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Effects of B. forsythus extracts on osteogenesis in vitro

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Effects of *B. forsythus* extracts on osteogenesis *in vitro*

by

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ABSTRACT

Effects of *Bacteroides forsythus* extracts on osteogenesis *in vitro*
Ravipan I. Smith, D.D.S.

Specific oral microorganisms have been implicated in the etiology and pathogenesis of periodontal diseases. *Bacteroides forsythus*, an oral bacterium, is considered to be a putative periodontal pathogen largely based on data showing its presence at diseased sites. However, the effects of its products on cells of the periodontium are less known. Therefore, this study was undertaken to evaluate the effects of extracts of *B. forsythus* on osteogenesis *in vitro*. Cultures of *B. forsythus* were grown for 7 days under anaerobic conditions and sonic extracts were prepared. Sonic extract concentrations of 0, 0.01, 0.1, 1, 10, 100 μg/ml media were subsequently added to confluent cultures of MG-63 cells (human osteosarcoma cell line.) The effects of the bacterial extracts on osteogenesis were evaluated at days 2, 4, 6 and 14 by measuring alkaline phosphatase (AP) activity and mineral production (inorganic phosphate and calcium.) The results showed the extracts stimulated bone formation at concentrations of 10 and 100 μg/ml. AP activity increased 15-fold in 2-day cultures ($p<0.001$), 6-fold in 4-day cultures ($p<0.001$) and 2.7-fold in 14 day cultures ($p<0.001$) in comparison to controls. Comparable results were found for mineral content. Extracts from *Porphyromonas gingivalis*, another putative periodontal pathogen, caused similar increases in osteogenic activity of MG-63 cells. While previous studies have shown that extracts of these microorganisms inhibit bone formation in other *in vitro* culture systems, these results show they may also play a role in stimulating bone formation. This suggests that these bacteria exhibit both inhibitory and stimulatory effects on bone formation and thus may play a role in bone remodeling in periodontal diseases.

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INTRODUCTION

I. PERIODONTAL DISEASE AND ASSOCIATED MICROORGANISMS

Periodontal disease is seen in systemically healthy patients but can also be associated with systemic diseases in both children and adults.[1, 2] In both circumstances, the primary etiology in disease is microbial plaque. Several microorganisms have been implicated in periodontal disease. Some oral microorganisms are considered etiologic agents, while most others have been shown to be moderately associated with disease progression. Socransky and co-workers[3] categorized microorganisms based on the presence of complexes or "communities" of such oral microorganisms. The "red complex", or the complex most strongly associated with disease, include *Porphyromonas gingivalis, Bacteroides forsythus,* and *Treponema denticola.*

Aggressive periodontitis is the new nomenclature for periodontal diseases formerly called localized and generalized juvenile periodontitis.[4] Certain oral bacteria have been associated with aggressive periodontitis. Microorganisms including *Eubacterium nodatum, Eubacterium timidum, Campylobacter recta,* and *T. denticola* have been isolated from diseased sites in aggressive periodontitis patients.[5] Aggressive periodontitis patients have been shown to harbor *Actinobacillus actinomycetemcomitans, Capnocytophaga sputigena, Eikenella corrodens, Prevotella intermedia* and *P. gingivalis.*[6] Van Steenbergen and co-workers[7], found that *A. actinomycetemcomitans, P. gingivalis, P. intermedia* and *C. rectus* were strongly associated with generalized aggressive periodontitis. The presence of *P. gingivalis, Fusobacterium nucleatum, Streptococcus intermedia* and *B. forsythus* was demonstrated in another study by Kamma

and co-workers.[8] Zambon and co-workers[9] found that a leukotoxic strain of *A. actinomycetemcomitans* was highly associated with localized aggressive periodontitis. Thus, these studies demonstrate that aggressive periodontal disease is the result of presence of several periodontopathogens.

Patients with chronic periodontitis have also been shown to harbor certain sets of microorganisms.[10] Epidemiological studies have been done demonstrating the presence of putative pathogens in patients with disease progression, while a substantially decreased number of pathogens were seen in disease-free patients.[11, 12] *P. gingivalis* was found to be highly prevalent in not only chronic periodontitis patients, but also in patients with aggressive periodontitis.[13, 14] *B. forsythus* has been isolated from subgingival plaque of patients with periodontal disease and from patients with no breakdown.[15] These studies demonstrate that although certain microorganisms are predominant in various forms of periodontal diseases, they are not pathognomonic for disease.

Prevalence of *B. forsythus*, *P. gingivalis* and *C. ochracea*. The prevalence of *B. forsythus* in diseased sites and the effects of antimicrobial therapy on eradication of *B. forsythus* have been studied. Several studies have found an increased prevalence of *B. forsythus* in diseased sites versus healthy sites. *B. forsythus* and *P. gingivalis* have been shown in several studies to be present at high levels in periodontally diseased sites, with *B. forsythus* typically appearing in diseased sites with advanced destruction. Christersson and co-workers[16] found that the likelihood that *B. forsythus* was present was 14 times greater and for *P. gingivalis* 6.3 times if pocket depths were greater than 5mm. Kamma and co-workers[8] examined the microflora of slight, moderate and severe periodontal lesions in young adults with aggressive periodontitis. In sites with

greater loss of attachment, there was a higher prevalence of *B. forsythus* and *P. gingivalis*. The implications of this are unclear. *B. forsythus* could be more virulent than *P. gingivalis* and thus it is more closely associated with advanced destruction. *B. forsythus* may be less virulent and colonize the lesion following initial destruction caused by *P. gingivalis* and other microbes, or the combination of *P. gingivalis* and *B. forsythus* may be more destructive than either individually.

Christersson and co-workers[16] demonstrated that *B. forsythus* was present in 44-54% of sites in diseased patients. After conventional periodontal treatment with and without adjunctive antibiotic therapy, decreases in *B. forsythus* and *P. gingivalis* levels were demonstrated. Winkel and co-workers[17, 18] found that *B. forsythus* and *P. gingivalis* were suppressed to below detectable levels after treatment with metronidazole and amoxicillin. Umeda and co-workers[19], found that *B. forsythus* and *P. gingivalis*, which were predominant in the acute phase of periodontitis, decreased significantly after application of minocycline gel without any debridement. These studies demonstrate that conventional treatment with adjunctive antibiotic therapy can reduce colonization by periodontopathogens to below detection levels. Other studies have shown that without treatment, sites harboring these periodontopathogens were more likely to have continued breakdown.

The majority of other oral microorganisms are less strongly associated with periodontal disease, including *C. ochracea*. Studies have been done investigating the prevalence of *C. ochracea* in diseased sites with varying results. A study of a family with a high prevalence of aggressive periodontitis was done by analyzing and comparing the subgingival flora in members of the family. *C. ochracea* was isolated in both an affected

and unaffected child at 2.5% of total isolates.[20] Haffajee and co-workers[21] found that 94% of subjects with and without periodontal destruction harbored *C. ochracea*. From these studies, the prevalence of *C. ochracea* varies greatly and appears to be present in both diseased and non-diseased sites.

II. PATHOGENIC POTENTIAL OF PERIODONTAL PATHOGENS

Only certain microorganisms are successful at colonizing the oral cavity. In order to colonize the oral cavity, bacteria must be able to withstand being flushed away by saliva. Components of saliva that protect the host from bacterial and viral invasion include IgA, lysozyme, lactoferrin, histatins and β-defensins. The immunoglobulin IgA is found in saliva and its main function is to protect mucous membranes from bacterial and viral invasion. IgA binds to the surface of the microorganism and inhibits their ability to adhere to oral tissues making them more susceptible to phagocytosis.[22]

Microorganisms such as *P. gingivalis* and *T. denticola* can produce proteases that destroy IgA when it attempts to bind the microorganism.[22, 23] Lysozyme also found in saliva can dissolve some bacterial cell walls.[22] Lactoferrin binds iron which microorganisms need in order to thrive. Other bactericidal proteins such as β-defensin and histatins also play a role in host protection. Oral microorganisms can minimize contact with these host-defense proteins by colonizing areas that are protected from salivary flow.

In addition, many bacteria possess fimbrae, which allow adherence to surfaces in the oral cavity. Some bacterial surface polysaccharides mimic host polysaccharides and thus are not recognized as foreign and allow the organism to evade phagocytosis.

Microorganisms such as *C. rectus* and *A. actinomycetemcomitans* also contain leukotoxin, which can kill neutrophils. If the host is successful at phagocytizing and killing the microorganisms, lipopolysaccharides which are endotoxins found in gram-negative cell walls, are extremely potent stimulators of the immune system and bone resorption. Many periodontopathogens are gram-negative and thrive in anaerobic conditions. Colonization occurs in subgingival environments that allow undisturbed growth due to protection from the flushing actions of saliva, antibacterial salivary components, and mechanical removal from brushing or flossing.

Periodontopathogens possess virulence factors and are often able to evade host defense systems and cause breakdown of periodontal tissues. Loomer and co-workers[24] demonstrated that sonicated extracts of *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans* caused decreased alkaline phosphatase activity and consequently decreased bone formation in a chick periosteal model compared to controls. This suggests that cellular components of these periodontopathogens are capable of inducing bone resorption *in vivo.* Morimoto and co-workers[25] demonstrated that proteinaceous factors in *A. actinomycetemcomitans* induced apoptosis (cell death) in the MG-63 human osteosarcoma cell line, suggesting its role in periodontal breakdown. Gadhavi and co-workers[26] also found that *A. actinomycetemcomitans* directly inhibited mitosis in the MG-63 cell line. Thus, by possessing such virulence factors, these periodontopathogens can not only thrive because of host factor evasion, but can mediate tissue breakdown allowing for more growth as tissues move apically during the progression of periodontitis.

Putative pathogens are present in diseased as well as healthy sites, however, virulent strains need to be present in order for disease to occur. Several studies have been

done to investigate potential virulence factors of *P. gingivalis* and *A. actinomycetemcomitans*. Zambon and co-workers[27] identified five serotypes of *A. actinomycetemcomitans* and found that serotype b was most frequently associated with aggressive periodontitis. Han and co-workers[28] identified 12 additional genomic clonal types of *A. actinomycetemcomitans*, but no association with disease was mentioned. Baker[29] compared three strains of wild-type *P. gingivalis* and one mutant strain lacking fimbrillin. Only one of the three wild-type strains did not induce bone loss in mice. Due to its fastidious nature, however, no studies have been done on *B. forsythus* and the number of virulent strains that exist.

III. MECHANISM OF BONE LOSS IN PERIODONTAL DISEASE

Osseous tissues continuously remodel by resorption then deposition of bone. The mechanism behind physiologic bone remodeling as described by Baker[29], is that the process is controlled primarily by osteoblasts. Parathyroid hormone, 1, 25-dihydroxyvitamin D3 and prostaglandin E_2, promote resorption and cause osteoblasts to upregulate the expression of a cell surface molecule that is an osteoclast differentiation inducing factor. These cell surface molecules bind to a receptor on osteoclast-precursor cells, which causes formation of multinucleated osteoclasts, and the resorptive process is initiated. In health, a resorptive phase of ten days is typically followed by a longer formative phase of several months. In disease, components of the bacterial cell are released into the subgingival environment and immune cells such as T-cells release proinflammatory cytokines such as interleukin-1β and prostaglandin E_2, which are potent

stimulators of bone resorption. In a cascade of events, collagenase released by fibroblasts and activated osteoclasts cause tissue breakdown and a net resorption of bone.

In periodontal disease, products of periodontopathogens stimulate bone resorption. The bone lining the socket wall is first to be resorbed. The resorptive patterns follow the course of vascular structures, allowing bacterial products and cytokines from immune cells in gingival connective tissue to penetrate the bone[30]. The cortical plate is porous allowing vessels to pass through from the connective tissue. Thus, bone is also resorbed from the crest apically as well as from the periodontal ligament laterally. As alveolar bone adjacent to the periodontal ligament space is lost, the periodontal ligament attached to the root is also lost. This continued loss of support continues to occur until minimal or no support remains and tooth loss ultimately results.

Putative pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* have been shown to cause bone resorption *in vitro* and in animal models. Iino and Hopps[31] demonstrated that lipopolysaccharides (LPS) from *A. actinomycetemcomitans*, *P. gingivalis* and *C. ochracea* induced bone resorption. *P. gingivalis* extracts, including proteinaceous as well as non-proteinaceous products such as LPS, caused inhibition of osteogenesis in a 17-day old chick periosteum osteogenesis model system.[24, 32] This was demonstrated by decreased alkaline phosphatase activity when the osteoblasts were treated with extracts of *P. gingivalis*. When these osteoblasts were exposed to the bacterial extracts from day 0 to day 2, alkaline phosphatase activity, calcium levels and amount of collagen was less than controls. This study suggests that once inhibition of osteogenic activity occurred, the formation of bony tissue was less than with osteoblasts that were not exposed to bacterial extracts during this period.

IV. BIOLOGY OF *BACTEROIDES FORSYTHUS*

A. Morphology. *Bacteroides forsythus* is a fastidious, anaerobic Gram-negative bacterium. It was initially isolated from advanced periodontal lesions and was found to be non-motile and fusiform in morphology.[33] Other morphological characteristics of *B. forsythus* include a filamentous form with a diameter of approximately 0.5 μm, and some have central swellings that can have diameters of up to 3 μm. The cell length can vary between 1 and 30 μm. The cell wall consists of an outer membrane, a distinctive external layer and the outermost cell wall which appear as dome-like subunits. The peptidoglycan layer between the inner and outer cell membranes seen in other *Bacteroides* species is not distinct in *B. forsythus*. This may be a consequence of their requirement for N-acetylmuramic acid, which is a precursor or component of peptidoglycan.

B. Growth characteristics. Growth of *B. forsythus* requires anaerobic conditions at 37°C. Whole blood and other nutrient supplements such as N-acetylmuramic acid and yeast extract are part of the culture media to ensure growth.[34] Colonies are usually not visible until 5-7 days.[35, 36] Growth in broth can be accomplished with trypticase soy broth or mycoplasma broth supplemented with the above nutrients, as well as hemin, menadione and dithiotreitol. *B. forsythus* strains can be identified in culture by positive API ZYM tests for trypsin-like protease and β-galactosidase activity. They are also N-benzoyl-DL-arginine naphthylamide (BANA) and CBZ-Arg-Arg-AFC positive.[33, 37]

C. Isolation of *B. forsythus*. In the study identifying *B. forsythus* as a separate species, DNA from 12 strains of *B. forsythus* showed approximately 75% homology to

one another.[33] These strains possessed minimal to no homology with DNA of other *Bacteroides* species, including *Prevotella* and *Porphyromonas* species, which indicated that *B. forsythus* was a single and separate species. Culturing *B. forsythus* is difficult due to its fastidious nature, and if conditions are not optimal, little or no growth will occur. This is problematic when trying to isolate *B. forsythus* from periodontal pockets, as cultures of the subgingival flora may falsely show absence of *B. forsythus*.

Studies on detection of *B. forsythus* with various molecular biological techniques have been developed. Several groups of researchers have used rapid PCR methods to identify *B. forsythus*.[36, 38, 39] Using PCR methods they were able to identify *B. forsythus* in 89% of the cases, whereas culturing only identified it in 38% of the cases.[38] Thus, this method has better sensitivity than conventional culturing. Umeda and co-workers[19] compared the accuracy of detecting periodontopathic bacteria from whole saliva versus periodontal pocket samples using PCR. In order to detect *B. forsythus* with good accuracy, the authors stated that both whole saliva and periodontal pocket samples might be required.

D. Clinical effects of *B. forsythus*. *B. forsythus* has not been linked to any particular form of periodontitis, but its prevalence in sites with periodontal breakdown has been demonstrated.[3] Studies of subgingival plaque composition have shown that, like other putative periodontal pathogens, *B. forsythus* is present in significantly higher concentrations in cases of severe periodontitis when compared with cases where no periodontal disease exists.[40] In their review of microbial agents in destructive periodontal diseases, Haffajee and Socransky[10] concluded that *P. gingivalis, T. denticola* and *B.*

forsythus are etiologic agents. Altered host response may play a role, and the presence of one or more periodontopathogen together may have a synergistic effect on periodontal breakdown.

In both animal and human studies, the presence of *B. forsythus* has been associated with enhanced tissue breakdown. In a study by Takemoto and co-workers[34] infection with *B. forsythus*, *P. gingivalis* and *F. nucleatum*, alone or combinations of *B. forsythus* with the other two microorganisms were analyzed. Rabbits were inoculated subperitoneally. Inoculation with just one pathogen did not result in abscess formation after 7 days. However, co-inoculation with *B. forsythus* and *P. gingivalis* resulted in abscess formation in 100% of the animals, whereas only 75% of the animals co-inoculated with *B. forsythus* and *F. nucleatum* had abscess formation. In this model, *B. forsythus* acted synergistically with other pathogens to induce tissue breakdown. This reinforces the idea that periodontal diseases are not caused by the presence of a single pathogen and how the presence of more than one periodontopathogen may be a critical factor in tissue breakdown.

Grossi and co-workers,[41, 42] found that patients were 2.45-2.52 times more likely to have bone loss if *B. forsythus* was present than in patients who did not harbor the pathogen. In another study, patients were 5.3 times more likely to have attachment loss if *B. forsythus* was present at five consecutive visits.[43] In an analysis of bacterial levels after various modalities of treatment, Cugini and co-workers[44] looked at clinical and microbiologic effects of scaling and root planing after 12 months. All clinical parameters improved, and levels of *P. gingivalis* and *B. forsythus* decreased significantly at 12 months after therapy. These studies indicate that high levels of periodontopathogens are

present with clinical signs of disease, and with treatment, clinical signs of disease may resolve and colonization by periodontopathogens can be reduced to below detectable levels.

When periodontopathogens are present, confounding factors such as smoking may increase the potential for further periodontal breakdown. Patients with a history of smoking have been shown to be more susceptible to periodontal breakdown. Kazor and co-workers[45] investigated the prevalence of BANA-hydrolyzing pathogens (including *B. forsythus*) in smokers and found that plaque samples from non-bleeding sites in smokers were 11 times more likely to have a positive BANA reaction than plaque samples from non-smokers. The authors suggest that smoking may increase the presence of BANA-positive periodontopathogens. The risk for periodontal disease was 2.3 times more likely if *B. forsythus* was detected in smokers, compared with non-smokers or former smokers.[46]

E. Effects of antimicrobial therapy on *B. forsythus*. Administration of various antibiotics has been shown to be effective in treating patients with aggressive periodontitis. A regimen of metronidazole and amoxicillin given as adjuncts to conventional therapy for subjects with aggressive periodontitis resulted in *A. actinomycetemcomitans* being reduced to below detectable levels by culture. This regimen has been recommended for treatment in patients who harbor periodontopathogens such as *B. forsythus*.[47] Winkel and co-workers[17] investigated the effects of metronidazole with subgingival debridement in "refractory" patients who were

B. forsythus-positive and *A. actinomycetemcomitans*-negative. *B. forsythus* was suppressed below detection levels in 17 out of 27 patients six months post-treatment.

Feres and co-workers[48] did similar studies using systemic doxycycline as an adjunct to root planing. Doxycycline (100mg) daily for 14 days did not significantly alter levels of *B. forsythus, P. gingivalis* or *T. denticola* at any time point up to 90 days post-treatment when compared with root planing alone. Wong and co-workers[49] investigated the effects of scaling and root planing in conjunction with tetracycline fibers at sites of recurrent periodontitis. Although levels of all organisms decreased, there was no significant difference between groups treated with and without tetracycline. Since local antimicrobial therapy has not completely eradicated these periodontopathogens, the benefits of local antimicrobial therapy as an adjunct to mechanical therapy may be limited.

F. Virulence factors. Perhaps because of its fastidious nature, few studies have thus far investigated the mechanisms by which *B. forsythus* contributes to periodontal disease. A *B. forsythus* cell-surface protein containing a leucine-rich repeat motif encoded by the gene *bspA* has been found to mediate adhesion to extracellular matrix components. Recombinant *bspA* binds strongly to fibronectin and fibrinogen, and in adult subjects from whom *B. forsythus* was isolated, expression of antibodies to *bspA* protein was observed.[50] This cell-surface protein may play a role in disease progression by mediating an inflammatory response, which may lead to tissue breakdown.

It is possible that certain cellular components that have been preserved in several bacterial species contribute to the initiation of disease. Animals immunized with killed

P. gingivalis produced antibodies to *P. gingivalis* that cross-reacted with *B. forsythus*, thus demonstrating that both pathogens possess similar antigens.[51] This study suggests that *B. forsythus* is able to induce immunologic responses similar to *P. gingivalis*.

Trypsin-like protease activity has been demonstrated in *T. denticola, P. gingivalis* and *B. forsythus*.[52] These proteases have been shown to activate PMN and fibroblast collagenases. Thus, tissue breakdown occurs as a result of endogenous collagenase production. In an investigation of the adherence of four laboratory strains and twelve clinical isolates of *B. forsythus* to host cells, it was found that only ATCC strain 43037 strongly adhered to neutrophils (PMN).[53] Adherence to such immune cells was weakened by the presence of L-histidine and L-arginine, and was enhanced by trypsin treatment of PMNs. There was also evidence of adherence to fibroblasts. This study provided additional evidence that *B. forsythus* can bind to host cells, and may impair the ability of PMNs to phagocytize microorganisms which may ultimately lead to cellular damage and tissue breakdown.

Periodontopathogens have been shown to interact with other microorganisms and may depend on this commensal relationship for survival. Yao and co-workers[54] investigated interspecies binding amongst *T. denticola, P. gingivalis* and *B. forsythus* with commensal organisms such as *Streptococcus crista*. It was found that *P. gingivalis* and *B. forsythus* had a protein-protein interaction, which was eliminated in the presence of serum. There was no interaction observed between *T. denticola* and the other periodontopathogens, however, similar interactions were observed between *T. denticola* and *S. crista*. This study helps explain how periodontopathic bacteria become established in the oral environment through interaction with other non-pathogenic species.

V. PURPOSE OF STUDY.

The most significant clinical aspect of periodontal disease is bone loss. The purpose of this study was to determine if extracts of *B. forsythus* affect bone formation processes *in vitro.* To investigate this, we studied the effects of sonicated *B. forsythus* extracts on osteogenesis using MG-63 human osteosarcoma cells. The following experiments were performed: (1) extracts of *B. forsythus* were obtained by sonication, (2) cultures of MG-63 cells with varying concentrations extracts of *B. forsythus, P. gingivalis* (as a positive control) or *C. ochracea* (as a negative control), and (3) the effects on osteogenic activity were determined using biochemical assays for acid phosphatase, alkaline phosphatase activity, calcium, inorganic phosphate, and protein in order to normalize other assay values to allow comparison between experiments.

VI. HYPOTHESIS.

Endogenous factors or enzymes released by *B. forsythus* act as destructive factors that inhibit osteogenesis by osteoblastic cells. The goal of this study was to gain a better understanding of the mechanisms of bone remodeling during periodontal breakdown. Further investigation of specific cellular components which affect osteogenesis may improve therapies directed at the treatment of periodontal disease.

MATERIALS AND METHODS

I. BACTERIAL CULTURE AND CONDITIONS. (See Appendix A) *B. forsythus* (ATCC 43037) was grown on laked rabbit blood-brucella (LRBB) agar plates supplemented with N-acetylmuramic acid and Isovitalex®. *P. gingivalis* (ATCC 33277) and *C. ochracea* (ATCC 27872) were grown on LRBB agar plates, without supplementation. All three strains were obtained from frozen stock cultures maintained by C. Hoover (University of California, San Francisco). After sufficient growth was achieved on culture plates, bacterial innocula were grown in tripticase soy broth for sonic extraction. All cultures were grown anaerobically at 37°C for 7 days. Purity of the broth cultures was determined by darkfield microscopy and subculture on LRBB agar plates prior to obtaining sonicated bacterial extracts.

II. PREPARATION OF BACTERIAL EXTRACTS. Bacteria were harvested from the broth (50ml) by centrifugation (8,000xg, 4°C, 10 minutes). The pellet was then resuspended with phosphate buffered solution (PBS, 25ml, pH 7.4) containing 0.01% dithiotreitol and centrifuged again as above. This step was repeated. The pellet was then resuspended with αMEM without nucleosides (2ml), and sonicated for a total of 5 minutes (10x30sec bursts, see Appendix B). The sonicated sample was analyzed under darkfield microscopy to ensure that most (>95%) of the cells had been lysed. The resultant suspension was then centrifuged (22,500xg, 4°C, 10 minutes). The supernatant was filter-sterilized (0.02μm pore size), aliquoted and stored at -70°C until use. Crude

protein concentration was determined for each of the sonicated bacterial extracts using the Bio-Rad protein assay (see Appendix C).

III. OSTEOBLASTIC CELL LINE. MG-63 osteosarcoma cells (ATCC #CRL-1427) were obtained from P. Johnson (University of California, San Francisco). MG-63 cells from frozen stock were incubated for 7 days, at 37°C in a humidified atmosphere containing 5% CO_2, in αMEM without nucleosides supplemented with 15% fetal calf serum (see appendix B). Co-cultures of MG-63 osteosarcoma cell lines and bacterial extracts were then placed in 24-well subculture plates. Four wells served as controls and were uninoculated and the other wells were inoculated with increasing concentrations of bacterial extracts obtained from *B. forsythus, P. gingivalis* and *C. ochracea.* The concentrations used for each bacterial sonicate extract were 0.01, 0.1, 1, 10, and 100 mg/ml. Experiments for each of the bacterial sonicate extracts were conducted for two weeks and media change was done every two days. Additional experiments were done with sonicate extracts of *B. forsythus* which were stopped at days 2, 4, and 6.

IV. BIOCHEMICAL ANALYSIS. All cultures were subjected to biochemical analysis to determine the effects of *B. forsythus, P. gingivalis* and *C. ochracea* extracts on osteogenesis. The following assays were performed: 1) alkaline phosphatase and acid phosphatase activity as a measure of mineralization and cell turnover, 2) calcium and phosphate accumulation as a measure of mineralization, and 3) protein production as an indicator of cell viability and to normalize other values to allow comparison of measurements between experiments (see Appendix C). Upon completion of an

experiment, the MG-63 osteosarcoma cells were lifted from the wells using a cell scraper, PBS (1ml) was added to each well and the resultant suspension was removed and then sonicated (30 seconds). The homogenate was centrifuged (22,500xg, 10 minutes, 4°C). The supernatant was used for protein, acid phosphatase and alkaline phosphatase assays. HCl (0.5N) was added to the pellet, stored overnight, and the resultant supernatant was used for the calcium and inorganic phosphate assays.

V. STATISTICAL ANALYSIS. Each experiment was repeated a minimum of three times and there were four wells for each extract concentration per experiment. Values from each set of measurements were used to calculate a mean and standard deviation for each experimental group as described above. Significance at the $P<0.05$ level was ascertained using one-way ANOVA between groups. Paired t-test assuming unequal variances was used to determine significance between controls and each group within each experiment.

RESULTS

Protein concentration of each sonicated bacterial extract were determined using the Bio-Rad protein assay. The amount of protein in sonicated extracts of *B. forsythus, P. gingivalis* and *C. ochracea* was 1.66 mg/ml, 3.35 mg/ml and 9.5 mg/ml respectively. The effects of sonicated *B. forsythus* extracts on the MG-63 cell line were evaluated at days 2, 4, 6 and 14, and effects of sonicated *P. gingivalis* and *C. ochracea* extracts on MG-63 cells were evaluated at day 14. Tables 1-18 present results of normalized values for the three experiments for each of the sonicated bacterial extracts at two weeks as well as effects of *B. forsythus* sonicate extracts on MG-63 cells at days 2, 4 and 6. Graphs 1-15 represent means of each experimental group for each assay, and Graphs 16-18 represent effects of *B. forsythus* on protein, alkaline phosphatase and acid phosphatase levels at days 2, 4, and 6.

B. forsythus extracts did not appear to have any significant dose-dependent effects on protein concentration (Graph 1) of MG-63 cells ($p>0.05$). Although a slight decrease in protein concentration was observed at higher extract concentrations, significance at the $p=0.05$ level was approached in only one of three trials (Table 1). Significant increases in alkaline phosphatase activity were noted at extract concentration of 100 μg/ml ($p<0.05$) for all three experiments (Table 2), which is also reflected in Graph 2, which represents mean values of all three experiments. Calcium and inorganic phosphate production also increased significantly at extract concentrations of 10 and 100 μg/ml ($p<0.05$) for all three experiments (Tables 4 and 5). Graph 4 demonstrates that calcium levels rose by 200-fold at 100μg/ml, whereas in Graph 5 phosphate levels rose by 9-fold

at 100μg/ml extract concentration. A dose-dependent decrease in acid phosphatase activity was seen in all three experiments (Table and Graph 3), which was significant at higher extract concentrations ($p<0.05$). Thus, *B. forsythus* sonicate extracts were not toxic to MG-63 cells and stimulated mineral deposition at high extract concentrations. Due to the discrepancy in proportions of calcium and inorganic phosphate and the low values of calcium at lower extract concentrations, it seemed that the calcium assay was not sensitive enough at low extract concentrations.

B. forsythus sonicate extracts (1, 10, and 100μg/ml) on days 2, 4 and 6, caused a significant dose-dependent decrease on protein concentration in MG-63 cells (Tables16-18 and Graphs 16-18), and the protein concentration did not change appreciably from day 2 to day 6. Alkaline phosphatase activity consistently increased with increasing sonicate extract concentrations ($p<0.05$). A significant increase in alkaline phosphatase activity was seen at 100μg/ml sonicate extract, and decreased from 14-fold over controls at day 2, 4-fold at day 4, to 6-fold by day 6. Acid phosphatase activity increased with increasing sonicate extract concentrations on days 2, 4 and 6, with the highest increase occurring on day 6 (Table 18), reaching six-times the control value at sonicate extract concentration of 100μg/ml. Although no apparent effects on protein concentration were observed after day 2, the sharp increase in alkaline phosphatase activity gradually decreased while acid phosphatase levels increased.

P. gingivalis sonicated extracts had dose-dependent effects in only one experiment and no trends were seen in the other two trials (Table 6). Graph 6 demonstrates a slight overall decrease in protein concentration with increasing extract concentration. A significant dose-dependent decrease in alkaline phosphatase activity

was seen in all experiments as shown in Table 7 ($p<0.05$). Experiment 2 had two "zero" values which could be due to operator error during assays. The dose-dependent decrease in alkaline phosphatase activity is apparent in Graph 7. In assaying acid phosphatase activity, significant differences between controls and sonicated extracts were demonstrated only in experiment 1 (Table 8), but no dose-dependent trend was observed. Graph 8 demonstrates that no dose-dependent trend can be observed. A significant dose-dependent increase in calcium and phosphate production was seen at concentrations of 0.1, 1 and 10μg/ml ([$p<0.05$] Tables 9 and 10). In addition, a significant increase in calcium and phosphate at 10 μg/ml extract concentration was seen in all experiments. Zero values in experiments 1 and 2 of Table 9 indicate possible operator error during assays or that the assay is not sensitive enough to detect low calcium levels and were not included in statistical analysis. Graphs 9 and 10 demonstrate the mean increase in mineral deposition for both calcium and phosphate. Thus, in spite of a dose-dependent decrease in alkaline phosphatase activity, there was a net increase in mineral deposition.

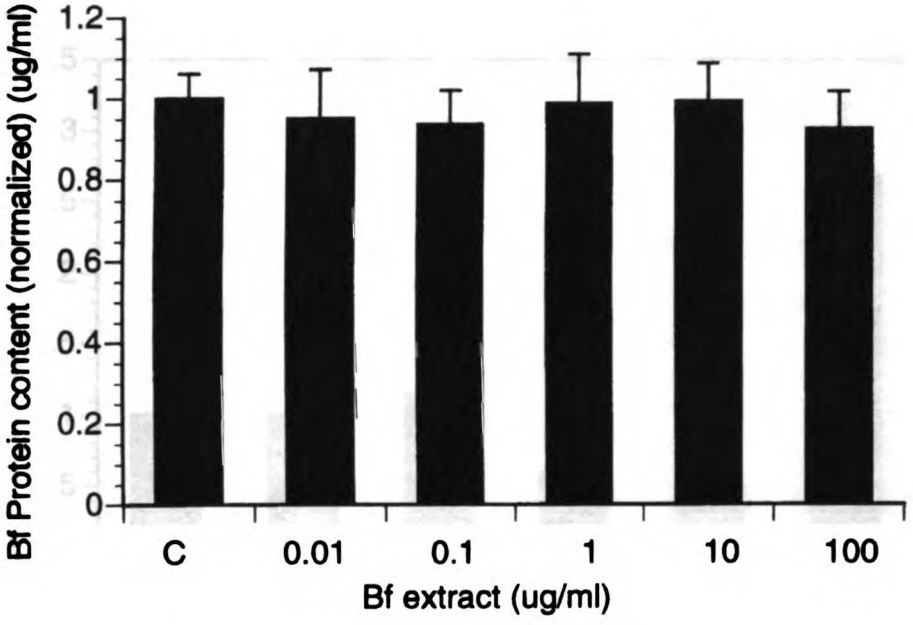
C. ochracea sonicate extracts had significant effects ($p<0.05$) on protein concentrations in MG-63 cells, however, no trends were noted (Table 11). Graph 11 demonstrates that the mean protein concentrations decrease initially and rise again at higher extract concentrations. No specific trends were noted in any of the experiments for alkaline phosphatase activity and there were large variations between values within each extract concentration (Table 12). Significant differences between controls and higher concentrations were seen in experiment 2 and 3. Graph 12 shows no consistent trend with alkaline phosphatase activity. Acid phosphatase activity increased and then decreased at higher extract concentrations in all three experiments (Table 13).

Significant differences between controls and middle values of extract concentration were observed in all experiments. This is also reflected in Graph 13. Similar increases and decreases were seen in calcium and phosphate production (Tables 14,15 and Graphs 14 and 15), with significance occurring between control groups and middle extract concentrations in all three experiments for both assays. Although *C. ochracea* extracts had an effect on enzyme activity and mineral deposition, no discernable trend could be observed.

Table 1. Sonicated *B. forsythus* extracts on protein content at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.09	0.99 ± 0.09	0.89 ± 0.05	1.08 ± 0.19	0.99 ± 0.09	0.97 ± 0.10
Exp 2	1 ± 0.02	0.95±0.07	1.04 ± 0.07	1.02 ± 0.05	1.09±0.10	0.92 ± 0.08
Exp 3	1 ± 0.07	0.91 ± 0.20	0.88 ± 0.14	0.85 ± 0.13 *	0.88±0.09 *	0.87 ± 0.09 *
Group mean + SD	1 ± 0.06	0.95 ± 0.14	0.94 ± 0.09	0.98 ± 0.12	0.99 ± 0.09	0.92 ± 0.09

Graph 1. Effects of sonicated *B. forsythus* extracts on protein concentration at two weeks.

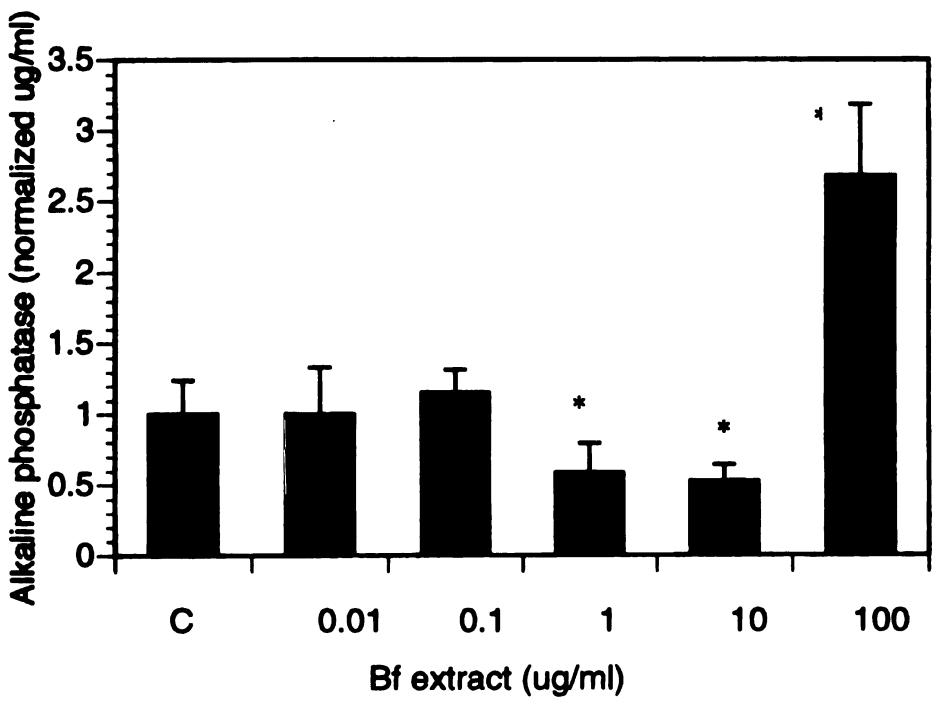


* - Denotes significance ($p<0.05$) between control and individual groups

Table 2. *B. forsythus* extracts on alkaline phosphatase activity at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.117	0.91 ± 0.12	1.10 ± 0.17	0.77 ± 0.18 *	0.82 ± 0.17 *	2.31 ± 0.23 *
Exp2	1 ± 0.133	0.89 ± 0.14	1.11 ± 0.10	0.60 ± 0.22 *	0.37 ± 0.13 *	2.49 ± 0.23 *
Exp 3	1 ± 0.466	1.20 ± 0.74	1.24 ± 0.22	0.37 ± 0.23 *	0.38 ± 0.04 *	3.24 ± 1.05 *
Group mean + SD	1 ± 0.24	1 ± 0.33	1.15 ± 0.16	0.58 ± 0.21 *	0.52 ± 0.11 *	2.68 ± 0.50 *

Graph 2. Effects of sonicated *B. forsythus* extracts on alkaline phosphatase activity at two weeks.

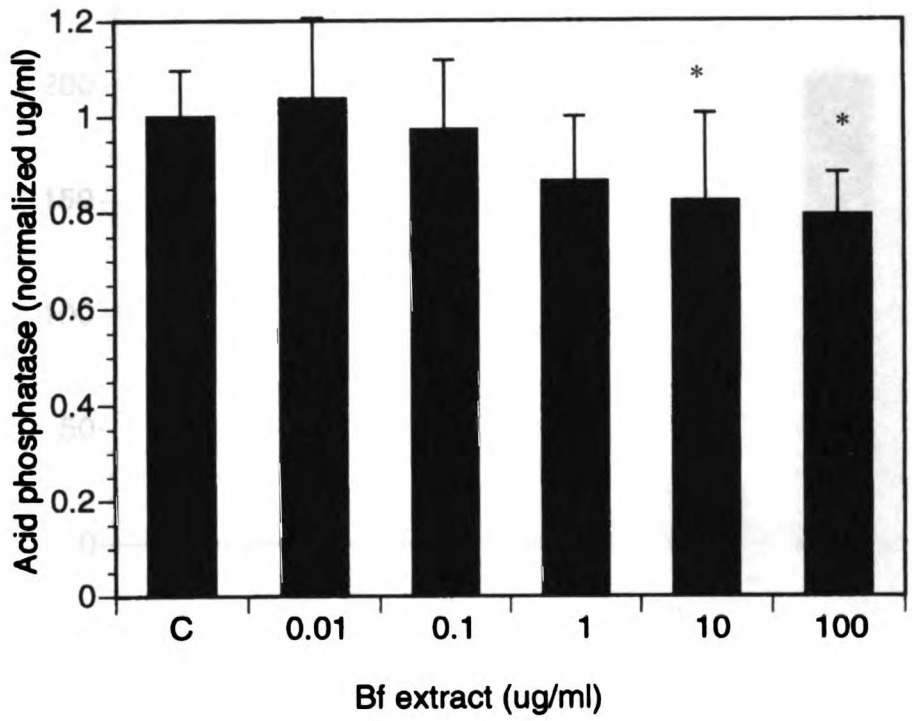


* - Denotes significance ($p<0.05$) between control and individual groups

Table 3. *B. forsythus* extracts on acid phosphatase activity at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.11	0.99 ± 0.12	1.06 ± 0.13	0.79 ± 0.11 *	0.81 ± 0.09 *	0.70 ± 0.09 *
Exp 2	1 ± 0.12	0.99 ± 0.14	0.92 ± 0.16	0.86 ± 0.13	0.77 ± 0.11 *	0.78 ± 0.1 *
Exp 3	1 ± 0.06	1.13 ± 0.25	0.94 ± 0.14	0.93 ± 0.16	0.89 ± 0.35	0.91 ± 0.08 *
Group mean + SD	1 ± 0.10	1.04 ± 0.17	0.97 ± 0.14	0.86 ± 0.13	0.82 ± 0.18 *	0.79 ± 0.09 *

Graph 3. Effects of sonicated *B. forsythus* extracts on acid phosphatase activity at two weeks.

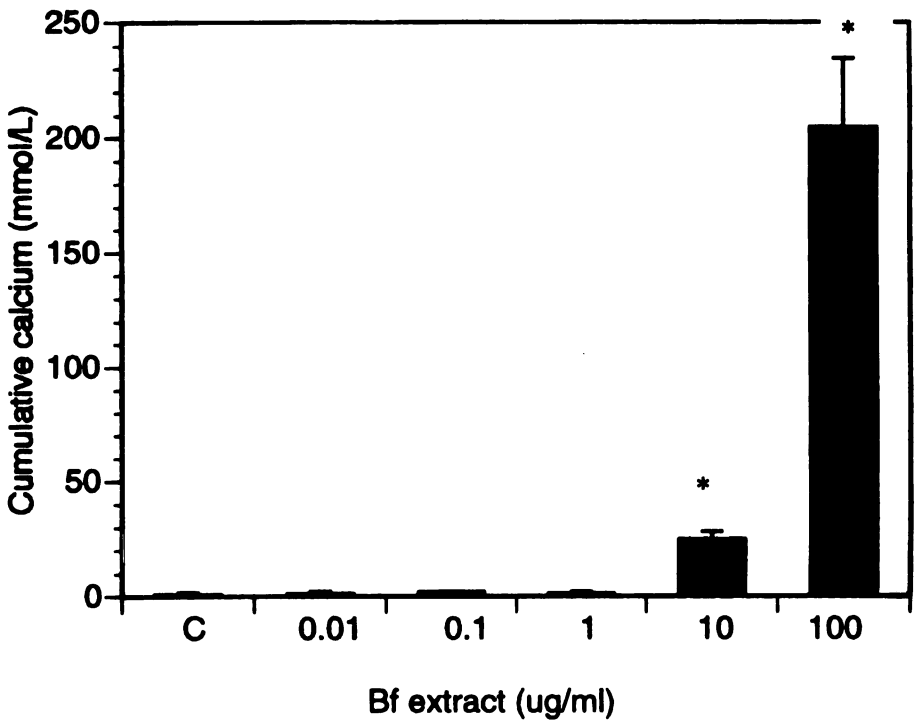


* - Denotes significance ($p<0.05$) between control and individual groups

Table 4. *B. forsythus* extracts on total calcium production at two weeks (mmol/L). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.88	1.34 ± 1.55	2.68 ± 0.70 *	1.79 ± 1.46	39.21 ± 5.42 *	356.7±41.53*
Exp 2	1 ± 0.77	0.87 ± 0.66	0.80 ± 0.31	0.49 ± 0.23	10.43 ± 1.42*	53.2 ± 17.29 *
Group mean + SD	1 ± 0.83	1.11 ± 01.11	1.74 ± 0.51	1.14 ± 0.85	24.82 ± 3.42 *	204.9 ± 29.4 *

Graph 4. Effects of sonicated *B. forsythus* extracts on total calcium production at two weeks.

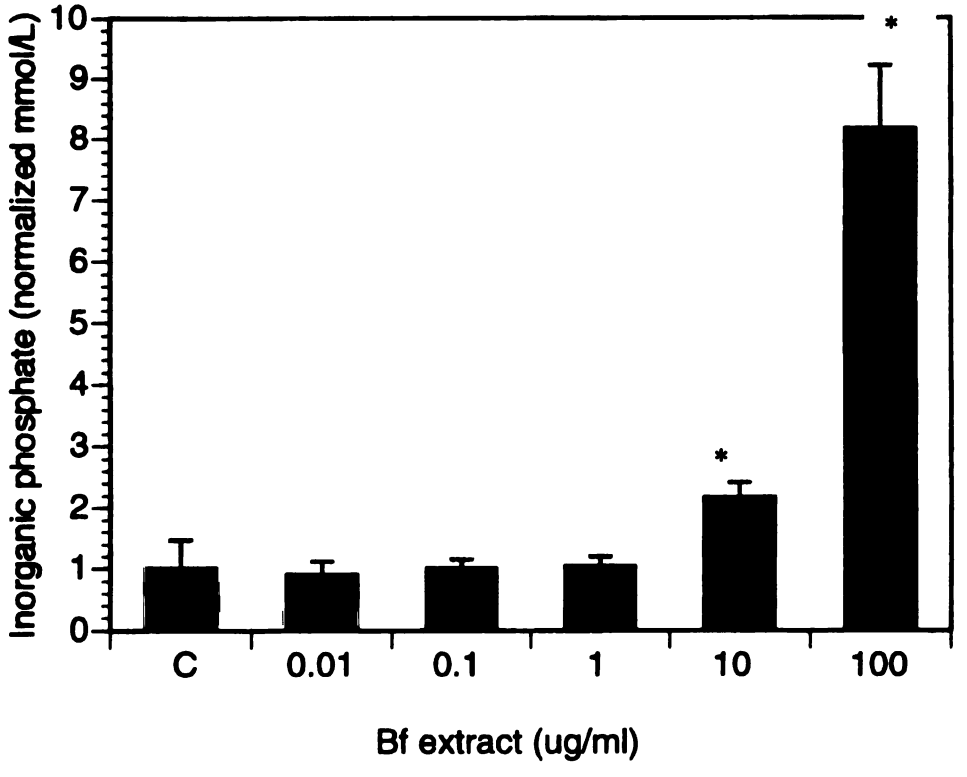


* - Denotes significance ($p<0.05$) between control and individual groups

Table 5. *B. forsythus* extracts on total inorganic phosphate at two weeks (mmol/L). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.43	1.12 ± 0.14	1.26 ± 0.08	1.21 ± 0.16	1.93 ± 0.15 *	9.02 ± 1.63 *
Exp 2	1 ± 0.46	0.58 ± 0.36	0.79 ± 0.23	0.81 ± 0.12	2.11 ± 0.14 *	7.76 ± 0.78 *
Exp 3	1 ± 0.53	0.99 ± 0.17	0.93 ± 0.14	1.11 ± 0.21	2.47 ± 0.46 *	7.73 ± 0.70 *
Group mean + SD	1 ± 0.47	0.90 ± 0.22	0.99 ± 0.15	1.04 ± 0.16	2.17 ± 0.25 *	8.17 ± 1.04 *

Graph 5. Effects of sonicated *B. forsythus* extracts on total inorganic phosphate production at two weeks.

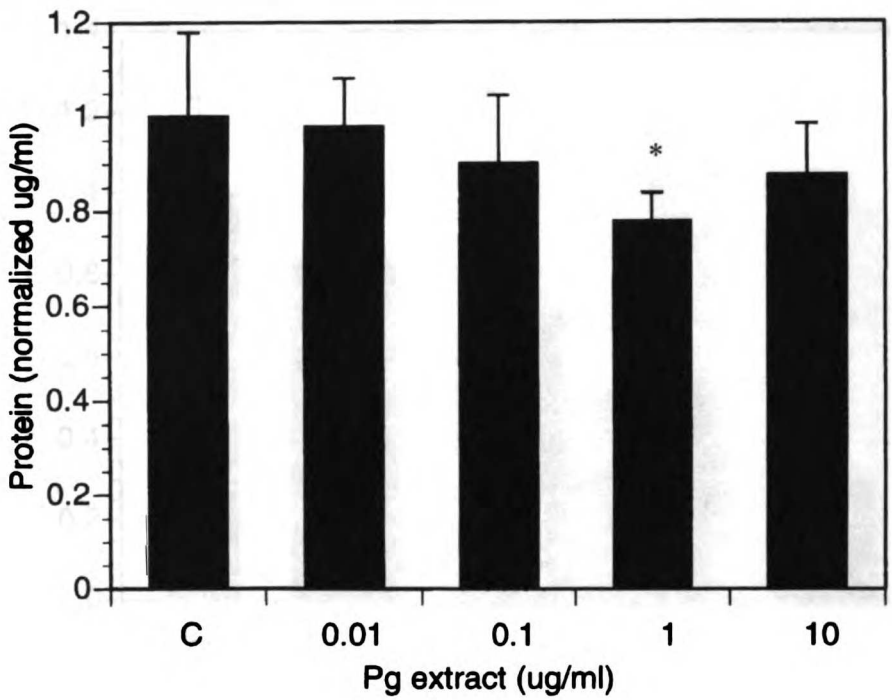


* - Denotes significance ($p<0.05$) between control and individual groups

Table 6. Sonicated *P. gingivalis* extracts on protein content at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10
Exp 1	1 ± 0.09	1.08 ± 0.19	1.17 ± 0.28	0.82 ± 0.07 *	1.08 ± 0.13
Exp 2	1 ± 0.38	0.90 ± 0.06	0.72 ± 0.07	0.79 ± 0.03	0.79 ± 0.08
Exp 3	1 ± 0.06	0.96 ± 0.06	0.81 ± 0.08 *	0.73 ± 0.08 *	0.75 ± 0.11 *
Group mean + SD	1 ± 0.18	0.98 ± 0.10	0.9 ± 0.14	0.78 ± 0.06 *	0.87 ± 0.11

Graph 6. Effects of sonicated *P. gingivalis* extracts on protein production at two weeks.

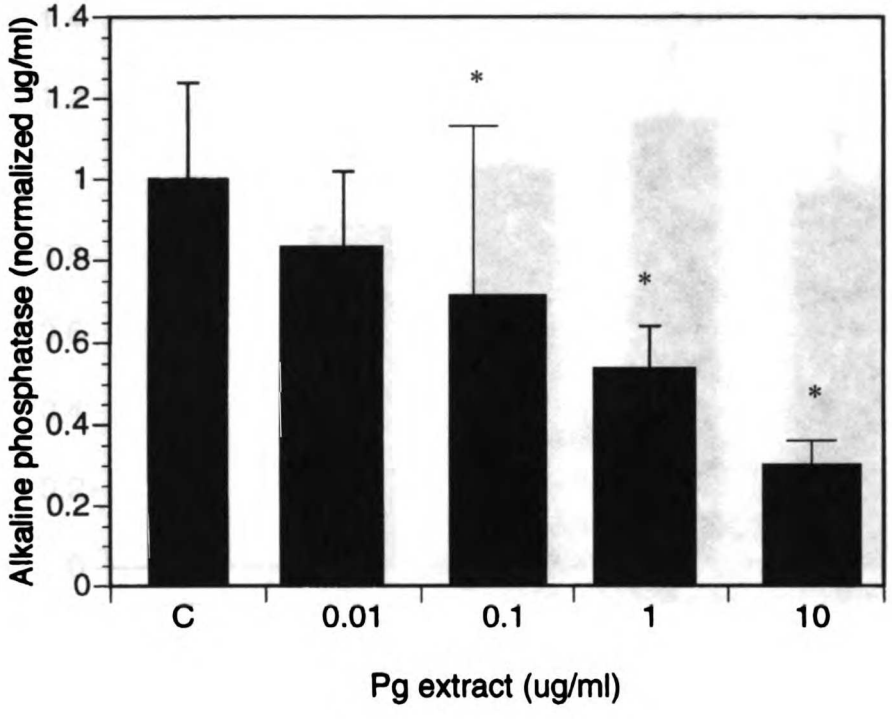


* - Denotes significance ($p<0.05$) between control and individual groups

Table 7. *P. gingivalis* extracts on alkaline phosphatase activity at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10
Exp 1	1 ± 0.09	0.73 ± 0.11 *	0.70 ± 0.09 *	0.81 ± 0.04 *	0.56 ± 0.05 *
Exp 2	1 ± 0.31	0.57 ± 0.39	0 #	0.24 ± 0.36 *	0 #
Exp 3	1 ± 0.31	0.89 ± 0.36	0.77 ± 0.51	0.14 ± 0.34 *	0.01 ± 0.03 *
Group mean + SD	1 ± 0.24	0.81 ± 0.24	0.74 ± 0.3 *	0.48 ± 0.19 *	0.29 ± 0.04 *

Graph 7. Effects of sonicated *P. gingivalis* extracts on alkaline phosphatase activity at two weeks.

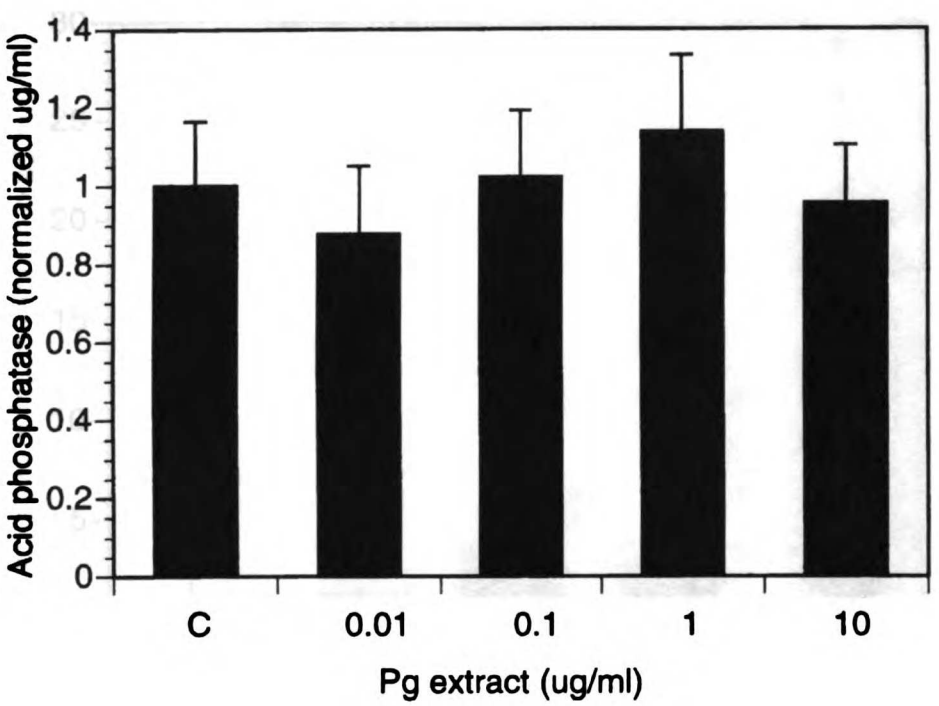


* - Denotes significance ($p<0.05$) between control and individual groups
# - Zero values not used in analysis

Table 8. *P. gingivalis* extracts on acid phosphatase activity at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10
Exp 1	1 ± 0.10	0.74 ± 0.12 *	0.76 ± 0.13 *	1.15 ± 0.16	0.79 ± 0.13 *
Exp 2	1 ± 0.17	1.04 ± 0.21	1.18 ± 0.21	1.07 ± 0.19	1.02 ± 0.116
Exp 3	1 ± 0.23	0.85 ± 0.19	1.13 ± 0.17	1.20 ± 0.23	1.06 ± 0.20
Group mean + SD	1 ± 0.17	0.88 ± 0.17	1.02 ± 0.17	1.14 ± 0.19	0.96 ± 0.15

Graph 8. Effects of sonicated *P. gingivalis* extracts on acid phosphatase activity at two weeks.

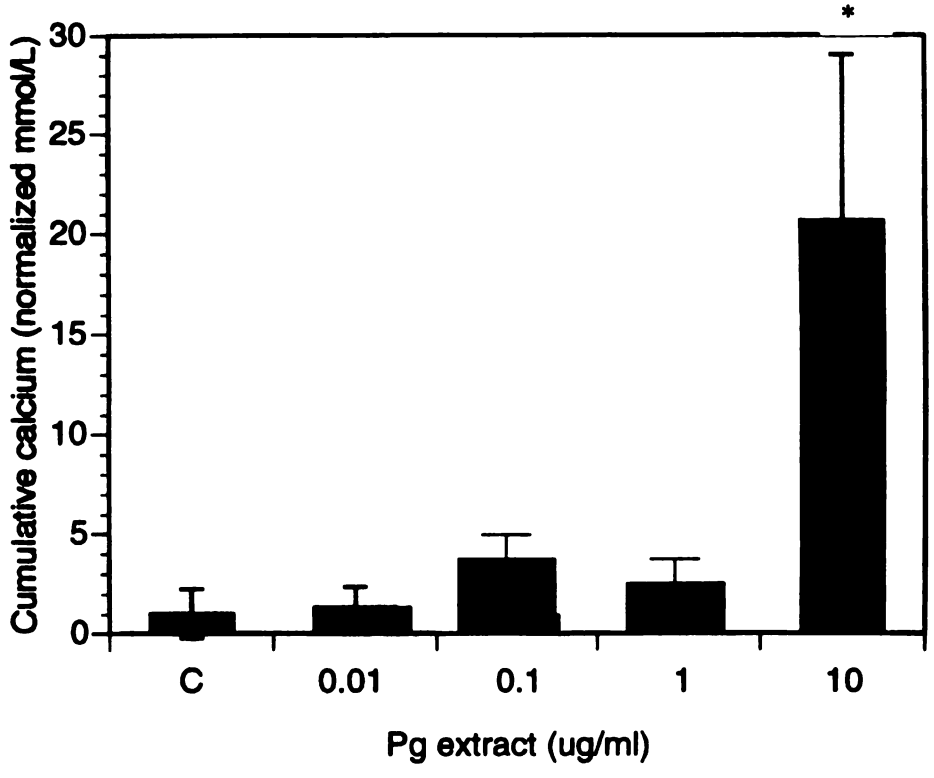


* - Denotes significance ($p<0.05$) between control and individual groups

Table 9. *P. gingivalis* extracts on calcium production at two weeks (mmol/L). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10
Exp 1	1 ± 2.45	1.11 ± 2.16	0 #	0 #	33.78 ± 19.10*
Exp 2	1 ± 0.88	0 #	0 #	0 #	18.30 ± 4.80 *
Exp 3	1 ± 0.34	2.75 ± 0.96 *	2.67 ± 0.34 *	1.87 ± 0.68 *	9.93 ± 1.18 *
Group mean + SD	1 ± 1.22	1.93 ± 1.56	2.67 ± 0.34	1.87 ± 0.68	20.67 ± 8.36 *

Graph 9. Effects of sonicated *P. gingivalis* extracts on total calcium production at two weeks.

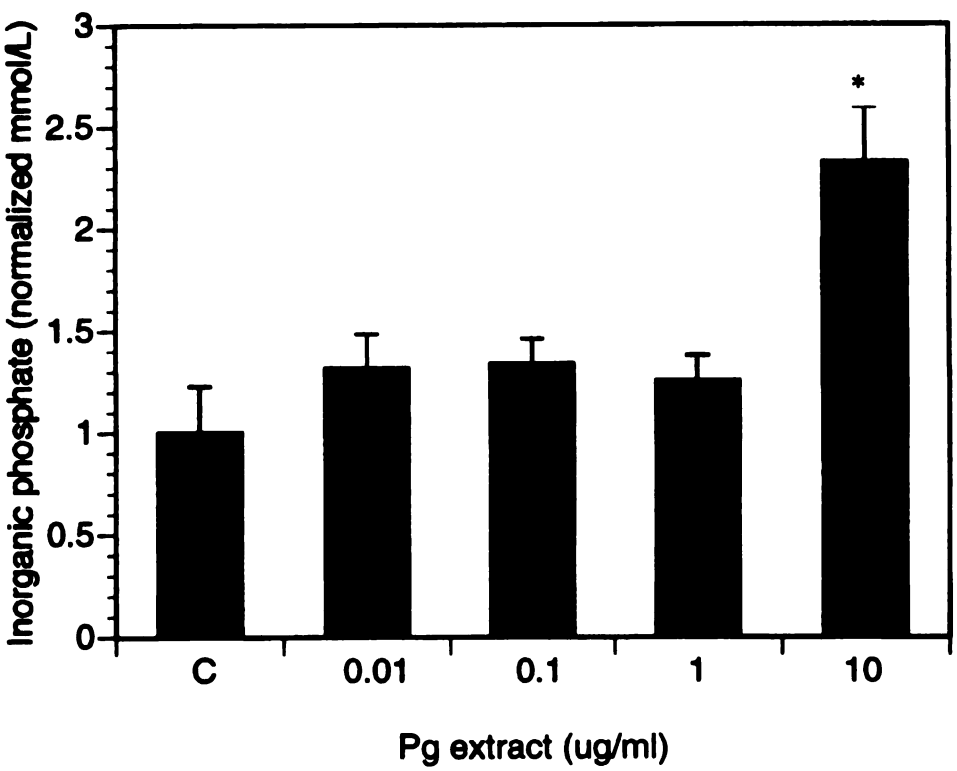


* - Denotes significance ($p<0.05$) between control and individual groups
# - Zero values not used in analysis

Table 10. *P. gingivalis* extracts on inorganic phosphate production at two weeks (mmol/L). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10
Exp 1	1 ± 0.16	1.24 ± 0.26	0.99 ± 0.09	1.14 ± 0.04	2.18 ± 0.34 *
Exp 2	1 ± 0.11	1.49 ± 0.17 *	1.62 ± 0.13 *	1.47 ± 0.17 *	2.18 ± 0.26 *
Exp3	1 ± 0.42	1.22 ± 0.07	1.40 ± 0.13	1.15 ± 0.16	2.61 ± 0.22 *
Group mean + SD	1 ± 0.23	1.32 ± 0.17	1.34 ± 0.12	1.25 ± 0.12	2.32 ± 0.27 *

Graph 10. Effects of sonicated *P. gingivalis* extracts on total inorganic phosphate production at two weeks.



* - Denotes significance ($p<0.05$) between control and individual groups

Table 11. Sonicated extracts of *C. ochracea* on protein content at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.02 *	0.88 ± 0.06 *	0.68 ± 0.02 *	0.67 ± 0.04 *	0.68 ± 0.04 *	0.73 ± 0.05 *
Exp 2	1 ± 0.11*	0.81 ± 0.08*	0.81 ± 0.13*	0.67 ± 0.13*	0.66 ± 0.07 *	0.74 ± 0.09 *
Exp 3	1 ± 0.07	0.92 ± 0.09*	0.65 ± 0.07*	0.67 ± 0.04*	0.82 ± 0.02 *	0.89 ± 0.08*
Group mean + SD	1 ± 0.07	0.87 ± 0.08 *	0.71 ± 0.07 *	0.67 ± 0.07 *	0.72 ± 0.04 *	0.79 ± 0.07 *

Graph 11. Effects of sonicated *C. ochracea* extracts on protein production at two weeks

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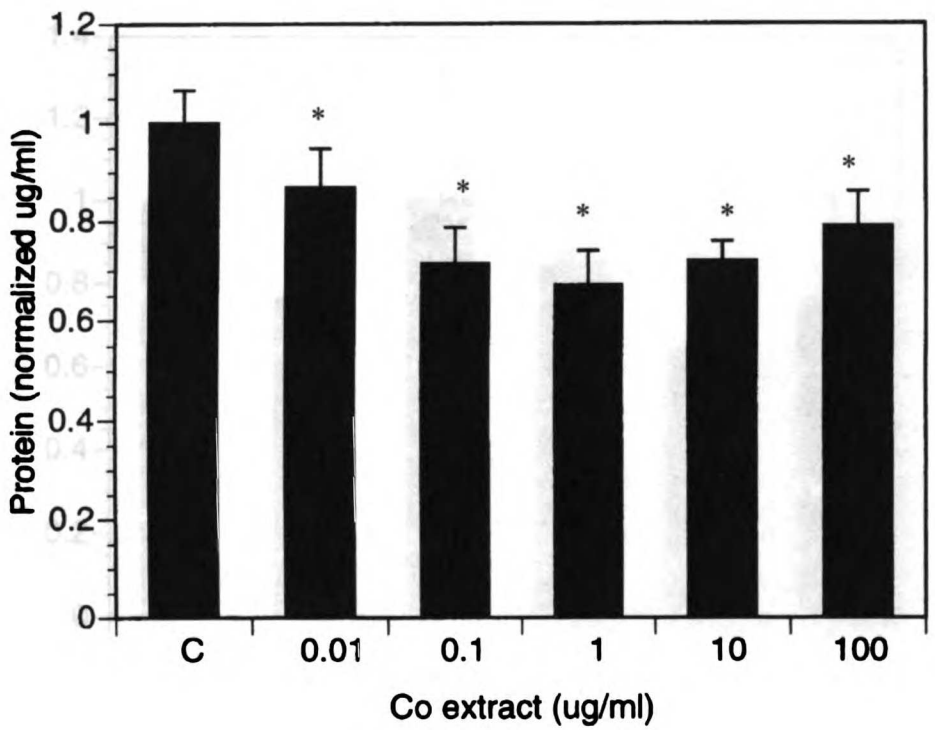
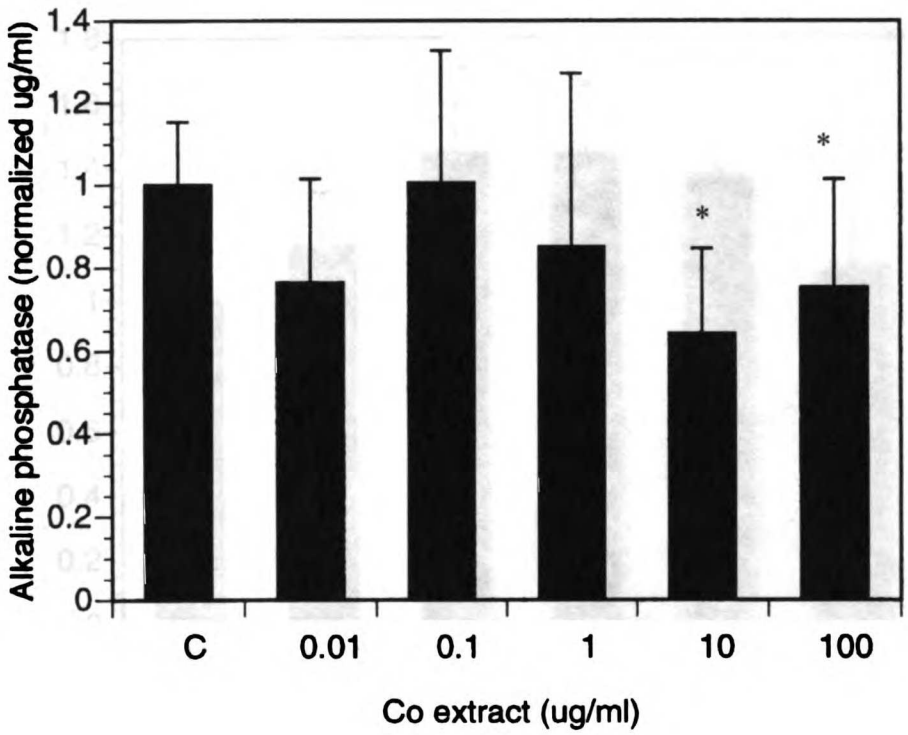


* - Denotes significance ($p<0.05$) between control and individual groups

Table 12. *C. ochracea* extract on alkaline phosphatase activity at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.11	0.81 ± 0.31	1.01 ± 0.34	1.15 ± 0.42	1.23 ± 0.11*	1.25 ± 0.39
Exp 2	1 ± 0.18	1.34 ± 0.36	1.14 ± 0.13	0.46 ± 0.28*	0.14 ± 0.18*	0.73 ± 0.18*
Exp 3	1 ± 0.18	0.15 ± 0.08*	0.87 ± 0.51	0.94 ± 0.57	0.56 ± 0.33*	0.28 ± 0.22*
Group mean + SD	1 ± 0.16	0.77 ± 0.25	1.01 ± 0.33	0.85 ± 0.42	0.64 ± 0.21 *	0.75 ± 0.26 *

Graph 12. Effects of sonicated *C. ochracea* extracts on alkaline phosphatase activity at two weeks.

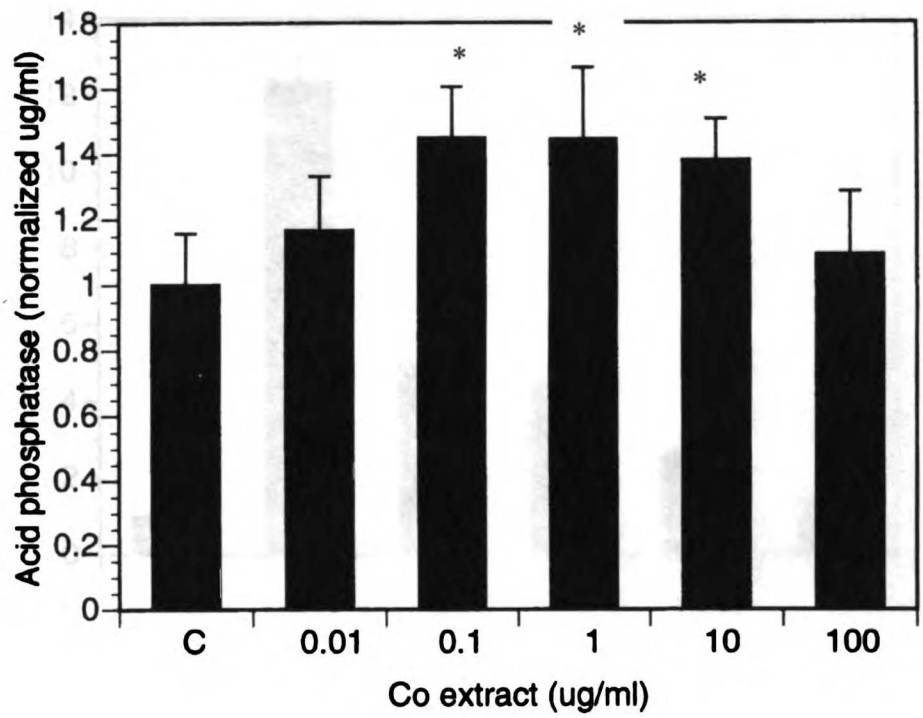


* - Denotes significance ($p<0.05$) between control and individual groups

Table 13. *C. ochracea* extract on acid phosphatase activity at two weeks (μg/ml). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.05	1.11 ± 0.18	1.49 ± 0.09*	1.53 ± 0.37*	1.50 ± 0.13*	1.09 ± 0.31
Exp 2	1 ± 0.33	1.31 ± 0.24	1.47 ± 0.23*	1.31 ± 0.14	1.09 ± 0.13	1.16 ± 0.16
Exp 3	1 ± 0.09	1.08 ± 0.08	1.38 ± 0.16*	1.49 ± 0.15*	1.55 ± 0.12*	1.03 ± 0.1
Group mean + SD	1 ± 0.16	1.17 ± 0.17	1.45 ± 0.16 *	1.44 ± 0.22 *	1.38 ± 0.13 *	1.09 ± 0.19

Graph 13. Effects of sonicated *C. ochracea* extracts on acid phosphatase activity at two weeks.

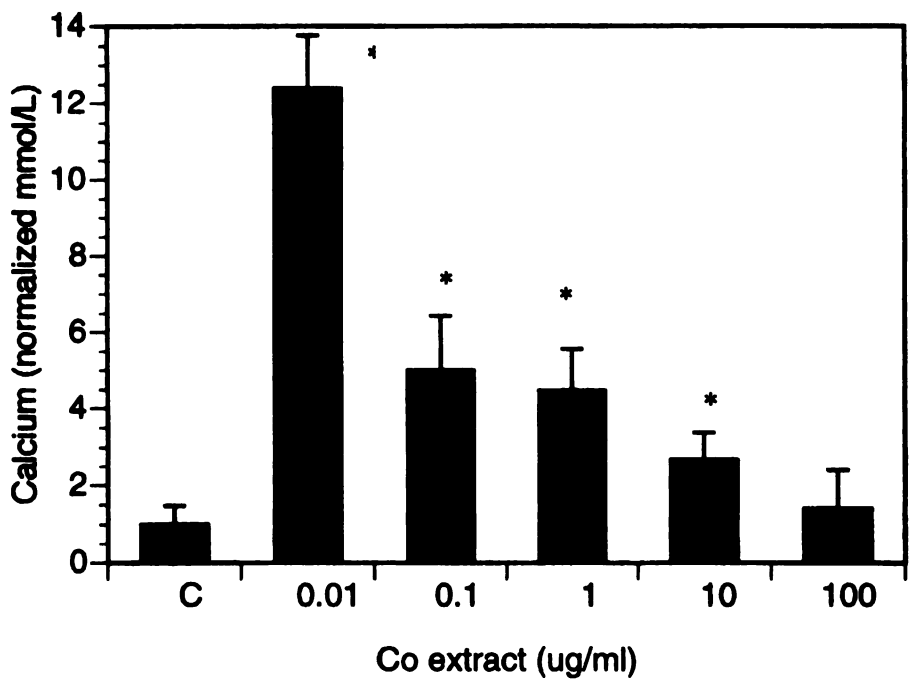


* - Denotes significance ($p<0.05$) between control and individual groups

Table 14. *C. ochracea* extract on total calcium production at two weeks (mmol/L). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.16	1.59 ± 0.16*	1.54 ± 0.13*	1.26 ± 0.13*	1.42 ± 0.16*	1.10 ± 0.19
Exp 2	1 ± 0.51	7.74 ± 0.79*	3.62 ± 0.97*	3.84 ± 1.15*	2.76 ± 0.42*	2.06 ± 1.36
Exp 3	1 ± 0.75	27.88 ± 3.11*	9.82 ± 3.20*	8.26 ± 2.04*	3.77 ± 1.57*	1.04 ± 1.42
Group mean + SD	1 ± 0.47	12.4 ± 1.35 *	4.99 ± 1.43 *	4.45 ± 1.11 *	2.65 ± 0.72 *	1.4 ± 0.99

Graph 14. Effects of sonicated *C. ochracea* extracts on total calcium production at two weeks.

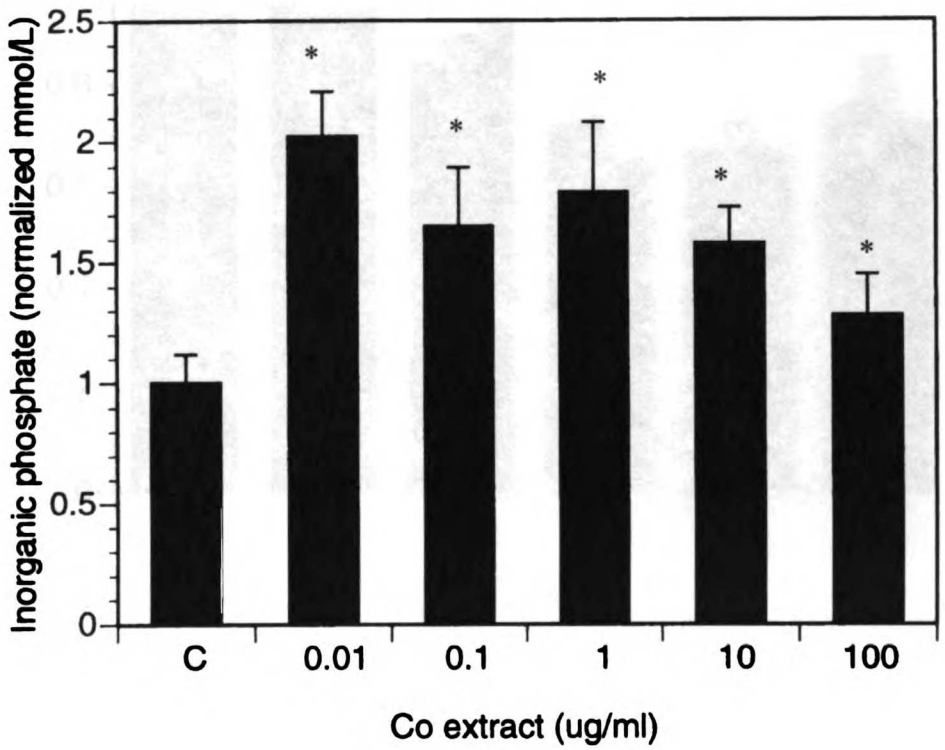


* - Denotes significance ($p<0.05$) between control and individual groups

Table 15. *C. ochracea* extract on inorganic phosphate production at two weeks (mmol/L). (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Exp 1	1 ± 0.16	1.59 ± 0.16*	1.54 ± 0.13*	1.26 ± 0.13*	1.42 ± 0.16*	1.10 ± 0.19
Exp 2	1 ± 0.15	2.47 ± 0.23*	1.55 ± 0.23*	2.01 ± 0.49*	1.64 ± 0.18*	1.53 ± 0.27*
Exp 3	1 ± 0.04	2.01 ± 0.17*	1.87 ± 0.37*	2.09 ± 0.25*	1.68 ± 0.11*	1.21 ± 0.06*
Group mean + SD	1 ± 0.12	2.02 ± 0.19 *	1.65 ± 0.24 *	1.79 ± 0.29 *	1.58 ± 0.15 *	1.28 ± 0.17 *

Graph 15. Effects of sonicated *C. ochracea* extracts on total inorganic phosphate production at two weeks.

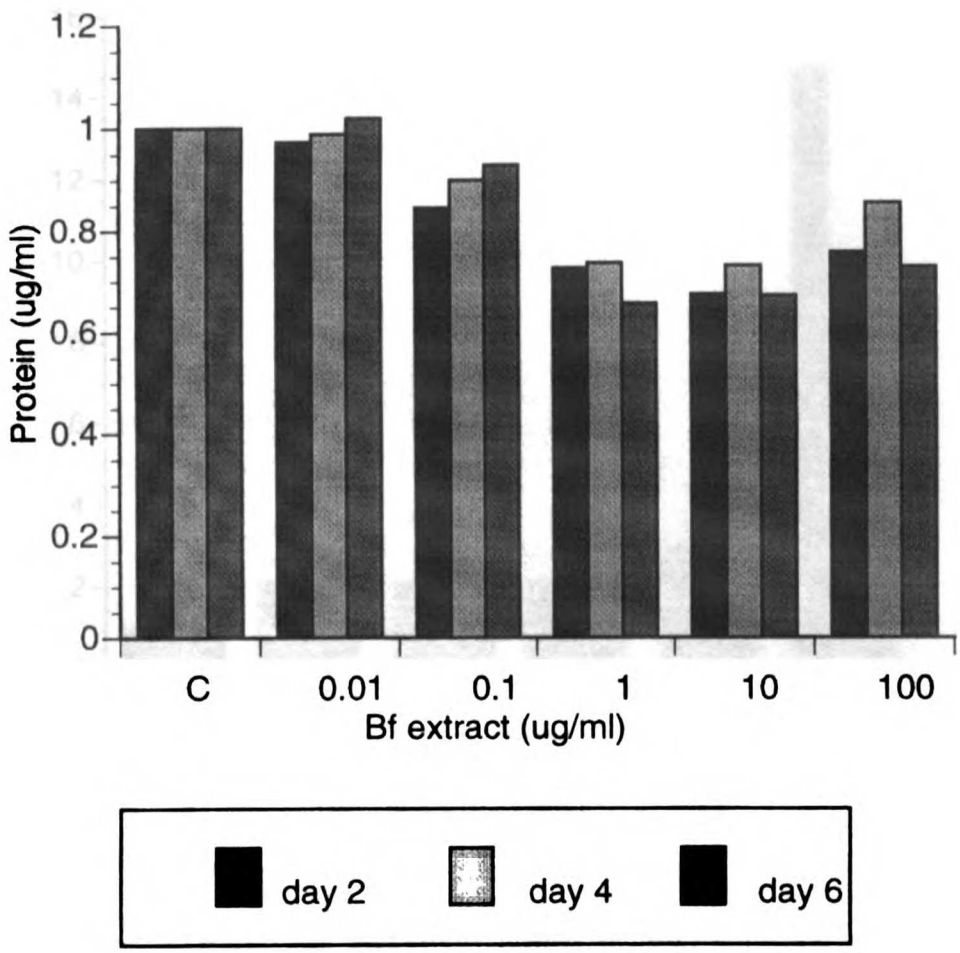


* - Denotes significance ($p<0.05$) between control and individual groups

Table 16. Effects of sonicated *B. forsythus* extracts on protein concentration at days 2, 4 and 6. (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Day 2	1 ± 0.05	0.97 ± 0.04	0.85 ± 0.18	0.73 ± 0.03*	0.67 ± 0.04*	0.76 ± 0.04*
Day 4	1 ± 0.05	0.99 ± 0.05	0.90 ± 0.13	0.74 ± 0.13*	0.73 ± 0.17*	0.85 ± 0.08*
Day 6	1 ± 0.03	1.02 ± 0.05	0.93 ± 0.07	0.65 ± 0.02*	0.67 ± 0.04*	0.73 ± 0.06*

Graph 16. Effects of sonicated *B. forsythus* extracts on protein production at days 2, 4 and 6.

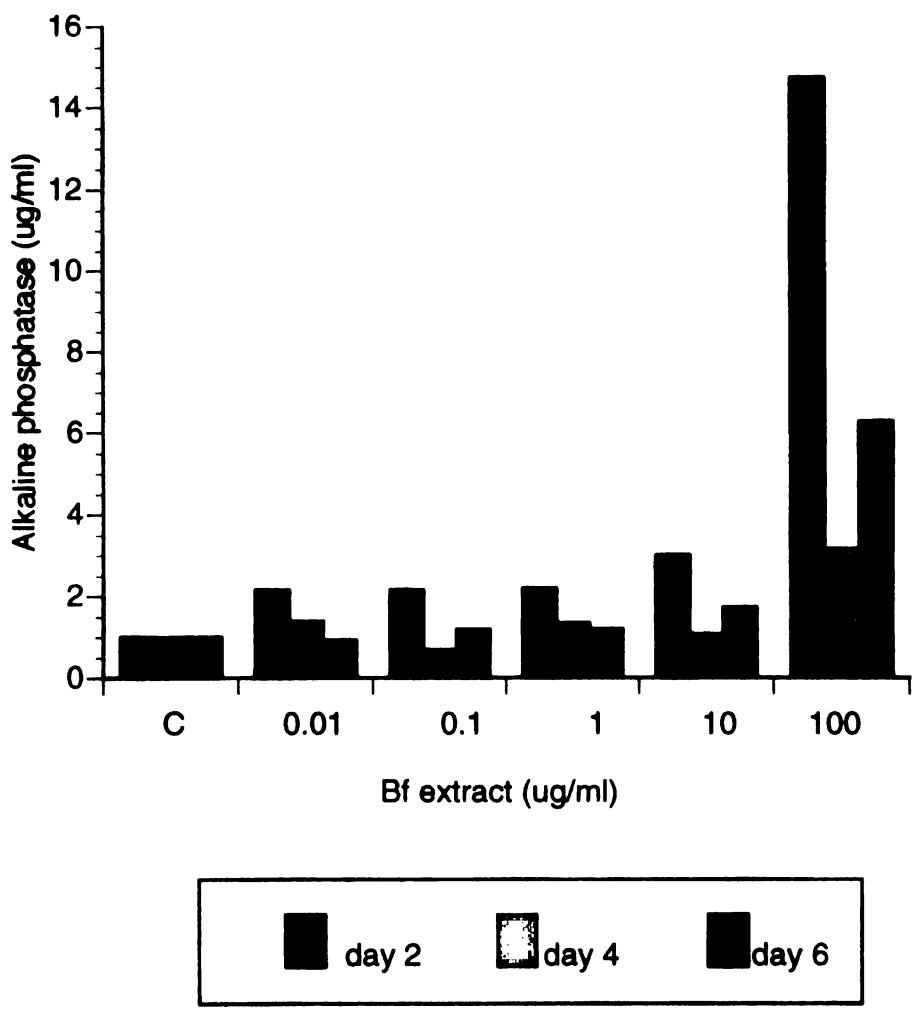


* - Denotes significance ($p<0.05$) between control and individual groups

Table 17. Effects of sonicated *B. forsythus* extracts on alkaline phosphatase activity at days 2, 4 and 6. (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Day 2	1 ± 0.53	2.15 ±0.67*	2.16 ±1.03*	2.18 ± 1.03*	2.99 ± 1.17*	14.7 ± 1.81*
Day 4	1 ± 0.58	1.39 ±0.48*	0.68 ± 0.32	1.34 ± 0.33	1.07 ± 0.38	3.16 ± 0.79*
Day 6	1 ± 0.08	0.93 ± 0.24	1.18 ± 0.34	1.64 ± 0.39	1.78 ± 0.21*	6.25 ± 0.89*

Graph 17. Effects of sonicated *B. forsythus* extracts on alkaline phosphatase activity at days 2, 4 and 6.

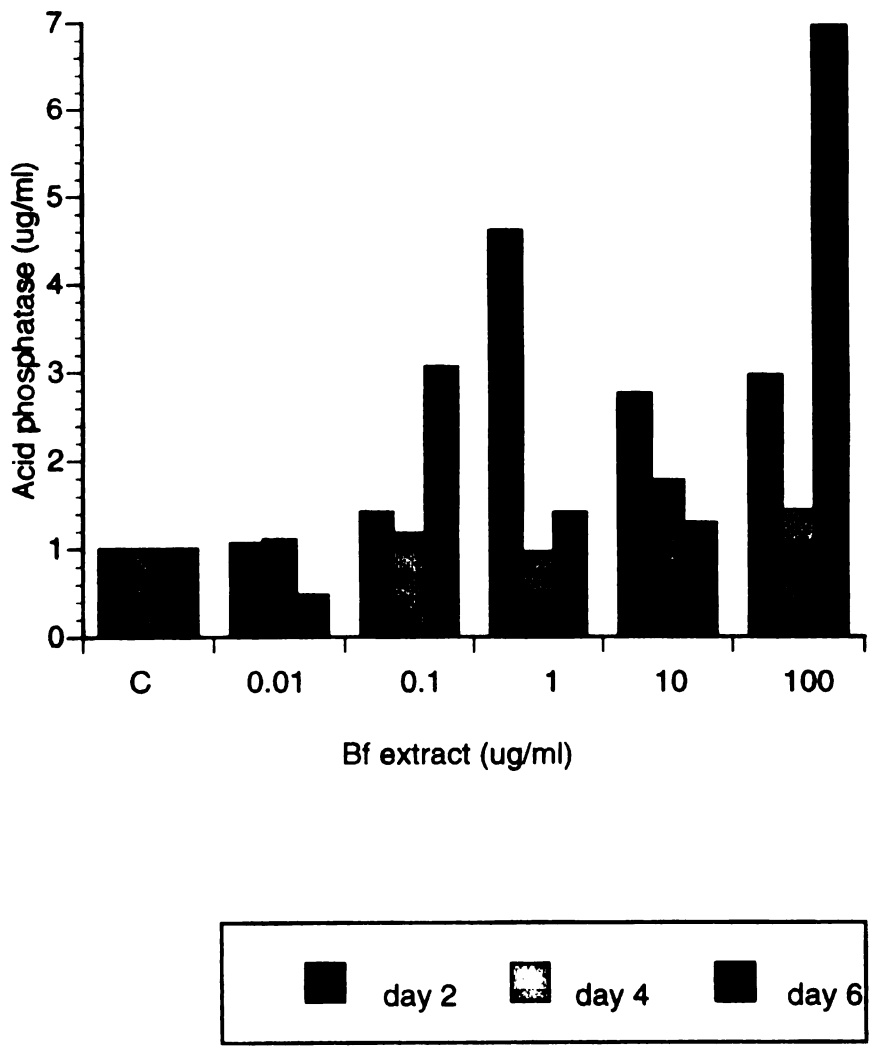


* - Denotes significance ($p<0.05$) between control and individual groups

Table 18. Effects of sonicated *B. forsythus* extracts on acid phosphatase activity at days 2, 4 and 6. (C=control, no bacterial sonicate added)

	C	0.01	0.1	1	10	100
Day 2	1 ± 1.40	1.06 ± 1.32	1.41 ± 0.68	1.59 ± 2.96	2.75 ± 2.53	2.95 ± 6.52
Day 4	1 ± 0.12	1.10 ± 0.11	1.17 ± 0.26	0.96 ± 0.19	1.77 ± 0.47*	1.42 ± 0.30*
Day 6	1 ± 2.31	0.47 ±0.45*	3.05 ± 1.28	1.40 ± 1.18	1.29 ± 1.63	6.94 ± 2.90*

Graph 18. Effects of sonicated *B. forsythus* extracts on acid phosphatase activity at days 2, 4, and 6.



* - Denotes significance ($p<0.05$) between control and individual groups

DISCUSSION

This study sought to investigate the effects of *B. forsythus* on osteogenesis in a cell culture model. MG-63 cells were grown and co-cultured with varying concentrations of sonicated *B. forsythus* extracts. Co-cultures of MG-63 and *B. forsythus* extracts were stopped at days 2, 4, 6 and 14 and assays were done to measure alkaline phosphatase activity as well as mineral deposition. In addition, since this cell line has not been previously used to investigate effects of bacterial extracts on osteogenesis, *P. gingivalis* was used as a positive control and *C. ochracea* as a negative control (these cultures were analyzed at day 14).

MG-63 osteosarcoma cell line. In this study, it was found that mineral production by MG-63 cells was stimulated by culture with both *B. forsythus* and *P. gingivalis* sonicate extracts. The results of the present study do not agree with the initial hypothesis that these sonicated bacterial extracts would inhibit osteogenic activity.

The MG-63 cell line has been used to investigate the effects of various experimental conditions on osteogenic activity. Barillé and co-workers[55] determined that MG-63 cells retained major osteoblastic functions, i.e., IL-6 and osteocalcin production. Schwartz and co-workers[56] found that MG-63 cell proliferation was enhanced on dentin surfaces pretreated with tetracycline or osteoclasts, and that there was increased alkaline phosphatase activity on these treated surfaces, compared to smooth surfaces. These two studies demonstrate that MG-63 cells respond similar to normal osteoblasts under the same experimental conditions.

Results of the present study demonstrated increased mineral deposition with the higher concentrations of sonicated bacterial extracts of *B. forsythus* and *P. gingivalis*.

However, Morimoto and co-workers[25] found that proteinaceous extracts of *A. actinomycetemcomitans* induced apoptosis in MG-63 cells at concentrations of 0.1-10μg/ml in a period of 12-48 hours. In addition, Gadhavi and co-workers[26] found that surface proteins of *A. actinomycetemcomitans* also inhibited mitosis of MG-63 cells. Unlike the present study, results of these studies imply that inhibition of mitosis would lead to an inhibition of mineralization. In addition, they used serum-free medium in their cell culture system, which may explain differences in mineral deposition as serum has been shown to inhibit bone resorption in the presence of stimulators of bone resorption.[31]

Effects of *B. forsythus* sonicated extracts on enzyme activity and mineral deposition. At days 2, 4, 6 and at two weeks, a significant increase in alkaline phosphatase activity was seen with *B. forsythus* extracts at a concentration of 100 μg/ml compared to controls. However, this increase in activity decreased from 14-fold at day 2, 6-fold at day 6 to 3-fold at two weeks. The increased activity at 100 μg/ml compared to controls is consistent with the amount of mineralization seen at two weeks. At day 14, a significant decrease in alkaline phosphatase activity was seen with sonicated extract concentrations of 1 and 10 μg/ml ($p<0.05$) and a significant increase with 100 μg/ml sonicated extracts. It was postulated that *B. forsythus* sonicated extracts would cause a decrease in alkaline phosphatase activity like other periodontopathogens such as *P. gingivalis*.[24] However, results of the present study show *B. forsythus* extracts inhibit alkaline phosphatase activity at lower concentrations, while stimulating alkaline phosphatase activity at high extract concentrations. Thus, these results suggest that at lower extract concentrations, which may be similar to what is present in the oral cavity, these periodontopathogens are mitogens for bone resorption. At higher extract

concentrations, they may help stimulate osteogenic activity through mechanisms that are not currently understood.

Effects of *P. gingivalis* sonicated extracts on enzyme activity and mineral deposition. A dose-dependent decrease in alkaline phosphatase activity was seen with *P. gingivalis* at two weeks (Table 7 and Graph 7). However, a net increase in mineralization was seen with *P. gingivalis* at sonicate extract concentrations of 0.1 to 10 μg/ml, which was significantly higher ($p<0.05$) than controls at 10 μg/ml (Table and Graph 10). At 100 μg/ml sonicated extract, MG-63 cells became non-viable. At this extract concentration, it is unclear if the sonicated extracts were inhibiting enzyme production, or if the sonicated extracts were toxic to the cells. This is consistent with results found by Loomer and co-workers,[32] where increasing concentrations of sonicated extracts of *P. gingivalis* caused a decrease in alkaline phosphatase activity. Murata and co-workers[57] investigated effects of extracts of *P. intermedia, A. actinomycetemcomitans*, *P. gingivalis* and *C. ochracea* on mineral formation in MC3T3 osteoblastic cells. They found that *P. gingivalis* and *C. ochracea* extracts did not decrease alkaline phosphatase activity even at high extract concentrations. Differences in culture media as well as length of time of co-incubation may explain discrepancies in results of these two studies.

Loomer and co-workers[24] analyzed alkaline phosphatase activity in chick periosteal osteogenesis (CPO) cultures incubated with unfractionated sonicated extracts of *P. gingivalis* at days 2, 4 and 6 and found that alkaline phosphatase activity increased from day 2 to day 6 compared to controls. Thus, sonicated extracts of *P. gingivalis* suppressed alkaline phosphatase activity initially and the enzyme activity increased and approached control levels by day 6. Alkaline phosphatase activity preceded mineral

deposition and the results of this and other studies suggest that *P. gingivalis* and *B. forsythus* extracts affect alkaline phosphatase kinetics differently.[24] That is, these sonicated extracts may cause differences in peak activity, which is why there is a dose-dependent decrease in activity with *P. gingivalis* and not with *B. forsythus* at two weeks.

Effects of *C. ochracea* sonicated extracts on enzyme activity and mineral deposition. Sonicated extracts of *C. ochracea* had no dose-dependent effect on alkaline phosphatase activity. There was an increase in activity with lower extract concentrations, which decreased as extract concentrations were increased. Since this pattern of alkaline phosphatase activity was consistent with the pattern of mineralization, results suggest that alkaline phosphatase activity must have remained relatively constant throughout the two weeks within each sonicate extract concentration. Thus, the present study suggests that sonicated extracts of *C. ochracea* do not affect osteogenesis in a dose-dependent manner. Ochiai and co-workers[58] investigated effects of *C. ochracea* extracts and found that proteinaceous factors caused dose-dependent suppression of spleen cells resulting in decreased lymphocyte proliferation, demonstrating suppressive effects on immune cells. In a study investigating effects of LPS from *C. ochracea*, Kaneko and co-workers[59] found that compared to other periodontopathogens, *C. ochracea* LPS induced the highest antibody response. Although some groups[21] consider that *C. ochracea* is a non-pathogenic microorganism, these two studies demonstrate that *C. ochracea* components affect immune cell proliferation and thus may be a periodontopathogen because of its effects on the immune response rather than as a mitogen for bone resorption factors.

There are few reported cases of osteogenesis or mineral deposition in the presence of an irritant or pathogen in the literature. Cayé-Thomasen and co-workers[60] found that

otitis media caused by *S. pneumoniae* initially resulted in bone resorption followed by formative activity, seen as "massive osteogenesis". In this situation, the formative activity is considered pathologic. In the dental literature, several clinical cases of osseous proliferation under pontic spaces have been reported. Burkes and co-workers[61] published a case series on nine patients who presented with osseous proliferation and attributed this to chronic irritation and possible genetic predisposition, although no conclusions about the etiology were made. Interestingly, all cases occurred in the posterior mandibular regions. Ruffin and co-workers[62] presented a case report of osseous proliferation in the lower premolar region and treatment by removal of the proliferative bone. Although no bacterial etiology was mentioned, the present study suggests *B. forsythus* sonicated extracts may act as an irritant to osseous tissues rather than a stimulator of bone resorption.

Other factors. It has been shown that bone-resorbing activity of LPS is inhibited by serum. Iino and Hopps[31] demonstrated bone resorption when cultured with LPS from all periodontopathogens, and that fetal calf serum inhibited bone-resorbing activity of LPS from *A. actinomycetemcomitans*, *P. gingivalis* and *C. ochracea*. Thus, it is possible that in the present study, no apparent negative effects were seen with mineralization with sonicated extracts from all three periodontopathogens because of the presence of fetal bovine serum in culture media. Loomer and co-workers[24] supplemented their media with serum and saw a decrease in osteogenic activity. Therefore, it is possible that bone remodeling in the presence of bacterial products may be inhibited by presence of serum. Therefore, other factors must be present in order for bone resorption to occur. Further

investigation is warranted to determine if absence of serum would result in bone resorption in the presence of sonicated bacterial extracts.

The present study did not investigate effects of *P. gingivalis* and *C. ochracea* sonicated extracts at days 2, 4, or 6. Future studies investigating this may help to understand effects of bacterial sonicated extracts on alkaline phosphatase enzyme kinetics, which may explain why a dose-dependent decrease in activity is seen with *P. gingivalis* and not with *B. forsythus*. Finally, cell viability or apoptosis was not investigated in this study. A measure of protein content is merely a crude measure of cell activity. By investigating cell viability, it could be determined whether bacterial sonicated extracts were toxic to MG-63 cells or if these extracts were affecting alkaline phosphatase activity and hence affecting mineralization.

CONCLUSION

Sonicated extracts of *B. forsythus* stimulated osteogenic activity *in vitro* in MG-63 cells. While previous studies have shown that extracts of other periodontopathogens inhibit bone formation in other *in vitro* culture systems, results of the present study show they may also play a role in stimulating bone formation. Overall, this study in combination with others suggest that these bacteria have both inhibitory and stimulatory effects on bone formation and thus may play a role in bone remodeling in periodontal diseases.

APPENDIX A – Reagents and culture media for bacteria

Hemin[63] (50 mg/%)

1. 50 mg of hemin to 100 ml distilled water
2. Adjust pH to 8.0 with 1N NaOH
3. Autoclave, cool, store at 4°C

Menadione (500 mg/%)

1. 250 mg of menadione to 50 ml of 95% ethanol
2. Filter sterilize, store at 4°C

Dithiotreitol (1%)

1. 1 g dithiotreitol in 100 ml distilled water
2. Filter sterilize, store at 4°C

N-Acetylmuramic acid[64] (NAM)

1. Dissolve 50 mg NAM in 5 ml distilled water
2. Filter sterilize
3. Aliquot into 0.5 ml portions and store in freezer

Tripticase Soy Broth (TSB)

Per 500 ml distilled water:

15 g tripticase soy broth
1.5 g yeast extract

After autoclaving:

2.5 ml hemin (50 mg/%)
0.5 ml menadione (500 mg/%)
5 ml dithiotreitol (1%)
5 ml N-acetylmuramic acid (1% - for *B. forsythus* broth only)
5 ml Isovitalex® (for *B. forsythus* broth only)

LRBB agar plates

Per 465 ml distilled water:

21 g Brucella agar

Autoclave, cool to 55°C

2.5 ml hemin (50 mg/%)
0.5 ml menadione (500 mg/%)
5 ml dithiotreitol (1%)
5 ml N-acetylmuramic acid (1% - for *B. forsythus* only)
5 ml Isovitalex® (for *B. forsythus* only)
25 ml laked rabbit blood

APPENDIX B – Culture media for MG-63 cells

Unsupplemented α-MEM without nucleosides culture media
Per 500 ml media:

15% fetal bovine serum

Supplemented α-MEM without nucleosides culture media
Per 500 ml media:

15% fetal bovine serum
25 mg ascorbic acid
10^{-8} M dexamethasone[65]
10 mM β-glycerophosphate[66]
5 ml fungizone (250 μg/ml)
5 ml penicillin-streptomycin (10,000 mcg/ml streptomycin; 10,000 units/ml for penicillin)

Freezing media for cell culture
Per 50 ml α-MEM:

10% DMSO (5 ml)
10% FBS (5 ml)

APPENDIX C – Reagents for assays and assay protocols

Bicarbonate buffer

1. 3 mM $NaHCO_3$
2. 15 mM NaCl
3. Combine both and adjust pH to 7.4.

Standard for acid and alkaline phosphatase assay

Serial dilutions with bicarbonate buffer:

1. 0.1 mM pNP (p-nitrophenol)
2. 1 ml (1) + 1 ml bicarbonate buffer = 0.05 mM
3. 1 ml (2) + 1 ml bicarbonate buffer = 0.025 mM
4. 1 ml (3) + 1 ml bicarbonate buffer = 0.0125 mM
5. 1 ml (4) + 1 ml bicarbonate buffer = 0.006 mM
6. 1 ml (5) + 1 ml bicarbonate buffer = 0.003 mM
7. 1 ml (6) + 1 ml bicarbonate buffer = 0.0015 mM
8. 1 ml bicarbonate buffer = 0 mM

Acid phosphatase reagent

1. 0.02 M pNPP (p-nitrophenyl phosphate)
2. 0.1 M sodium acetate
3. Mix (1) and (2) at 3:7 ratio and adjust pH to 5.0.

Acid phosphatase assay

1. Place 50 ul standard/blank/sample in 96-well plate
2. Add 50 ul acid phosphatase reagent to each well
3. Let sit at room temperature for several hours or at 37°C for 30 min to 1 hr.
4. Stop experiment by adding 50 ul 0.2 N NaOH
5. Read at 405 nm.

Alkaline phosphatase reagent

1. 0.02 M pNPP
2. 0.1 M sodium barbital
3. Mix (1) and (2) at 3:7 ratio and adjust pH to 9.3.

Alkaline phosphatase assay

1. Place 50 ul standard/blank/sample in 96-well plate
2. Add 100 ul alkaline phosphatase reagent to each well
3. Let sit Let sit at room temperature for several hours or at 37°C for 30 min to 1 hr.
4. Read at 405 nm.

Standard for protein assay (Bio-Rad)

1. 100 μg/ml
2. 1 ml (1) + 1 ml bicarbonate buffer = 50 μg/ml
3. 1 ml (2) + 1 ml bicarbonate buffer = 25 μg/ml
4. 1 ml (3) + 1 ml bicarbonate buffer = 12.5 μg/ml
5. 1 ml (4) + 1 ml bicarbonate buffer = 6.25 μg/ml
6. 1 ml (5) + 1 ml bicarbonate buffer = 3.125 μg/ml
7. 1 ml (6) + 1 ml bicarbonate buffer = 1.6 μg/ml
8. 1 ml bicarbonate buffer = 0 μg/ml

Protein assay (Bio-Rad)

1. Place 100 μl standard/blank/sample in 96-well plate
2. Add 30 μl BioRad protein assay reagent to each well
3. Mix well and let stand a minimum of five minutes, up to one hour
4. Read at 562 nm.

Calcium assay (SIGMA, procedure no. 587)

1. Prepare standards (provided in kit)
2. Place 10 μl standard/sample/blank in centrifuge tubes
3. Add 1 ml calcium reagent working solution to each tube
4. Mix well and let stand a minimum of three minutes, up to ten minutes
5. Read at 575 nm.

Inorganic phosphate assay (SIGMA, procedure no. 670)

1. Prepare standards (provided in kit)
2. Place 50 μl of sample in centrifuge tubes
3. Add 250 μl of deionized water and 200 μl 20% trichloroacetic acid and let stand 10 minutes.
4. Take 200 μl of above mixture and add 300 μl deionized water and 100 μl acid molybdate solution
5. Place 200 μl of standard/blank in centrifuge tubes and add 300 μl deionized water and 100 μl acid molybdate solution
6. Mix well and add 25 μl Fiske & Subba Row solution to all standards and samples
7. Let stand 10 minutes
8. Place samples into 96-well plate and read at 600±40 nm.

REFERENCES

1. Chapple ILC. Hypophosphatasia: dental aspects and mode of inheritance. *J Clin Periodontol* 1993; 20: 615-622.

2. Izumi Y, Sugiyama S, Shinozuka O, Yamazaki T, Ohyama T, Ishikawa I. Defective neutrophil chemotaxis in Down's syndrome patients and its relationship to periodontal destruction. *J Periodontol* 1989; 60: 238-242.

3. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998; 25: 134-144.

4. Armitage GC. Development of a classification for periodontal diseases and conditions. *Ann Periodontol,* 1999; 4: 1-6.

5. Moore WEC, Holdeman LV, Cato EP, et al. Comparative bacteriology of juvenile periodontitis. *Infect Immun* 1985; 48: 507-519.

6. Watanabe K. Prepubertal periodontitis: a review of diagnostic criteria, pathogenesis, and differential diagnosis. *J Periodont Res* 1990; 25: 31-48.

7. van Steenbergen TJ, van der Velden U, Abbas F, de Graaf J. Microbiological and clinical monitoring of non-localized juvenile periodontitis in young adults: a report of 11 cases. *J Periodontol* 1993; 64: 40-47.

8. Kamma JJ, Nakou M, Manti FA. Microbiota of rapidly progressive periodontitis lesions in association with clinical parameters. *J Periodontol* 1994; 65: 1073-1078.

9. Zambon JJ. Periodontal diseases: microbial factors. *Ann Periodontol* 1996; 1(1): 879-932.

10. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal disease. *Periodontol 2000* 1994; 5: 78-111.

11. Slots J, Reynolds HS, Genco RJ. *A. actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. *Infect Immun* 1980; 29: 1013-1020.

12. Newman MG, Socransky SS, Savitt ED, Propas DA, Crawford A. Studies of the microbiology of periodontosis. *J Periodontol* 1976; 47: 373-379.

13. Slots J and Dahlén G. Subgingival microorganisms and bacterial virulence factors in periodontitis. *Scand J Dent Res* 1985; 93: 119-27.

14. Socransky SS and Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 1992; 63: 322-331.

15. Di Murro C, Paolantonio M, Pedrazzoli V, Lopatin DE, Cattabriga M. Occurrence of *P. gingivalis, B. forsythus* and *Treponema denticola* in periodontally healthy and diseased subjects as determined by an ELISA technique. *J Periodontol* 1997; 68: 18-23.

16. Christersson LA, Fransson CL, Dunford RG, Zambon JJ. Subgingival distribution of periodontal pathogenic microorganisms in adult periodontitis. *J Periodontol* 1992; 63: 418-425.

17. Winkel EG, Van Winkelhoff AJ, Timmerman MF, Vangsted T, Van der Velden U. Effects of metronidazole in patients with "refractory" periodontitis associated with *Bacteroides forsythus. J Clin Periodontol* 1997; 24: 573-579.

18. Winkel EG, van Winkelhoff AJ, van der Velden U. Additional clinical and microbiological effects of amoxicillin and metronidazole after initial periodontal therapy. *J Clin Periodontol* 1998; 25: 857-864.

19. Umeda M, Contreras A, Chen C, Bakkler I, Slots J. The utility of whole saliva to detect the oral presence of periodontopathic bacteria. *J Periodontol* 1998; 69: 828-833.

20. Page RC, Vandesteen GE, Ebersole JL, Williams BL, Dixon IL, Altman LC. Clinical and laboratory studies of a family with a high prevalence of juvenile periodontitis. *J Periodontol* 1985; 56: 602-610.

21. Haffajee AD, Socransky SS, Smith C, Dibart S. The use of DNA probes to examine the distribution of subgingival species in subjects with different levels of periodontal destruction. *J Clin Periodontol* 1992; 19: 84-91.

22. Botta GA, Arzese A, Minisini R, Trani G. Role of structural and extracellular virulence factors in gram-negative anaerobic bacteria. *Clin Infect Dis* 1994; 18: S260-4.

23. Berne RM, Levy MN. *Physiology.* 3rd edition, St. Louis: Mosby-Year Book 1992; pp.336-337.

24. Loomer PM, Ellen RP, Tenenbaum HC. Characterization of inhibitory effects of suspected periodontopathogens on osteogenesis *in vitro. Infect Immun* 1995; 63: 3287-3296.

25. Morimoto Y, Morimoto H, Murata T, Kobayashi S, Ohba T, Haneji T. Extracts of *Actinobacillus actinomycetemcomitans* induce apoptotic cell death in human osteoblastic MG-63 cells. *J Dent Res* 1999; 78: 735-742.

26. Gadhavi A, Wilson M, Tabona P, Newman HN, Henderson B, Bennett JH. Inhibition of mitosis and induction of apoptosis in MG-63 human osteosarcoma-derived cells *in*

vitro by surface proteins from *A. actinomycetemcomitans*. *Arch Oral Biol* 2000; 45: 707-711.

27. Zambon JJ, Christersson LA, Slots J. *A. actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. *J Periodontol* 1983; 54: 707-711.

28. Han N, Hoover CI, Winkler JR, Ng CY, Armitage GC. Identification of genomic clonal types of *Actinobacillus actinomycetemcomitans* by restriction endonuclease analysis. *J Clin Microbiol* 1991, 29: 1574-1578.

29. Baker PJ. The role of immune responses in bone loss during periodontal disease. *Microb Infect* 2000; 2: 1181-1192.

30. Soames JV, Entwisle DN, Davies RM. The progression of gingivitis to periodontitis in the beagle dog: a histological and morphometric investigation. *J Periodontol* 1976; 47: 435-439.

31. Iino Y, Hopps RM. The bone-resorbing activities in tissue culture of lipopolysaccharides from the bacteria *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis* and *Capnocytophaga ochracea* isolated from human mouths. *Arch Oral Biol* 1984; 29: 59-63.

32. Loomer PM, Sigusch B, Sukhu B, Ellen RP, Tenenbaum HC. Direct effects of metabolic products and sonicated extracts of *Porphyromonas gingivalis* 2561 on osteogenesis *in vitro*. *Infect Immun* 1994; 62: 1289-1297.

33. Tanner ACR, Listgarten MA, Ebersole JL, Strzempko MN. *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. *Int J Sys Bacteriol* 1986; 36: 213-221.

34. Takemoto T, Kurihara H, Dahlen G. Characterization of *Bacteroides forsythus* isolates. *J Clin Microbiol* 1997; 35: 1378-1381.

35. Maiden MFJ, Tanner A, Macuch PJ. Rapid characterization of periodontal bacterial isolates by using fluorogenic substrate tests. *J Clin Microbiol* 1996; 34: 376-384.

36. Guillot E and Mouton C. A PCR-DNA probe assay specific for *Bacteroides forsythus*. *Mol Cell Probes* 1996; 10: 413-421.

37. Yoshimura F, Nishikata M, Suzuki T, Hoover CI, Newbrun E. Characterization of a trypsin-like protease from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. *Arch Oral Biol* 1984; 29: 559-564.

38. Meurman JH, Wahlfors, J, Korhonen A, et al. Identification of *Bacteroides forsythus* in subgingival dental plaque with the aid of a rapid PCR method. *J Dent Res* 1997; 76: 1376-1380.

39. Slots J, Ashimoto A, Flynn MJ, Li G, Chen C. Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin Infect Dis* 1995; (20 Suppl 2): S304-S307.

40. Haffajee AD, Socransky SS, Dzink JL, Taubman MA, Ebersole JL. Clinical, microbiological and immunological features of subjects with refractory periodontal diseases. *J Clin Periodontol* 1988; 15: 390-398.

41. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* 1994; 65: 260-267.

42. Grossi SG, Genco RJ, Machtei EE, et al. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. *J Periodontol* 1995, 66: 23-29.

43. Tran SD, Rudney JD, Sparks BS, Hodges JS. Persistent presence of *Bacteroides forsythus* as a risk factor for attachment loss in a population with low prevalence and severity of adult periodontitis. *J Periodontol* 2001; 72: 1-10.

44. Cugini MA, Haffajee AD, Smith C, Kent RL Jr., Socransky SS. The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results. *J Clin Periodontol* 2000, 27: 30-36.

45. Kazor C, Taylor GW, Loesche WJ. The prevalence of BANA-hydrolyzing periodontopathic bacteria in smokers. *J Clin Periodontol* 1999; 26: 814-821.

46. Zambon JJ, Grossi SG, Machtei EE, Ho AW, Dunford R, Genco RJ. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *J Periodontol* 1996; 67: 1050-1054.

47. van Winkelhoff AJ, Rodenburg JP, Goene RJ, Abbas F, Winkel EG, de Graaff J. Metronidazole plus amoxicillin in the treatment of *Actinobacillus actinomycetemcomitans* associated periodontitis. *J Clin Periodontol* 1989; 16: 128-131.

48. Feres M, Haffajee AD, Goncalves C, et al. Systemic doxycycline administration in the treatment of periodontal infections (I). Effect on the subgingival microbiota. *J Clin Periodontol* 1999; 26: 775-783.

49. Wong MY, Lu CL, Liu CM, Hou LT. Microbiological response of localized sites with recurrent periodontitis in maintenance patients treated with tetracycline fibers. *J Periodontol* 1999; 70: 861-868.

50. Sharma A, Sojar HT, Glurich I, Honma K, Kuramitsu HK, Genco RJ. Cloning, expression and sequencing of a cell surface antigen containing a leucine-rich repeat motif from *B. forsythus* ATCC 43037. *Infect Immun* 1998; 60: 5703-5710.

51. Vasel D, Sims TJ, Bainbridge B, Houston L, Darveau R, Page RC. Shared antigens of *Porphyromonas gingivalis* and *Bacteroides forsythus*. *Oral Microbiol Immunol* 1996; 11: 226-235.

52. Sorsa T, Ingman T, Suomalainen K, et al. Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. *Infect Immun* 1992; 60: 4491-5.

53. Munemasa T, Takemoto T, Dahlén G, et al. Adherence of *Bacteroides forsythus* to host cells. *Microbios* 2000; 101: 115-126.

54. Yao ES, Lamont RJ, Leu SP, Weinberg A. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. *Oral Microbiol Immunol* 1996; 11: 35-41.

55. Barillé S, Collette M, Bataille R, Amiot M. Myeloma cells upregulate interleukin-6 secretion in osteoblastic cells through cell-to-cell contact but downregulate osteocalcin. *Blood* 1995; Oct 15, 86: 3151-3159.

56. Schwartz Z, Lohmann CH, Wieland M, et al. Osteoblast proliferation and differentiation on dentin slices are modulated by pretreatment of the surface with tetracycline or osteoclasts. *J Periodontol* 2000; 71: 586-597.

57. Murata T, Ansai T, Takahara T, Kobayashi S, Haneji T. Extracts of *Prevotella intermedia* and *A. actinomycetemcomitans* inhibit alkaline phosphatase activity in osteoblastic cells in vitro. *Oral Diseases* 1997; 3: 106-112.

58. Ochiai K, Senpuku H, Kurita-Ochiai T. Purification of immunosuppressive factor from *Capnocytophaga ochracea*. *J Med Microbiol* 1998; 47: 1087-1095.

59. Kaneko T, Hara Y, Yoshimura A, Kato I. Induction of anti-thymocyte/T-lymphocyte antibodies in mice injected with lipopolysaccharides from periodontopathic bacteria. *J Periodontol Res* 1999; 34: 105-112.

60. Cayé-Thomasen P, Tos M. Adaptive bone modeling and remodeling in acute otitis media caused by non-typeable or type B *Haemophilus influenzae* or *Moraxella catarrhalis*. *Acta Otolaryngol* 2000; 120: 815-820.

61. Burkes EJ Jr., Marbry DL, Brooks RE. Subpontic osseous proliferation. *J Prosth Dent* 1985; 53: 780-785.

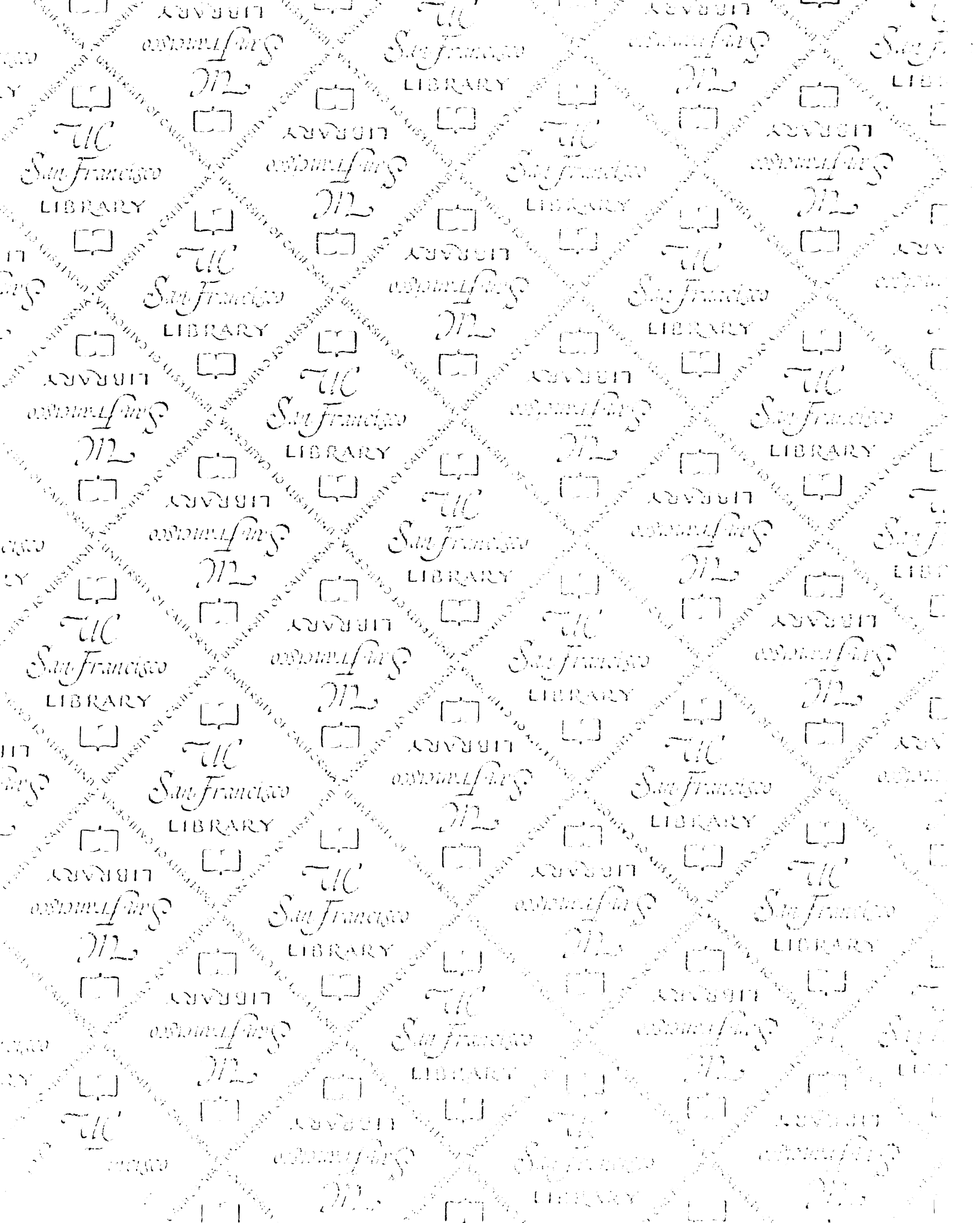
62. Ruffin SA, Waldrop TC, Aufdemorte TB. Diagnosis and treatment of subpontic osseous hyperplasia. Report of a case. *Oral Surg Oral Med Oral Pathol* 1993; 76: 68-72.

63. Wyss C, Hunziker P, Klauser S. Support of peptide-dependent growth of *Bacteroides forsythus* by synthetic fragments of haemoglobin or fetuin. *Arch Oral Biol* 1993; 38: 979-984.

64. Wyss C. Dependence of proliferation of *Bacteroides forsythus* on exogenous N-acetylmuramic acid. *Infect Immun* 1989; 57: 1757-1759.

65. Tenenbaum HC, Heersche JNM. Dexamethasone stimulates osteogenesis in chick periosteum *in vitro*. *Endocrinology* 1985; 117: 2211-2217.

66. Tenenbaum HC, Kamalia N, Sukhu B, Torontali M. Osteogenic phase-specific co-regulation of collagen synthesis and mineralization by β-glycerophosphate in chick periosteal cultures. *Bone* 1992; 13: 129-138.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