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Host-modulating therapeutic approaches in periodontal disease: histological evaluations

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HOST-MODULATING THERAPEUTIC APPROACHES IN PERIODONTAL DISEASE: HISTOLOGICAL EVALUATIONS

by

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HOST-MODULATING THERAPEUTIC APPROACHES IN PERIODONTAL DISEASE: HISTOLOGICAL EVALUATIONS

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ABSTRACT

Periodontal disease is an oral inflammatory disease often associated with damage to the soft tissues and hard structures that support teeth. Within the subgingival biofilm, multiple microorganisms initiate inflammatory responses that can lead to a chronic oral inflammatory state, in which periodontal ligament may be damaged leading to alveolar bone loss. In recent studies, amnion-derived cellular cytokine solution (ACCS) and cytokines secreted by the amnion-derived multipotent progenitor cells (AMPs) obtained from the placenta illustrated evidence of wound healing, promotion of macrophage activity, and infected tissue repair. Furthermore, ACCS were capable of preventing periodontal inflammation induced by *Porphyromonas gingivalis* (*P. gingivalis*) in an experimental model, suggesting its role in modulating host's inflammatory response in order to arrest, if not eradicate, inflammation in periodontal tissues.. This study aimed to investigate a novel combination of ACCS that specifically targeted the bone tissues. Whereas in previous study, ACCS was tested to identify the efficacy of its ability to reduce inflammation and to repair damages in gingiva and reduce alveolar bone loss, ACCS-B was tested here to determine its impact on bone turnover.

The study used a periodontal model of rabbit jaws to induce periodontitis. Periodontitis-specific pathogen *P. gingivalis* was applied in a carboxymethyl cellulose

(CMC) slurry topically applied to the mandibular second premolars at the site of silk ligatures.. The silk ligatures were placed to serve as the retention for the *P. gingivalis* slurry. The protocol followed two phases, the disease induction phase (6 weeks) and the treatment phase (6 weeks). In the disease induction phase, 24 New-Zealand White rabbits were induced with topical *P. gingivalis* every other day on the mandibular premolars. At the end of this phase, 3 rabbits were randomly selected and sacrificed to serve as the baseline disease group. Of the remaining 21 rabbits, 3 rabbits were kept untreated and served as untreated-control group and the remaining 18 rabbits were randomly assigned to 3 different treatment groups: Placebo Treatment (Saline), ACCS-U treatment, and ACCS-B treatment. During the treatment phase, each treatment group received its respective treatments to the ligated sites 3 times a week in an every-other-day fashion. After 6 weeks, the rabbit jaws were evaluated histologically.

At the end of treatment phase, the untreated group exhibited significant progression of periodontal disease evident by the destruction of soft and hard tissue on the site of interest. ACCS-U and ACCS-B showed substantial reduction of tissue inflammation and crestal bone loss compared to those in baseline disease, untreated, and saline-treated groups, however there were no statistically significant differences between ACCS-B and ACCS-Utreated sites. . Histological findings, specifically Masson's Trichrome stained sections, revealed that collagen deposition and new bone formations were actively present in the alveolar bone of ACCS-treated groups. Moreover, quantitative assessments of inflammatory cell activity and osteoclastic activity at the crestal and along the bone surfaces of the ligated sites by Hematoxylin-Eosin stains and

tartrate-resistant acid phosphatase stains respectively, confirmed the anti-inflammatory activity by the ACCS-treatments. The osteocalcin stained cells also revealed the initiation of bone formation and bone repair in the ACCS-treated groups.

These results state a clear indication of control of inflammatory disease and as a result, bone formation and tissue repair in both ACC-U and ACC-B treated groups.. As for the untreated group and the baseline disease group, significant disease progression is highlighted by bone loss and high level of inflammation in the site of interest. Although ACCS-B was inferior to previously tested ACCS-U with regard to anti-inflammatory effects and bone formation it clearly demonstrated its future use as potential treatment for periodontal disease, along with restoration of damaged tissues. These results further solidify findings that ACCS acts through host-modulated anti-inflammatory actions in the inflammatory conditions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACCS ..... Amnion-Derived Cytokine Solution

AMPs ..... Amnion-derived multipotent progenitor cells

ANOVA ..... Analysis of variance

BRONJ ..... Bisphosphonate-related osteonecrosis of the jaws

CMC ..... Carboxymethylcellulose

CFU ..... Colony forming unit

EDTA ..... Ethylenediaminetetraacetic acid

H&E ..... Hematoxylin & Eosin

H_2O_2 ..... Hydrogen Peroxide

IACUC ..... Institutional Animal Care and Use Committee

IBM ..... International Business Machines Corporation

IHC ..... immunohistochemistry

IM ..... Intramuscular

LSD ..... Least significant differences

M-W-F ..... Monday-Wednesday-Friday

NHANES ..... National Health and Nutrition Examination Survey

NZW ..... New Zealand White

OD ..... Optic density

PDGF ..... platelet-derived growth factor

P. gingivalis ..... *Porphyromonas gingivalis*

SD ..... Standard deviation

SPSS ..... Statistical Product and Service Solutions

TGF-β2 .....transforming growth factor beta 2

TRAP .....Tartrate-resistant acid phosphatase

VEGF .....vascular endothelial growth factor

INTRODUCTION

Periodontal disease is defined as a chronic inflammatory condition that leads to the destruction of the supporting structures of the teeth (Laudenbach & Simon, 2014). It is understood that as periodontal disease progresses, connective tissue, periodontal ligament and alveolar bone are at risk of destruction, tissue damage, which can ultimately lead to tooth loss.. While it is evident that the etiology of periodontal diseases is defined by multiple species of bacteria residing in the oral cavity, it is important to note that the greater cause of damaging both the soft and the hard tissues of the gingiva and alveolar bone is the inflammatory response that the host modulates (Genco, 1992). Past few decades of research and effort were spent solely on anti-bacteria and anti-infective therapies given the distinct etiology of this disease. However, recent studies illustrate that the subgingival environmental changes, due to the presence of oral bacteria, are the result of host-modulated inflammatory response (Bartold et al., 2013). Thus greater emphasis now rests on the search to effectively alleviate, if not fully control, the host's response.

The progression of periodontal disease can be detected by measuring the depth of the periodontal pockets around the affected teeth.. Increase in the amount of plaque and the pathogenic microorganisms that secrete toxins in turn irritates the periodontal tissues and stimulates inflammatory response. As teeth and bone structures break down by the process, gingival tissues are separated from the teeth forming pockets. The extension of inflammation leads to the pathologic deepening of the gingival sulcus (Reddy, 2008). A sulcus depth beyond 3 mm is considered gingivitis and increasing pocket depth and attachment loss mark the transition into periodontitis (Figure 1).

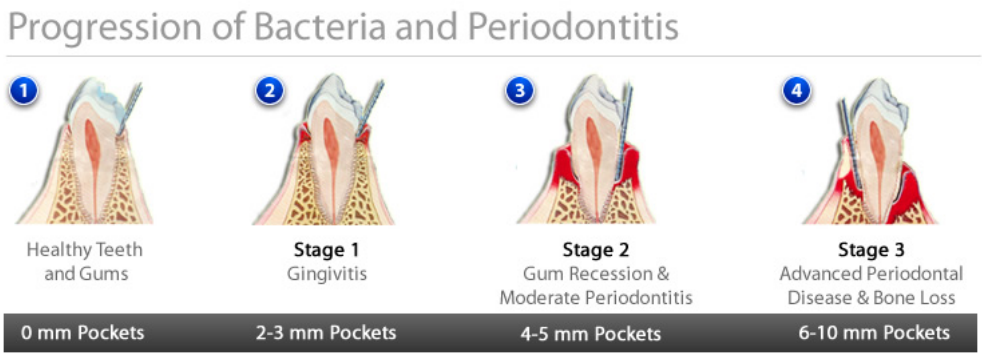

Figure 1: Progression of Periodontal Disease
(http://eicondental.com/services/periodontal-disease/)

Epidemiology of Periodontal Disease

According to the data from the National Health and Nutrition Examination Survey (NHANES), it was estimated that 46% of United States adults, which represents 64.7 million people in its category, suffer from periodontitis and of those 64.7 million people, 8.9% are in advance periodontitis stage. Overall this disease prevalence was positively associated with males and increasing age (Eke et al., 2015). Treatments may include barrier membranes, graft materials, and application of bioactive molecules, however, their effects are modest at best (Hughes et al., 2010). Despite the long history and the awareness of periodontal disease, a treatment method enabling the health of patient's oral cavity to return to its nourishing environment still remains to be seen. As statistics from NHANES indicate, periodontal disease becomes a burden to many patients and especially to those over 30 years old, primarily due to multiple implications that the disease may inflict on patients' health. Recent studies show strong bidirectional relationship between

diabetes and periodontal disease, rheumatoid arthritis and cardiovascular disease. Data also shows strong associations between periodontal disease and oral cancer (Bascones-Martínez et al., 2014; Yao et al., 2014). Furthermore, the inflammatory cytokines, host's response to bacteria, can be elevated in the systemic circulation and induce disruption of protein levels in the liver namely, CRP, fibrinogen and serum amyloid A, that promote atherosclerosis, and may also cause adverse pregnancy outcomes (Figure 2) (Hajishengallis, 2014). These findings, along with bidirectional relationships above, illustrate the importance of controlling, maintaining, and treating periodontal disease. Periodontal disease affects millions of patients globally and still lacks a fundamental treatment, thereby significantly raising its need for research in this field.

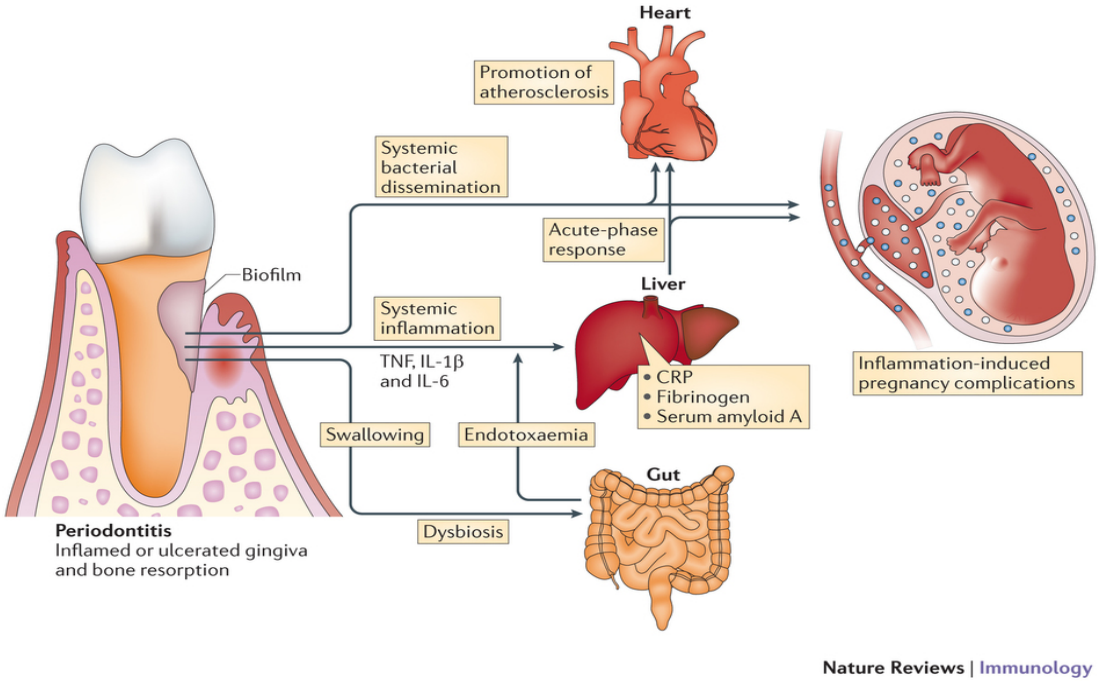

Figure 2: Biologically plausible mechanisms linking periodontitis to systemic inflammation and disease.
(http://www.nature.com/nri/journal/v15/n1/fig_tab/nri3785_F3.html)

Etiology of Periodontal Disease

It is well established that dental bacterial plaque plays a crucial role in both gingivitis and periodontitis. Whereas the former precedes periodontitis and is a reversible inflammatory reaction, the latter is irreversible in that the soft tissues and the alveolar bone itself are damaged. The accumulation of dental bacteria plaque is necessary for periodontal disease to develop, however periodontitis requires the susceptibility of the host's inflammatory system to fully develop (Tatakis et al., 2005).

Dental bacteria plaque follows several phases of different formation, but it ultimately results in a condition known as biofilm. At the gingival margin, the microbial biofilm is formed in order to epitomize the development and growth of bacteria's colony (Pöllänen et al., 2013). The biofilm increases its mass through a process known as auto-aggregation, where same species are attracted to each other, and also through co-aggregation, where different species are attracted (Kolenbrander, 2000). Multispecies biofilms then contribute to local inflammation in periodontium by elevating levels of IL-1α, IL-6 and other various pro-inflammatory and chemokines (Peyyala et al., 2013). Biofilm is a complex, organized, living system specifically designed to protect itself and grow exponentially.

Despite such complex structure being difficult to analyze, studies show that biofilm achieves its goals through cell-cell communication, gene transfer, antimicrobial resistance, gene expression regulation, lipopolysaccharide membrane, presence of heat shock proteins and extracellular proteolytic enzymes (Tatakis et al., 2005). One promising approach to intervene in the formation of microbial multi-biofilms is through

inhibition of adhesion between conglomerated mass of bacterial colony, thus impeding the interactions and recruitment of other bacterial species (Demuyser et al., 2014).

Each periodontal disease has a unique profile of bacteria that is associated with them. This statement is aligned with the fact that different bacteria have their distinctive characteristic in both the causation and progression of the disease (Tatakis et al., 2005). All bacteria are in some extent involved with different periodontal diseases due to the nature of oral cavity but, studies have shown some of the more recognized species involved with specific periodontal disease (Table 1).

Table 1. Bacterial species associated with periodontal health and disease.
Data taken from Tatakis et al., 2005.

Condition	Associated Microorganisms
Periodontal Health	Gram (+) anaerobe *Atopobium rimae* Gram (+) facultative *Streptococus sanguis* *Streptococous mitis* Gram (-) anaerobe *Bacteroiedes* sp oral clone BU063 *Veillonella* spp *Gemella* spp *Capnocytophaga* spp
Gingivitis	Gram (+) anaerobe *Actinomyces sanguis* *Peptostreptococcus micros* Gram (+) facultative *Streptococus* spp Gram (-) anaerobe *Campylobacter gracilis* *Fusobacterium nucleatum* *Prevotella intermedia*

	Veillonella parvula
Chronic Periodontitis	Gram (+) anaerobe *Peptostreptococcus micros* Gram (-) anaerobe *Porphyromonas gingivalis* *Tannerella forsythia* *Campylobacter rectus* *Eikenella corrodens* *Fusobacterium nucleatum* *Actinobacillus Actinomycetemcomitans* *Treponema* spp *Filifactor alocis* *Megasphaera* sp oral clone BB166 *Deferribacteres* sp oral clone BH017 *Desulfobulbus* sp oral clone R004 *Bacteroides* sp oral clone AU 126
Aggressive Periodontitis	Gram (-) anaerobe *Actinobacillus Actinomycetemcomitans* *Porphyromonas gingivalis* *Campylobacter rectus* *Eikenella corrodens*
Acute Necrotizing Gingivitis	Gram (-) anaerobe *Treponema* spp *Prevotella intermedia* *Rothia dentocariosa* *Fusobacterium* spp *Achromobacter* spp *Propionibacterium acnes* *Capnocytophaga* spp
Periodontal Abscess	Gram (+) anaerobe *Peptostreptococcus micros* Gram (-) anaerobe *Fusobacterium nucleatum* *Prevotella intermedia* *Tannerella forsythia* *Campylobacter rectus*

Rationale for Animal Models

Researchers have always addressed the importance of animal models, despite their limitations. Different animal models are essential for testing diverse biological significances that otherwise will be left to question. Recent studies have shown that animal-based studies provide framework in which mechanisms of periodontitis can be studied and clarified (Struillou et al., 2010). Moreover, these studies illustrate that dysbiosis, rather than individual bacteria species, is etiologically significant for initiating host inflammatory response that results in periodontal bone loss and soft tissue damage. However, the most noteworthy intake from these studies is how animal models contribute to the testing of possible therapeutic targets (Hajishengallis et al., 2015). While experiments conducted through animal models may not be directly applicable to humans, they have proven to be of a vital use, often replacing in vitro experiments (Oz et al., 2011). In addition, there is no animal model where all aspects of human life and disease are directly correlated, due to the complex nature of human body.. For example, Salk was able to successfully create a vaccine against strains of polio through Rhesus monkey cross-contamination studies (Shampo & Kyle, 1998). Specific animal models, thus, provide insight to particular disease mechanism that otherwise would be difficult to identify.

Rabbit models are ideal for studying periodontal disease and its association with host inflammatory response. Because of their size, easy handling, short life span, and economical aspects in purchasing and sustaining, rabbits are a first-hand choice in regards to sampling and sustaining in a lab environment (Stübinger et al., 2013). In

addition to handling, rabbits are perfect in that previous work have succeeded in inducing controllable and reproducible periodontitis through application of specific species of bacteria, *P gingivalis*, and silk ligatures (Hasturk et al, 2006, 2007, Zenobia et al., 2014). Furthermore, rabbit models are constantly being used due to their simplicity as mentioned above, its similarity to human systems and lastly their response to certain irritants. Hence, rabbit models have been used for other inflammatory diseases and other human diseases as a consequence; atherosclerosis (Fan et al., 2015), implant dentistry and related tissue regeneration (Stübinger et al., 2013), lung disease and stem cell therapy (Kamaruzaman et al., 2013), and pathophysiology of rhinosinusitis (Perez et al., 2014). Therefore due to their straightforwardness and resemblance to human response, rabbits are an ideal model to study mechanisms involving periodontal tissue regeneration and alveolar bone gain and repair.

Application of ACCS

Successfully inducing controlled and reproducible periodontitis in rabbits may shed light on the mechanisms surrounding the host's inflammatory response towards infection; however, it does not present a therapeutic treatment. Hence, identifying the best therapeutic solution to not only alleviate host's inflammatory action, but also to chronic wound healing in the subgingival ligament and alveolar bone regeneration is necessary to eradicate periodontal disease. Amnion-derived multipotent progenitor cells (AMPs) secrete cytokines known as amnion-derived cellular cytokine solution (ACCS). AMPs are acquired from the placenta and despite it being a stem cell it is non-

tumorigenic and non-immunogenic (Banas et al., 2008). Moreover, human amnion-derived cells are more reliable, in that they are less likely to contain genetic aberrations and can be programmed earlier for more efficiency, characteristics that the multipotent stem cells did not exhibit (Saito et al., 2012). Moreover, the secreted ACCS contain cytokines vital for acute and chronic wound healing (Franz et al., 2008), promote migration of macrophages into the wound-site (Uberti et al., 2011), arthritis (Tramontini et al, 2004), and finally, have substantial anti-inflammatory and anti-fibrotic properties (Silini et al., 2013).

ACCS contain proteins such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiogenin, and transforming growth factor beta 2 (TGF-β2) and they provide pathways eventually contributing to wound healing (Steed et al., 2008). In addition to providing these proteins, ACCS also play a role in controlling keratinocyte and fibroblast migration to further enhance wound healing in different parts of the body (Uberti et al., 2010). Recent studies show that ACCS was not only capable of preventing periodontitis in experimental model, but also able to repair damaged tissues and alveolar bone suggesting its role in reversing the severity of inflammation response initiated by the host (Hasturk et al., 2013).

SPECIFIC AIMS

The primary aim of this present study was to examine the effectiveness of a novel formulation of ACCS compared to the formulation previously tested for periodontal tissue regeneration (Hasturk et al., 2013). The previous study used original ACCS-U whereas in this study, a new formulation of ACCS designed to target the bone tissue, ACCS-B, was tested for therapeutic purposes. This study used a model of inducing periodontitis in rabbit premolars by placing silk ligatures and topical application of specific bacterial species, *P. gingivalis*. After two phases each lasting 6 weeks, histological analysis was performed to evaluate the efficacy of the new ACCS-B formulation.

METHODS

Animal Model

In phase 1, disease induction, periodontal disease was induced in 24 adult (3 months old) male New Zealand White rabbits. At the end of the phase 1, the pathogenic microorganism, *P. gingivalis*, application was halted and 3 rabbits were randomly selected and subsequently sacrificed to serve as a baseline disease group. The baseline disease group is established to compare the results in the phase 2, the treatment phase. The remaining 21 rabbits are randomly assigned into 3 groups of 6, each group representing test group, positive control group, and negative control group. The last 3 rabbits are assigned into untreated group. The 3 groups aforementioned are entered into phase 2 lasting 6 weeks of two different amnion-derived cellular cytokine solutions and placebo (saline).

Phase 1: Disease Induction

The protocol was reviewed and approved by the Forsyth Institute Institutional Animal Care and Use Committee (Forsyth IACUC) prior to study. Specific pathogen-free NZW rabbits were purchased from Covance Research Products, Inc. (Denver, PA), equilibrated for 7 days prior to experiments and housed for the duration of the study at the rabbit facility at the Forsyth Institute (1st Floor Rabbit Facility-Holding Room 2). Experimental periodontitis were induced using previously published methodology for rabbit periodontitis model (Serhan at al 2003; Hasturk et al, 2006, 2007, 2009). Briefly,

on the day of experiment, the rabbits were anesthetized using xylazine (5 mg/kg, IM) and ketamine (35 mg/kg, IM). A ligature (3-0 braided silk suture) was placed around the 2nd premolars of both sides of the mandible followed by *P. gingivalis* slurry application (as described below). After baseline ligature placement, animals were anesthetized using inhalation anesthesia (isoflurane; 3-4% for induction of sedation in induction box; 2.5% for continuation of sedation for oral applications) every other day (Monday, Wednesday and, Friday) to receive CMC slurry (1 ml) containing *P. gingivalis* for the first 6 weeks. At these times, the sutures were checked, and lost or loose sutures were replaced (at average once or twice for the total period). (Figure 3). All experimental procedures were conducted in the procedure room dedicated for the rabbit experiments (1st floor Rabbit Facility).

P. gingivalis (strain A7436; Shapira et al, 1998) was grown using standard procedures (Olsen and Socransky, 1981; Doan et al, 1999; Socransky et al, 1994). Briefly, bacteria were cultured on agar plates containing trypticase soy agar supplemented with 0.5% (w/v) yeast extract (Gibco Industries, Los Angeles, CA), 5% defibrinated sheep red blood cells, 5 μg hemin, and 1 μg/ml vitamin K (Sigma-Aldrich, St. Louis, MO). Plates were incubated for 3 days at 37°C in an anaerobic chamber maintained by hydrogen gas mixture (hydrogen/nitrogen) that is circulated through a heated palladium catalyst. Colonies were randomly selected and anaerobically cultured overnight at 37°C in Wilkins-Chalgren Anaerobe Broth. Colonies were randomly selected and anaerobically cultured overnight at 37°C in growth medium (Schaedler's broth) supplemented with vitamin K and hemin. Bacterial numbers were

spectrophotometrically determined at 600 nm and 10^9 colony-forming units (CFU) (0.8 OD) in growth medium (1 ml) and mixed with carboxymethylcellulose (CMC) to form a thick slurry, which was applied topically to the ligated teeth to induce periodontitis for 6 weeks.

Phase 2. Treatment

At the end of Phase 1, 3 rabbits were sacrificed to form the baseline disease group (Reference group) at 6 weeks. The remaining 21 rabbits randomly assigned into 4 groups entered in Phase 2 for a period of six weeks: 3 treatment groups as well as an untreated control group as shown below:

Study groups:

1. Test (ACCS-B) treatment group (n=6)
2. Positive control (ACCS-U) treatment group (n=6)
3. Placebo/Sham (Saline) treatment group (n=6)
4. Untreated (Control) group (n=3)
5. Reference (baseline periodontal disease at 6 weeks) group (n=3)

P. gingivalis application has ceased from this point on, but the ligatures were kept for topical applications. Test and placebo products were topically administered every-other-day (Mon-Wed-Fri) in 10 microliters/tooth/animal (in total 20 microliters per rabbit) using a blunt tipped 10 μL Hamilton syringe (Model 1701 Gastight Point Style 3-Cat# 14-813-125;Fischer Scientific) to the ligated sites, on the gum line, for 6 weeks. During this period lost sutures were replaced, if lost (once or twice per rabbit) (Figure 3).

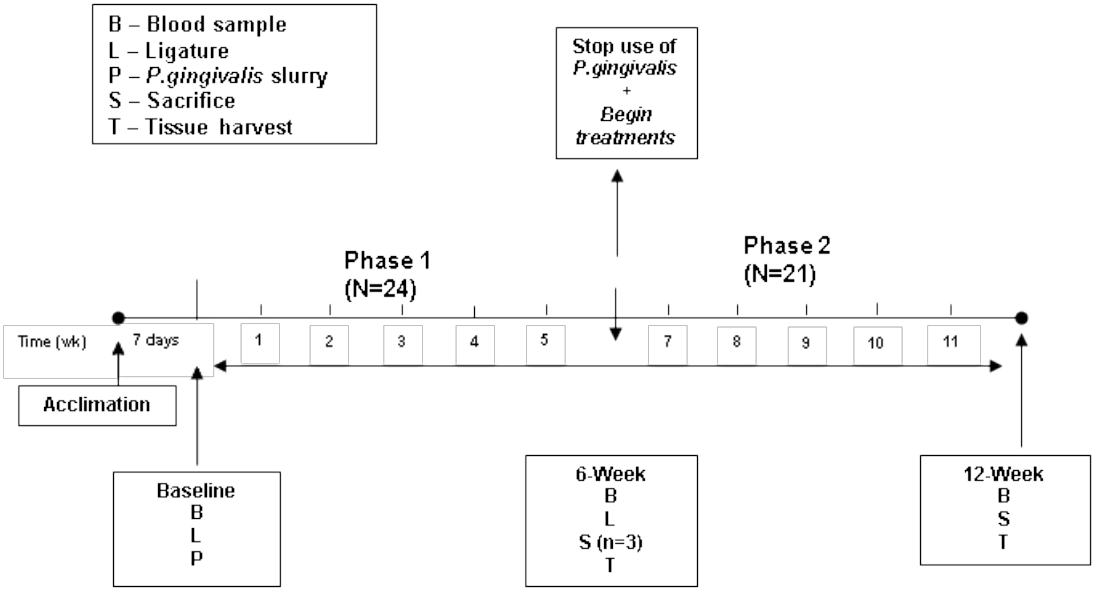

Figure 3. Timeline of experimental design.

Phase 1: 3-0 silk ligatures were tied to second premolars in mandibular quadrants at baseline in all groups and *P. gingivalis* was applied in methylcellulose slurry via a 3 ml plastic syringe without needle three times per week (M-W-F) for six weeks. At six weeks, 3 animals were sacrificed and the extent of disease determined as baseline periodontitis (represented by red bar in the following figures).

Phase 2: *P. gingivalis* application was ceased at this point and the other animals continued for a second six-week with treatment: 1) ACCS-B, 2) ACCS-U, and 3) Saline (6 rabbits/group). An additional group of 3 rabbits were kept untreated and served as negative control. All treatment solutions were topically delivered to the ligated site at the gum line via a blunt tipped 10µL Hamilton syringe. All treatment/untreated groups were sacrificed at 12 weeks and the extent of disease determined for comparison to baseline periodontitis and health (historical data).

Tissue Analysis

For histological analysis, the other half of the mandible was immersed in a volume of 10% EDTA solution equal to at least 20 times the size of section; solution was replaced every 2-3 days for 8 weeks while continuous gentle shaking was provided for stimulation of the process (Skinner et al 1997). X-rays determined the end point of the decalcification. After decalcification, specimens were dehydrated using xylene and embedded in paraffin. Thin sections (5 μm) were cut and sections were either conventionally stained with hematoxylin-eosin (H&E) to identify the cellular composition of the inflammatory infiltrates or with tartrate-resistant acid phosphatase (TRAP) to detect the osteoclastic activity. Some sections from each specimen were also stained with Masson's Trichrome for collagen deposition and new bone formation. Lastly, to compliment the findings with osteoclastic activity, immunohistochemistry staining was performed for detection of osteocalcin positive cells.

The cellular infiltrate was quantified using a method originally developed in our laboratories (Uzel et al, 2001) to assess the density of the inflammatory cells. Leukocytes were counted and the results presented as cells/unit area mm^2. This measurement was performed using a standard area under the microscope ($0.09mm^2$); repeated for 3 different slides for each specimen, and expressed as the average of 3 slides to assess the extent of inflammatory infiltrate.

To evaluate the osteoclastic activity (osteoclastogenesis) during inflammatory changes in the periodontal tissues, osteoclast-like cells were identified using tartrate resistant acid phosphatase (TRAP) staining. TRAP-positive cells were counted in the

coronal 1/3 of the root on each section (cells/mm^2) using Image J software program and calculated for both mesial and distal crestal alveolar bone of the ligated tooth (second premolar).

To evaluate the osteoblastic activity or bone formation activity, osteocalcin positive cells were detected by anti-osteocalcin antibody (OC4-30) (Abcam®; Catalog No: ab13418) using immunohistochemistry (IHC) technique for paraffin embedded sections. Similarly, osteocalcin- positive cells were counted in the coronal 1/3 of the root on each section (cells/mm2) using Image J software program and calculated for both mesial and distal crestal alveolar bone of the ligated tooth (second premolar).

Collagen fibers at the supracrestal areas and between tooth and bone were evaluated on Masson's trichrome stained sections. Specifically, the collagen deposition and the organization of collagen fibers were evaluated.

In order to quantify the changes in bone, the mean value (± standard deviation) of the linear distance for bone loss were calculated for each group. Previously developed measurement technique (Hasturk et al, 2006) was used to calculate the bone changes at three different sections of the root using the image analysis software (Image J). The linear measurements were made at three levels each corresponding to one-third of the root and alveolar bone interface: crestal, mid, and apical. Linear distance is reported as the distance from the base of the epithelium to the alveolar crest border at the three chosen levels, the apical, middle, and the coronal third of the root and is expressed as the difference between ligated and non-ligated sites. Results were presented at the crestal and mid-level (Figure 2).

Data Analysis

In this study, we investigated the potential effects of the ACCS-B solution in periodontal tissue regeneration compared to ACCS original, placebo and control (untreated). Based on our previous study with ACCS original (Hasturk et al,2013)), for intrabony defect changes, the mean difference between the ACCS original and ACCS irradiated was 0.4 mm with a standard deviation of 0.4. Hence, assuming mean difference of 0.4 mm with SD of 0.4 and alpha=0.05, and 80% power, a sample size of 6 per group for the test groups (ACCS-B, ACCS original and placebo) was sufficient. Due to large differences between the test groups and both baseline disease and untreated groups, the animals' numbers were reduced to 3 rabbits each. In addition, we used our previously generated data (historical) to increase the sample size in these groups, if needed.

The data obtained by direct measurements during morphologic assessment and by histomorphometric measurements were used in multiple statistical analyses. Mean values for linear and area measurements were utilized to determine the changes in bone level in reference disease group compared to treatment groups at 12 weeks. The ratio calculations were also used and multiple comparisons within groups were made using analysis of variance (ANOVA) with *post hoc* tests Bonferroni or LSD correction.

RESULTS

Histological Analysis

As illustrated in Figure 4, the histological analyses were performed on paraffin-embedded decalcified hemi-mandibles. Thin sections (5μm) were taken from each specimen-paraffin block (up to 30 sections). Consecutive sections at the middle of the block were stained with:

- Hematoxylin and eosin (H&E) for descriptive analysis of characteristics of the tissue, quantification of inflammatory cell infiltrate and histomorphometric assessments for alveolar bone levels.
- Tartrate acid phosphatase (TRAP) for the determination of osteoclastic cell activity (osteoclastogenesis).
- Masson's trichrome for the determination of collagen activity ad collagen fibers at the supracrestal level of the dento-alveolar unit.
- Osteocalcin for the analysis of osteoblastic cell activity at the ligated sites

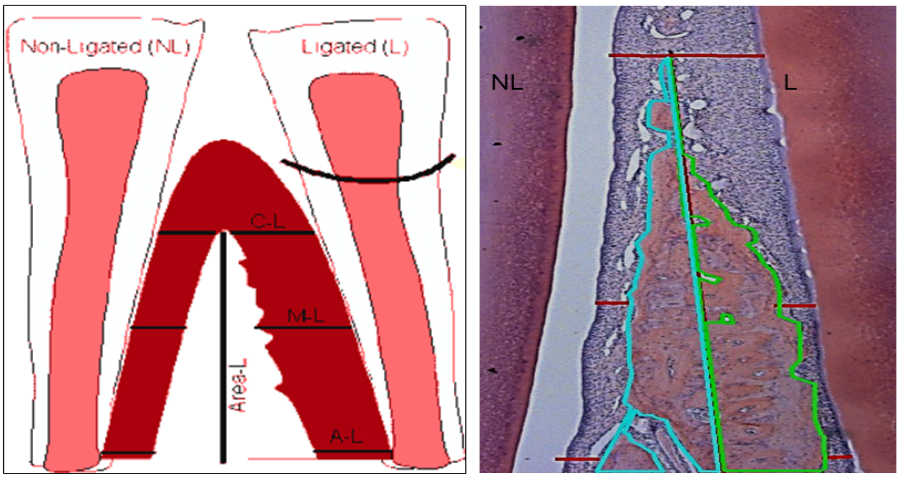

Figure 4. Schematic illustration of histomorphometric measurements.

5 µm paraffin-embedded sections stained with H&E staining were used for histometric analysis. Three measurements were made for each area (crestal, middle and apical) and averages are taken for each representative site (NL-non-ligated; L-ligated).

Histologic assessments of the sections from each group were performed under light microscope at 25X, 100X, 200X and 400X magnifications for the details of tissue characteristics. As reported previously (Hasturk et al 2007, 2009), every-other-day topical *P. gingivalis* slurry application at the ligated sites of mandible resulted in experimental periodontitis induction during the first 6 weeks of the experiment indicated by inflammatory changes at both soft tissue and alveolar bone levels. Soft tissue exhibited typical characteristics of periodontal pocket formation with increased inflammatory cell infiltration. (Figure 5A).

In the second phase of the study, the *P. gingivalis* applications were stopped and in the untreated group, the progression of disease was assessed. The analyses of sections from the untreated specimens showed significant inflammatory cell infiltration in connective tissue and bone resorption, with irregular bone surfaces and resorptive lacunae at the ligated sites (Figure 5B). The treated groups with ACCS-U and ACCS-B however showed significantly less inflammatory changes with an intact bone surface and a healthy connective tissue; the inflammatory changes and bone loss were clearly diminished in these groups (Figure 5D and E, respectively). Saline treatment did not result in any significant changes in tissue inflammation or bone loss (Figure 5C) from baseline; the group exhibited more inflammatory changes and bone loss indicating disease progression.

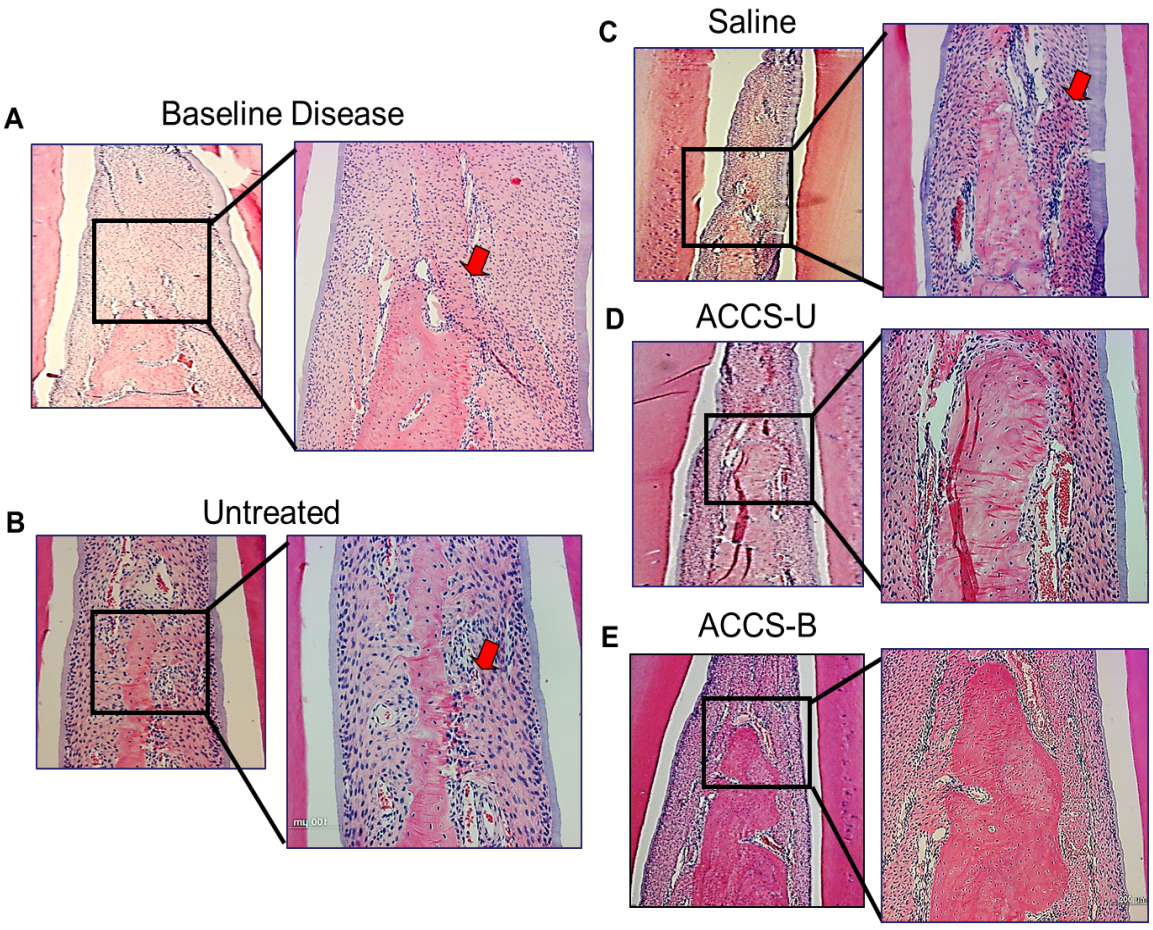

Figure 5. Histological Evaluations.

A. Baseline disease shows characteristics of inflammatory changes in connective tissue and crestal bone loss.
B. When the baseline disease left untreated, the progression of inflammation and bone loss were apparent. The inflammatory cell infiltration was dense, bone loss was detected with resorptive lacunae at the crestal and interproximal sites.

C. Saline treatment did not affect the disease process; the inflammatory cell infiltration was quite obvious as well as the alveolar bone loss at both crestal and interproximal sites.
D and E. ACCS-U and ACCS-B treatment groups exhibited the least inflammatory changes and bone loss at the ligated sites (Images shown-H&E 25x and 100x magnifications; red arrow depicts the inflammatory cell infiltration at the disease-ligated site).

Tables 2-4 show multiple comparisons between groups with regard to histological bone loss/gain, the counts of inflammatory cell, TRAP+ cell and osteocalcin + cell at crest and middle portion of the alveolar bone. Histomorphometric measurements showed similar findings as clinical evaluations; ACCS treated tissues demonstrated significantly less bone loss, less inflammation in soft tissue and less osteoclastogenic activity compared to untreated and placebo groups confirming the potential impact of ACCS-U on the treatment of periodontal diseases. The tested compound, ACCS-B demonstrated comparable results to ACCS-U in histological assessments of periodontal tissues with significant reduction in periodontal inflammation and breakdown of alveolar bone and periodontal tissues (Figures 6-8). Masson's Trichrome staining also showed more collagen deposition and well organized collagen fiber orientations at the supracrestal level in the ACCS-treated specimens compared to baseline disease, untreated and saline-treated groups (Figure 9). Osteoblastic activity was also evaluated by immunohisto-chemistry in sections stained with osteocalcin as a marker of new bone formation (Table 5, Figure 10). ACCS-treated groups exhibited greater osteoblastic activity along the alveolar bone (both crestal and interproximal at the ligated sites) compared to other groups (baseline, untreated and saline treated).

Table 2. Multiple comparisons for histological bone loss between groups

The data obtained by direct measurements during morphologic assessment and by histomorphometric measurements were used in multiple statistical analyses. Mean values for linear and area measurements were utilized to determine the changes in bone level in reference disease group compared to treatment groups at 12 weeks. The ratio calculations were also used and multiple comparisons within groups were made using analysis of variance (ANOVA) with *post hoc* tests Bonferroni or LSD correction.

Dependent Variable	ID	ID	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Crest	ACCS-U	ACCS-B	-.0266	.0263	.322	-.081	.028
		Saline	-.1433*	.0263	.000	-.198	-.089
		Untreated	-.1565*	.0322	.000	-.223	-.090
		Baseline	-.1605*	.0322	.000	-.227	-.094
		ligature alone	-.0415	.0294	.172	-.103	.019
	ACCS-B	ACCS-U	.0266	.0263	.322	-.028	.081
		Saline	-.1166*	.0263	.000	-.171	-.062
		Untreated	-.1299*	.0322	.001	-.197	-.063
		Baseline	-.1339*	.0322	.000	-.201	-.067
		ligature alone	-.0149	.0294	.618	-.076	.046
	Saline	ACCS-U	.1433*	.0263	.000	.089	.198
		ACCS-B	.1166*	.0263	.000	.062	.171
		Untreated	-.0132	.0322	.685	-.080	.054
		Baseline	-.0173	.0322	.597	-.084	.050
		ligature alone	.1017*	.0294	.002	.041	.163
	Untreated	ACCS-U	.1565*	.0322	.000	.090	.223
		ACCS-B	.1299*	.0322	.001	.063	.197
		Saline	.0132	.0322	.685	-.054	.080
		Baseline	-.0040	.0372	.915	-.081	.073
		ligature alone	.1150*	.0348	.003	.043	.187
	Baseline	ACCS-U	.1605*	.0322	.000	.094	.227
		ACCS-B	.1339*	.0322	.000	.067	.201
		Saline	.0173	.0322	.597	-.050	.084
		Untreated	.0040	.0372	.915	-.073	.081
		ligature alone	.1190*	.0348	.002	.047	.191
	ligature alone	ACCS-U	.0415	.0294	.172	-.019	.103
		ACCS-B	.0149	.0294	.618	-.046	.076
		Saline	-.1017*	.0294	.002	-.163	-.041
		Untreated	-.1150*	.0348	.003	-.187	-.043
		Baseline	-.1190*	.0348	.002	-.191	-.047

Table 2. continued. Multiple comparisons for histological bone loss between groups

Dependent Variable	ID	ID	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Middle	ACCS-U	ACCS-B	-.0058	.0440	.896	-.097	.085
		Saline	-.0407	.0440	.365	-.132	.051
		Untreated	-.1117	.0539	.050	-.223	.000
		Baseline	-.1579*	.0539	.008	-.270	-.046
		ligature alone	-.0206	.0492	.680	-.123	.081
	ACCS-B	ACCS-U	.0058	.0440	.896	-.085	.097
		Saline	-.0348	.0440	.437	-.126	.056
		Untreated	-.1059	.0539	.062	-.218	.006
		Baseline	-.1521*	.0539	.010	-.264	-.040
		ligature alone	-.0147	.0492	.767	-.117	.087
	Saline	ACCS-U	.0407	.0440	.365	-.051	.132
		ACCS-B	.0348	.0440	.437	-.056	.126
		Untreated	-.0710	.0539	.201	-.183	.041
		Baseline	-.1172*	.0539	.041	-.229	-.005
		ligature alone	.0201	.0492	.687	-.082	.122
	Untreated	ACCS-U	.1117	.0539	.050	.000	.223
		ACCS-B	.1059	.0539	.062	-.006	.218
		Saline	.0710	.0539	.201	-.041	.183
		Baseline	-.0462	.0622	.466	-.175	.083
		ligature alone	.0911	.0582	.132	-.030	.212
	Baseline	ACCS-U	.1579*	.0539	.008	.046	.270
		ACCS-B	.1521*	.0539	.010	.040	.264
		Saline	.1172*	.0539	.041	.005	.229
		Untreated	.0462	.0622	.466	-.083	.175
		ligature alone	.1373*	.0582	.028	.017	.258
	ligature alone	ACCS-U	.0206	.0492	.680	-.081	.123
		ACCS-B	.0147	.0492	.767	-.087	.117
		Saline	-.0201	.0492	.687	-.122	.082
		Untreated	-.0911	.0582	.132	-.212	.030
		Baseline	-.1373*	.0582	.028	-.258	-.017

*. The mean difference is significant at the 0.05 level.

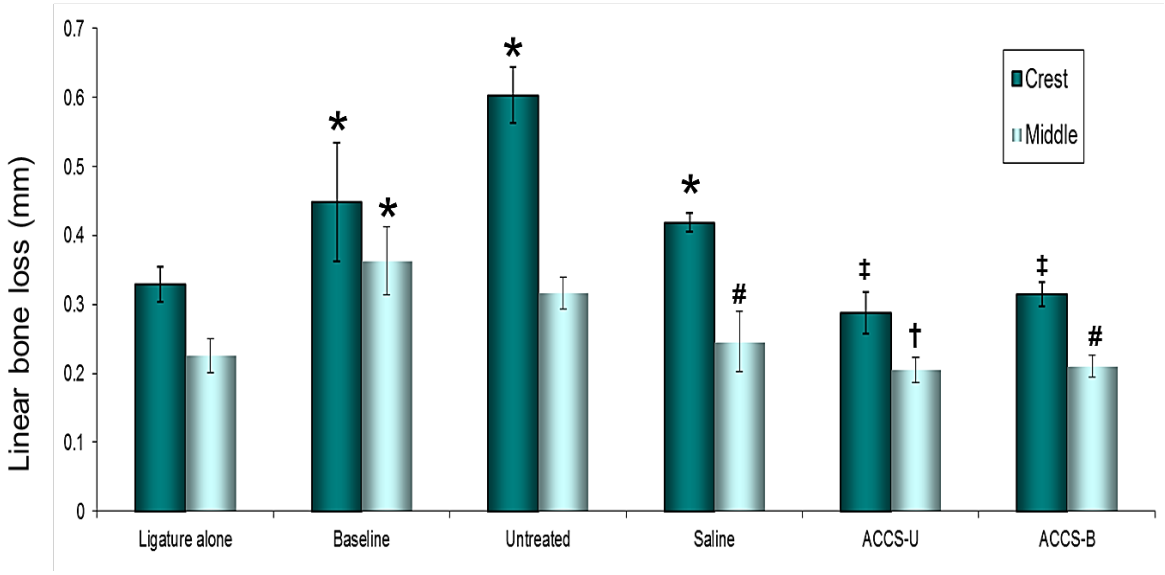

Figure 6. Histomorphometric bone level.

Linear distance was measured as the distance from the base of the epithelium to the alveolar crest border at the three chosen levels, the apical, middle, and the coronal third of the root and is expressed as the difference between ligated and non-ligated sites. Results were presented at the crestal and mid-level as described elsewhere (Figure 4). Similar to clinical findings *P. gingivalis* application resulted in significant bone loss compared to ligature alone group (historical data) at both crestal and mid-level of the alveolar bone confirming the disease model used in this study ($p=0.002$ and $p=0.028$, respectively). The untreated and saline treated groups showed typical characteristics of the disease, progression of bone loss, at crestal level compared to baseline disease; however the difference was not statistically significant. While both groups showed statistically significant bone loss compared to ligature alone group ($p=0.02$ and $p=0.03$, respectively). Both ACCS-treated groups dramatically reduced the histological bone loss indicating bone gain at crestal level compared to all other disease groups ($p<0.001$); ACCS-U treatment also showed significant bone gain at mid-level compared to baseline and untreated groups ($p=0.05$ and $p=0.008$, respectively), while both saline and ACCS-B treatment resulted in more bone at mid-level compared to baseline disease ($p=0.41$ and $p=0.01$, respectively). *Statistically significant bone loss compared to ligature alone and ACCS groups ($p<0.05$); $^{\#}$Statistically significant bone loss compared to baseline disease group ($p<0.05$); ‡Statistically significant compared to baseline, untreated and saline groups; †Statistically significant compared to baseline and untreated groups (ANOVA, LSD *post hoc* test; $p<0.05$).

Table 3. Multiple comparisons for inflammatory cell infiltration between groups

Thin sections (5 µm) were cut and sections were either conventionally stained with hematoxylin-eosin (H&E) to identify the cellular composition of the inflammatory infiltrates. The ratio calculations were also used and multiple comparisons within groups were made using analysis of variance (ANOVA) with *post hoc* tests Bonferroni or LSD correction

ID	ID	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
ACCS-U	ACCS-B	-1.971	8.662	.822	-20.10	16.16
	Saline	-23.526*	8.662	.014	-41.66	-5.40
	Untreated	-23.232*	10.609	.041	-45.44	-1.03
	Baseline	-27.261*	10.609	.019	-49.47	-5.06
ACCS-B	ACCS-U	1.971	8.662	.822	-16.16	20.10
	Saline	-21.555*	8.662	.022	-39.68	-3.42
	Untreated	-21.261	10.609	.060	-43.47	.94
	Baseline	-25.290*	10.609	.028	-47.49	-3.09
Saline	ACCS-U	23.526*	8.662	.014	5.40	41.66
	ACCS-B	21.555*	8.662	.022	3.42	39.68
	Untreated	.294	10.609	.978	-21.91	22.50
	Baseline	-3.735	10.609	.729	-25.94	18.47
Untreated	ACCS-U	23.232*	10.609	.041	1.03	45.44
	ACCS-B	21.261	10.609	.060	-.94	43.47
	Saline	-.294	10.609	.978	-22.50	21.91
	Baseline	-4.029	12.250	.746	-29.67	21.61
Baseline	ACCS-U	27.261*	10.609	.019	5.06	49.47
	ACCS-B	25.290*	10.609	.028	3.09	47.49
	Saline	3.735	10.609	.729	-18.47	25.94
	Untreated	4.029	12.250	.746	-21.61	29.67

*. The mean difference is significant at the 0.05 level.

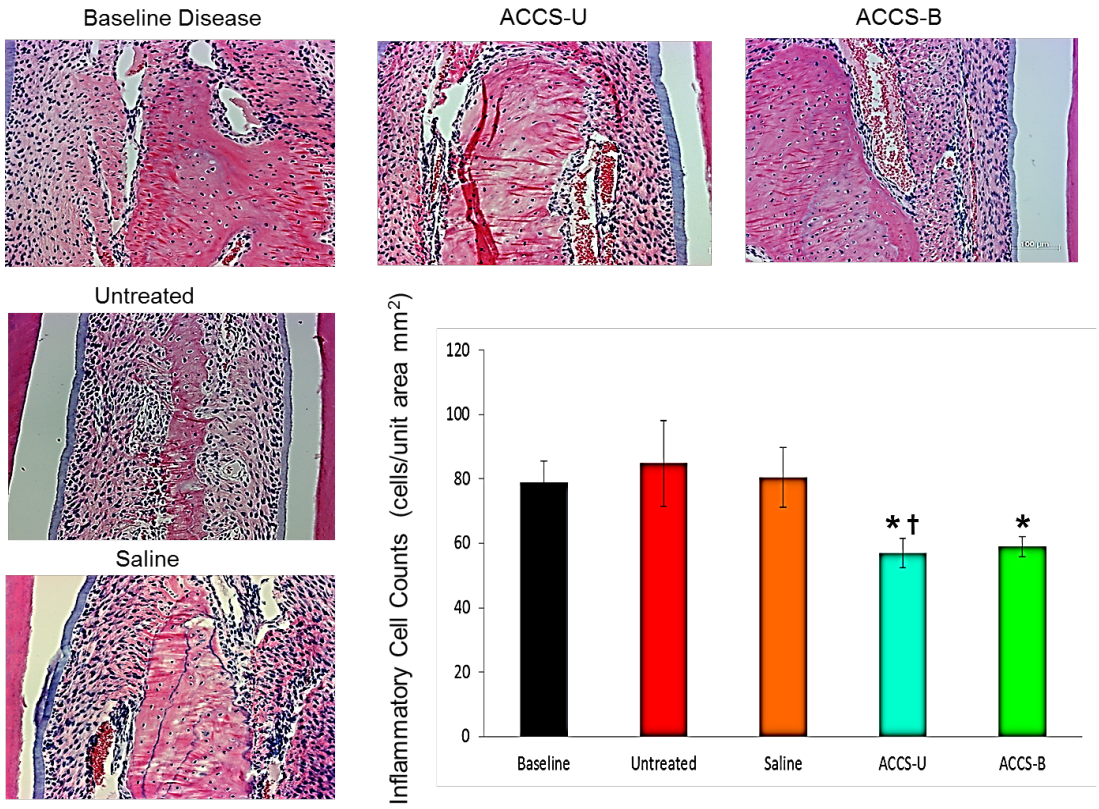

Figure 7. Inflammatory cell infiltrate.

Inflammatory infiltrate was quantified by cell counting per unit area (0.09 mm^2) at supracrestal and interproximal surfaces at the ligated sites (3 sections per specimen). Baseline, untreated and saline groups showed significantly more inflammatory cell counts compared to ACCS-U treated groups. Although ACCS-U treatment resulted in the least counts compared to ACCS-B treated group the difference was not statistically significant. *Statistically significant reduction in inflammatory cell infiltration compared to untreated and saline groups. †Statistically significant reduction in inflammatory cell infiltration compared to baseline and untreated groups (Mean ±SD; $p<0.05$; ANOVA and LSD *post hoc* test). (Images shown- H&E, 200X).

Table 4. Multiple comparisons for osteoclastic activity (TRAP+ cell counts) between groups

The osteoclast-like cells were identified using tartrate resistant acid phosphatase (TRAP) staining. TRAP-positive cells were counted in the coronal 1/3 of the root on each section (cells/mm^2) using Image J software program and calculated for both mesial and distal crestal alveolar bone of the ligated tooth (second premolar). The ratio calculations were also used and multiple comparisons within groups were made using analysis of variance (ANOVA) with *post hoc* tests Bonferroni or LSD correction.

		Mean			95% Confidence Interval	
ID	ID	Difference	Std. Error	Sig.	Lower Bound	Upper Bound
ACCS-U	ACCS-B	-.344	1.736	.845	-3.98	3.29
	Saline	-7.915*	1.736	.000	-11.55	-4.28
	Untreated	-5.350*	2.126	.021	-9.80	-.90
	Baseline	-8.791*	2.126	.001	-13.24	-4.34
ACCS-B	ACCS-U	.344	1.736	.845	-3.29	3.98
	Saline	-7.571*	1.736	.000	-11.20	-3.94
	Untreated	-5.005*	2.126	.029	-9.46	-.56
	Baseline	-8.447*	2.126	.001	-12.90	-4.00
Saline	ACCS-U	7.915*	1.736	.000	4.28	11.55
	ACCS-B	7.571*	1.736	.000	3.94	11.20
	Untreated	2.565	2.126	.242	-1.88	7.02
	Baseline	-.876	2.126	.685	-5.33	3.57
Untreated	ACCS-U	5.350*	2.126	.021	.90	9.80
	ACCS-B	5.005*	2.126	.029	.56	9.46
	Saline	-2.565	2.126	.242	-7.02	1.88
	Baseline	-3.441	2.455	.177	-8.58	1.70
Baseline	ACCS-U	8.791*	2.126	.001	4.34	13.24
	ACCS-B	8.447*	2.126	.001	4.00	12.90
	Saline	.876	2.126	.685	-3.57	5.33
	Untreated	3.441	2.455	.177	-1.70	8.58

*. The mean difference is significant at the 0.05 level.

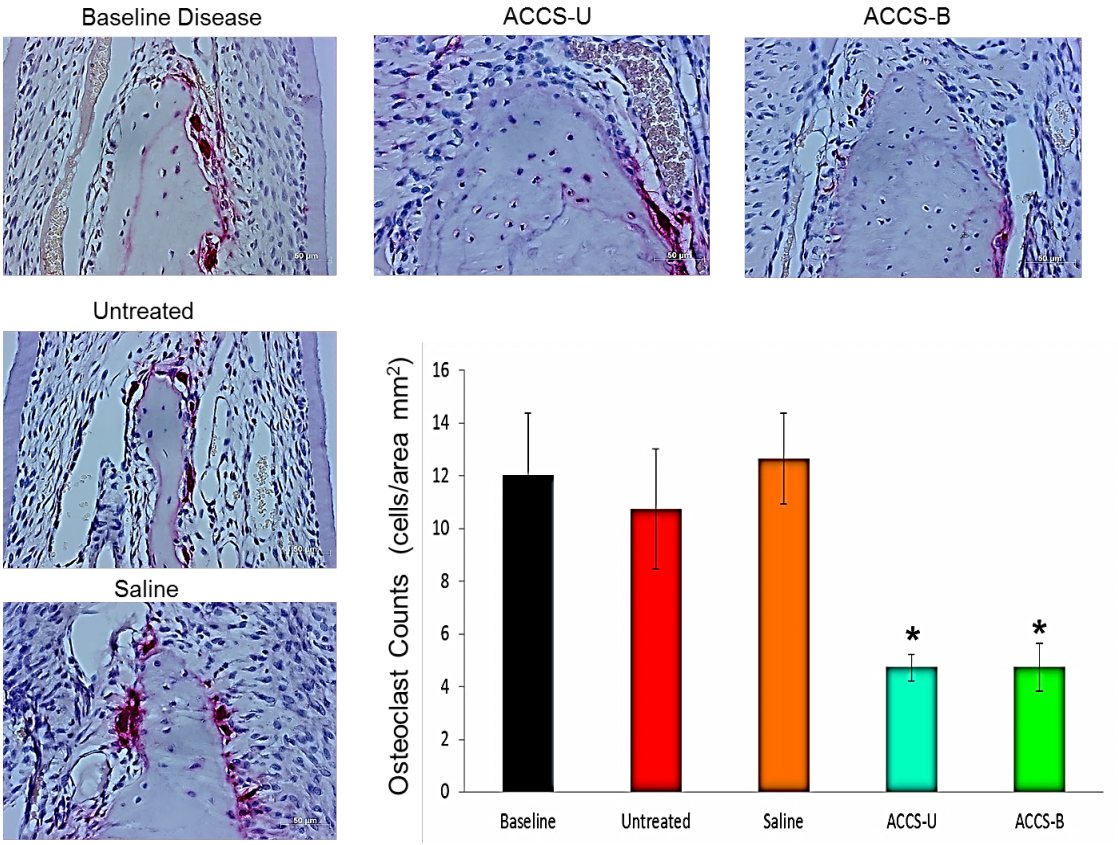

Figure 8. Osteoclastogenesis.

TRAP + cells were counted using Image J software program at crestal and middle portions of the alveolar bone using a standard area (0.09 mm^2) for each site (first and second premolar ligated sites), three sections/specimen. The results are shown as the average of two sites and total area of crestal and middle portions of each site. ACCS treated groups showed fewer osteoclasts around alveolar bone and the difference between all other groups was statistically significant confirming the histological findings with inflammatory cells and bone loss. ACCS-B resulted in reduced number of osteoclastic activity similar to ACCS-U group with no statistical difference. All other disease groups including baseline, untreated and saline-treated groups demonstrated elevated number of osteoclasts residing in the lacunas of alveolar crest and interproximal areas, *Statistically significant reduction in osteoclast counts compared to baseline disease, untreated and saline-treated groups (Mean ±SD; $p<0.05$; ANOVA and LSD *post hoc* test). (Images shown- TRAP, 400X).

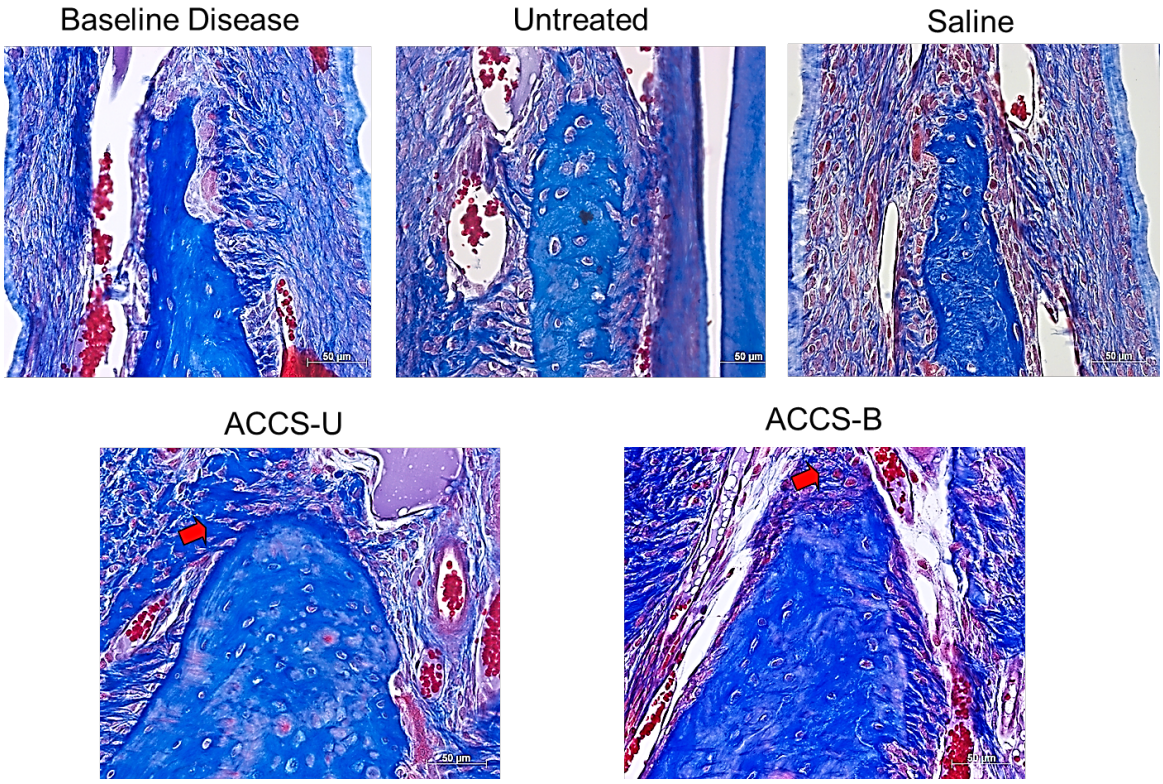

Figure 9. Evaluation of collagen deposition.

Collagen depositions around alveolar crest and interproximal areas were evaluated by Masson's trichrome staining (collagen fibers: blue; new bone deposition: red). Baseline disease and untreated groups as well as saline-treated group showed destruction in the collagen fibers at the crestal and interproximal areas. Conversely, the ACCS-treated groups showed dense and well organized collagen fibers at the supracrestal and interproximal area (depicted by red arrow). ACCS-B treated site showed activity of new bone formation with new collagen fiber deposition at the crestal site confirming the clinical findings (depicted by red arrow). (Images shown-Masson's Trichrome, 400X).

Table 5. Multiple comparisons for osteoblast-like cell (Osteocalcin+ cells) counts between groups

The osteocalcin- positive cells were counted in the coronal 1/3 of the root on each section (cells/mm2) using Image J software program and calculated for both mesial and distal crestal alveolar bone of the ligated tooth (second premolar). The ratio calculations were also used and multiple comparisons within groups were made using analysis of variance (ANOVA) with *post hoc* tests Bonferroni or LSD correction.

ID	ID	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
ACCS-U	ACCS-B	.156	1.095	.888	-2.14	2.45
	Saline	7.884*	1.095	.000	5.59	10.18
	Untreated	9.198*	1.341	.000	6.39	12.01
	Baseline	8.447*	1.341	.000	5.64	11.25
ACCS-B	ACCS-U	-.156	1.095	.888	-2.45	2.14
	Saline	7.727*	1.095	.000	5.43	10.02
	Untreated	9.041*	1.341	.000	6.23	11.85
	Baseline	8.290*	1.341	.000	5.48	11.10
Saline	ACCS-U	-7.884*	1.095	.000	-10.18	-5.59
	ACCS-B	-7.727*	1.095	.000	-10.02	-5.43
	Untreated	1.314	1.341	.340	-1.49	4.12
	Baseline	.563	1.341	.679	-2.24	3.37
Untreated	ACCS-U	-9.198*	1.341	.000	-12.01	-6.39
	ACCS-B	-9.041*	1.341	.000	-11.85	-6.23
	Saline	-1.314	1.341	.340	-4.12	1.49
	Baseline	-.751	1.549	.633	-3.99	2.49
Baseline	ACCS-U	-8.447*	1.341	.000	-11.25	-5.64
	ACCS-B	-8.290*	1.341	.000	-11.10	-5.48
	Saline	-.563	1.341	.679	-3.37	2.24
	Untreated	.751	1.549	.633	-2.49	3.99

*. The mean difference is significant at the 0.05 level.

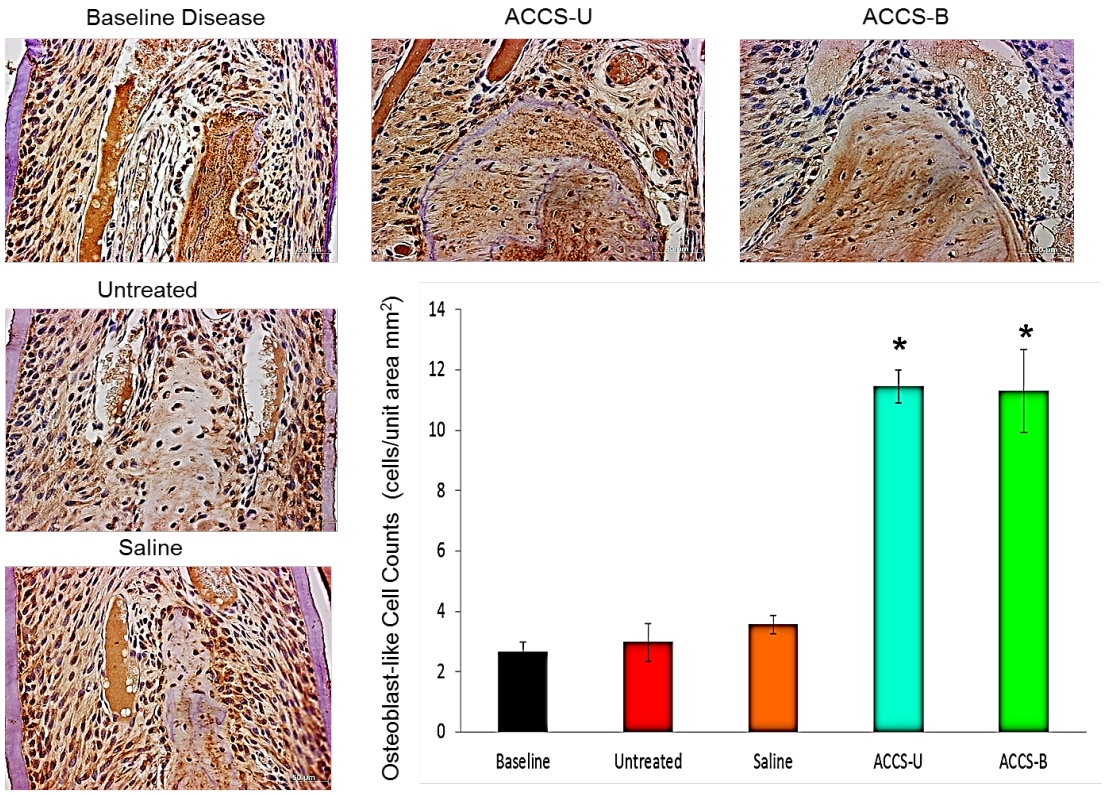

Figure 10. Osteoblastic activity.

Osteocalcin + cells were counted using Image J software program at crestal and middle portions of the alveolar bone using a standard area (0.09 mm^2) for each site (first and second premolar ligated sites), three sections/specimen. The results are shown as the average of two sites and total area of crestal and middle portions of each site. ACCS treated groups showed a statistically significant osteoblastic activity confirming the bone repair and formation seen in clinical and histological assessments compared to baseline, untreated and saline groups ($p=0.0001$). *Statistically significant compared to baseline disease, untreated and saline-treated groups (Mean ±SD; $p<0.001$; ANOVA and LSD post hoc test). (Images shown- Osteocalcin, 400X).

DISCUSSION

This study clearly demonstrates the role of ACCS in its application for the treatment of periodontal disease and restoration of damaged tissues. In addition, although the new formulation tested here, ACCS-B, did not statistically differ in its efficacy for treating experimental periodontal disease in rabbits from previously tested formulation, ACCS-U, it showed a clear potential in reducing bone loss during the progression of periodontitis. The results also reaffirm ACCS's mechanism of action by stimulating hosts' anti-inflammatory reactions, to achieve its goals. ACCS treated tissues showed significant reduction in both alveolar bone loss and inflammation in soft tissues. This was evident by the osteoclast-like cell counts, osteoblast-like cell counts, and inflammatory cell counts analyzed in TRAP stains, immunohistochemistry sections stained with oesteocalcin, and H&E slides respectively. In both groups of rabbits treated with ACCS-U and ACCS-B, the number of osteoblast-like cells showed clear proliferation compared to other groups untreated, saline, and disease base groups, while the number of osteoclast-like cells and inflammatory cells showed clear reduction compared to rabbits in other groups. Osteoblasts are involved in alveolar bone growth whereas osteoclasts are involved in bone reabsorption. Therefore these results show that ACCS treated tissues demonstrate not only a reduction in bone loss and inflammation, but also a proliferation in bone gain. Although there was no clear statistical difference between two ACCS groups, this study supports the potential of using these ACCS formulations as a therapeutic intervention for one of the most prominent oral health diseases to date.

In the field, there is a greater recognition of the need to suppress inflammatory response versus treatment of chronic periodontitis; however, no clear method was presented to reduce hosts' inflammatory response. There is growing use of antihypertensive and immunosuppressant drugs to dampen the development of chronic periodontitis; however, none of the drugs evaluated to date - anti-inflammatory steroids, non-steroidal drugs, anti-Tumor Necrosis Factor -alpha agents - have emerged as a potentially curative treatment for periodontal disease (Heasman et al, 2014). Moreover there is evidence that patients prescribed drugs to diminish development of gingival inflammation, such as bisphosphonates and anti-platelet drugs, are in great danger due to the risk of side effects. One of the side effects includes bisphosphonate-related osteonecrosis of the jaws (Janovská, 2012). With the NHANES report aforementioned describing patients with periodontal disease over 30 years old, the complications that drugs bring to treatment strategies puts serious pressure on patients. Furthermore, some of these drugs place patients at risk for more invasive dental procedures involving extractions and surgical treatments unlike ACCS treatments, which alleviates periodontitis while restoring damaged teeth and tissues to its original health (Heasman et al, 2014). With these reasons, increased effort and research in ACCS is required to treat periodontal disease as it is a far better treatment method than those previously attempted.

Periodontitis is often associated with other systematic inflammatory diseases. Pro-inflammatory cytokines and other agents drive their pathogenesis in multidirectional matter eventually reaching other organs of the body notably, the heart, lungs, and the liver (El-Shinnawi et al, 2013). Moreover, due to the effect that periodontitis has on the

levels of proteins, cytokines, and other agents, it is possible that other diseases such as osteoporosis, cancer, diabetes, heart disease, and lung disease may stem from periodontal disease. This puts a greater emphasis on treating periodontitis, but it also shows that a body works as a whole; oral health does affect systemic health. With its significant impact on controlling inflammation ACCS provides a potential for novel therapeutic approaches for periodontitis, but also for other inflammatory disease as well. Inflammatory diseases that involve bones, such as arthritis, can benefit potentially from ACCS' as it may be useful in treating similar diseases by reducing inflammation as well as bone absorption. It is not simply limited to inflammatory diseases with bones but also to other inflammatory disease, because as this study illustrated, ACCS is capable of significantly reducing inflammatory response of the host by modulating cytokines and other agents. It is evident that ACCS has potential to shed light on discovering possible solutions to many inflammatory diseases still finding a cure today. Nonetheless, it will be very difficult to attempt to emulate the success of ACCS with periodontal disease in different parts of the body, due to vastly different environment that human body possesses. However, the findings of this study warrant the use of ACCS in further preclinical and clinical studies in periodontal disease and in other systemic inflammatory conditions.

LIST OF JOURNAL ABBREVIATIONS

Am J Dent .....American Journal of Dentistry

Ann Plas Surg .....Annals of Plastic Surgery

Annu Rev Microbiol ..... Annual Review of Microbiology

Brit Dent J ..... British Dental Journal

Curr Mol Med .....Current Molecular Medicine

Faseb J..... Faseb Journal

Hum Immunol ..... Human Immunology

Infect Immun.....Infection and Immunity

Int J Mol Sci..... International Journal of Molecular Science

J Biomed Biotechnol.....Journal of Biomedicine and Biotechnology

J Dent Res ..... Journal of Dental Research

J Histotechnol..... Journal of Histotechnology

J Immunol .....Journal of Immunology

J Invest Surg..... Journal of Investigative Surgery

J Periodonto .....Journal of Periodontology

Mayo Clin Proc .....Mayo Clinic Proceedings

Med Clin N Am ..... Medical Clinic of North America

Nat Rev Immunol..... Nature Reviews Immunology

Periodontol 2000.....Periodontology 2000

Pharmacol Therapeut .....Pharmacology & Therapeutics

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