamin B_{12}	mcg/kg	25
Vitamin D_3	IU/kg	1000
Vitamin E	IU/kg	91.0
Vitamin K_1 (Phylloquinone)	mg/kg	1.0

Ingredients

Soy Flour, Soybean Oil, Corn Syrup, Sucrose, Casein, Cellulose, Corn Starch, Wheat Bran, Molasses, Mineral Mix, Banana Flakes, Acacia Gum, Bacon Flavor, Glycerin, Vitamin Mix, DL-Methionine, Choline Bitartrate, BHA

These are typical amounts of nutrients calculated from available information. Actual assay results may vary.
For more information contact Jaime Lecker, Ph.D.
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Table 2 Weight measurements weekly for all subjects

		01/08/15	01/13/15	01/27/15	02/03/15	02/19/15	02/05/00	03/05/15	03/16/15	03/23/15	Average
Cage # 1											
1	No Hole	23.79	23.81	26.18	27.62	31.87	36.24	33.36	34.53	34.45	30.21
2	Right Hole	22.28	22.08	24.81	25.68	28.13	28.56	30.28	32.71	30.91	27.27
3	Left Hole	25.02	24.50	27.71	29.14	31.13	31.03	33.61	34.78	29.43	29.59
4	Both	22.32	22.40	24.96	25.20	27.24	30.60	28.34	29.22	24.00	26.03
Cage # 2											
1	No Hole	23.13	22.99	25.08	26.65	27.13	32.97	28.99	31.91	30.05	27.66
2	Right Hole	23.61	23.55	27.05	27.41	29.84	29.65	31.12	33.54	32.42	28.69
3	Left Hole	23.60	25.10	27.51	29.16	31.35	31.27	35.19	36.12	37.62	30.77
4	Both	24.89	24.40	28.72	29.55	31.76	24.81	34.31	35.71	36.65	30.09
Avg Group 1		23.58	23.60	26.50	27.55	29.81	30.64	31.90	33.57	31.94	28.79
Cage # 3											
1	No Hole	24.70	24.60	30.53	32.32	32.96	31.44	36.79	38.12	38.90	32.26
2	Right Hole	23.29	24.90	28.95	26.30	32.99	28.09	29.26	31.12	29.48	28.26
3	Left Hole	24.79	23.35	28.13	29.15	29.51	29.45	31.56	31.22	32.42	28.84
4	Both	23.87	24.01	28.02	28.51	32.43	32.75	31.23	33.79	32.09	29.63
Cage # 4											
1	No Hole	25.40	25.90	29.45	30.80	26.98	28.07	34.11	36.12	37.05	30.43
2	Right Hole	23.47	23.25	28.02	28.11	24.60	31.20	30.34	31.43	31.71	28.01
3	Left Hole	23.23	23.82	27.42	29.67	31.51	32.57	32.64	33.71	34.83	29.93
4	Both	15.59	14.62	20.45	22.42	34.50	32.25	25.33	27.92	26.01	24.34
Avg Group 2		23.04	23.06	27.62	28.41	30.69	30.73	31.41	32.93	32.81	28.97
Cage # 5											
1	No Hole	25.03	25.60	28.49	30.98	35.14	32.54	35.86	36.91	39.08	32.18
2	Right Hole	25.36	24.80	28.85	30.02	28.25	28.96	34.45	34.83	35.66	30.13
3	Left Hole	24.13	25.40	27.10	28.81	30.58	33.37	31.31	32.56	31.96	29.47
4	Both	24.70	25.40	28.86	31.08	29.56	27.76	33.25	34.41	35.45	30.05
Cage # 6											
1	No Hole	22.29	23.20	24.92	25.47	32.17	30.21	28.41	30.31	28.83	27.31
2	Right Hole	20.70	23.80	22.35	23.50	29.51	28.01	25.92	27.13	25.97	25.21
3	Left Hole	23.59	20.50	27.28	28.35	30.63	32.82	32.48	34.48	32.45	29.18
4	Both	26.09	26.71	29.96	31.62	24.50	32.73	36.66	38.73	37.27	31.59
Avg Group 3		23.99	24.43	27.23	28.73	30.04	30.80	32.29	33.67	33.33	29.39
Cage # 7											
1	No Hole	24.59	25.70	25.37	30.15	31.31	34.48	32.16	34.91	32.53	30.13
2	Right Hole	22.60	23.09	28.36	26.11	27.60	33.59	28.95	30.82	29.54	27.85
3	Left Hole	24.68	25.06	27.68	30.03	32.45	30.61	33.11	36.12	34.33	30.45
4	Both	23.82	24.70	27.60	29.84	32.21	33.19	33.28	35.41	32.73	30.31
Cage # 8											
1	No Hole	22.69	23.15	25.65	26.81	27.42	27.34	27.95	30.72	28.31	26.67
2	Right Hole	24.67	25.40	27.89	29.67	31.97	25.40	31.30	33.45	32.18	29.10
3	Left Hole	22.93	23.41	28.06	29.78	34.01	32.15	32.91	34.26	33.01	30.06
4	Both	24.18	24.57	27.72	29.41	30.91	35.60	32.32	33.71	32.15	30.06
Avg Group 4		23.77	24.39	27.29	28.98	30.99	31.55	31.50	33.68	31.85	29.33

Table 3.Linear analysis average measurements for Group 1

Sample	Side	CEJ to bone crest at			CEJ to bone crest .2mm fro				Averag	Avg Ma	Avg Gr	SEM
		1st M-I	2nd M-	2nd M-	1st M-I	2nd M-	2nd M-	3rd M-M				
Group 1												
1	right	0.16	0.15		0.13	0.17			0.1525			
1	left	0.20	0.13		0.14	0.17			0.1600			
										0.1563		
2	right	0.19	0.09		0.13	0.13			0.1350			
2	left	0.15	0.12		0.14	0.17			0.1450			
										0.1400		
3	right	0.17	0.11		0.16	0.13			0.1425			
3	left	0.20	0.15		0.15	0.17			0.1675			
										0.1550		
4	right	0.19	0.15		0.15	0.17			0.1650			
4	left	0.17	0.13		0.18	0.19			0.1675			
										0.1663		
5	right	0.19	0.11		0.14	0.16			0.1500			
5	left	0.19	0.13		0.15	0.14			0.1525			
										0.1513		
6	right	0.23	0.09		0.15	0.14			0.1525			
6	left	0.16	0.12		0.14	0.14			0.1400			
										0.1463		
7	right	0.15	0.13		0.12	0.18			0.1450			
7	left	0.16	0.11		0.09	0.16			0.1300			
										0.1375		
8	right	0.23	0.14		0.15	0.17			0.1725			
8	left	0.20	0.13		0.18	0.17			0.1700			
										0.1713		
Group											0.1529	0.0037

Table 4 Linear analysis average measurements for Group 2

Group 2												
9	right	0.22	0.15		0.24	0.22			0.2075			
9	left	0.25	0.21		0.22	0.24			0.2300			
										0.2188		
10	right	0.36	0.23		0.26	0.27			0.2800			
10	left	0.24	0.23		0.20	0.20			0.2175			
										0.2488		
11	right	0.38	0.20		0.22	0.26			0.2650			
11	left	0.32	0.31		0.30	0.30			0.3075			
										0.2863		
12	right	0.28	0.28		0.24	0.24			0.2600			
12	left	0.26	0.19		0.23	0.23			0.2275			
										0.2438		
13	right	0.30	0.30		0.27	0.27			0.2850			
13	left	0.44	0.24		0.37	0.27			0.3300			
										0.3075		
14	right	0.26	0.23		0.19	0.24			0.2300			
14	left	0.42	0.35		0.40	0.28			0.3625			
										0.2963		
15	right	0.25	0.26		0.25	0.24			0.2500			
15	left	0.28	0.25		0.24	0.26			0.2575			
										0.2538		
16	right	0.40	0.34		0.44	0.26			0.3600			
16	left	0.45	0.33		0.15	0.19			0.2800			
										0.3200		
Group											0.2718	0.0085

Table 5 Linear analysis average measurements for Group 3

Group 3												
17	right	0.19	0.15		0.17	0.20			0.1775			
17	left	0.25	0.19		0.17	0.18			0.1975			
										0.1875		
18	right	0.26	0.13		0.17	0.21			0.1925			
18	left	0.20	0.14		0.21	0.20			0.1875			
										0.1900		
19	right	0.18	0.13		0.17	0.18			0.1650			
19	left	0.20	0.13		0.15	0.17			0.1625			
										0.1638		
20	right	0.19	0.12		0.18	0.14			0.1575			
20	left	0.19	0.12		0.14	0.16			0.1525			
										0.1550		
21	right	0.20	0.14		0.14	0.19			0.1675			
21	left	0.17	0.11		0.14	0.16			0.1450			
										0.1563		
22	right	0.15	0.12		0.13	0.18			0.1450			
22	left	0.18	0.14		0.16	0.17			0.1625			
										0.1538		
23	right	0.16	0.12		0.15	0.17			0.1500			
23	left	0.20	0.11		0.16	0.17			0.1600			
										0.1550		
24	right	0.17	0.12		0.15	0.14			0.1450			
24	left	0.18	0.12		0.13	0.16			0.1475			
										0.1463		
Group											0.1634	0.0039

Table 6. Linear analysis average measurements for Group 4

Group 4												
25	right	0.21	0.16		0.23	0.23			0.2075			
25	left	0.37	0.29		0.25	0.29			0.3000			
										0.2538		
26	right	0.29	0.29		0.25	0.29			0.2800			
26	left	0.26	0.20		0.25	0.21			0.2300			
										0.2550		
27	right	0.24	0.23		0.23	0.26			0.2400			
27	left	0.23	0.22		0.24	0.24			0.2325			
										0.2363		
28	right	0.30	0.27		0.19	0.30			0.2650			
28	left	0.33	0.25		0.21	0.25			0.2600			
										0.2625		
29	right	0.38	0.29		0.23	0.29			0.2975			
29	left	0.48	0.31		0.28	0.31			0.3450			
										0.3213		
30	right	0.28	0.24		0.27	0.27			0.2650			
30	left	0.36	0.32		0.26	0.26			0.3000			
										0.2825		
31	right	0.31	0.26		0.28	0.33			0.2950			
31	left	0.31	0.37		0.30	0.30			0.3200			
										0.3075		
32	right	0.30	0.29		0.29	0.35			0.3075			
32	left	0.30	0.32		0.28	0.34			0.3100			
										0.3088		
Group											0.2784	0.0065

Table 7. Volumetric analysis average measurements for all groups

	Right	Left	Average
1	76.77	52.33	64.55
2	83.79	92.54	88.17
3	69.30	62.13	65.72
4	78.88	86.68	82.78
5	65.31	72.35	68.83
6	77.53	83.80	80.67
7	67.41	72.11	69.76
8	69.55	58.40	63.98
Group # 1 Average			73.06
9	28.28	35.24	31.76
10	18.43	34.74	26.59
11	34.26	34.66	34.46
12	31.17	37.25	34.21
13	26.63	19.06	22.85
14	28.32	12.84	20.58
15	29.87	24.58	27.23
16	22.75	51.74	37.25
Group # 2 Average			29.36
17	64.68	73.22	68.95
18	64.42	78.54	71.48
19	81.55	66.39	73.97
20	78.74	73.31	76.03
21	79.87	80.28	80.08
22	79.50	84.28	81.89
23	60.01	73.28	66.65
24	83.72	74.52	79.12
Group # 3 Average			74.77
25	55.69	36.41	46.05
26	39.58	49.31	44.45
27	35.96	41.69	38.83
28	21.08	18.41	19.75
29	38.86	29.49	34.18
30	23.98	26.72	25.35
31	52.16	48.07	50.12
32	32.54	40.87	36.71
Group # 4 Average			36.93

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