Determination of the antibacterial activity of simvastatin against periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: An *in vitro* study

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Abstract

Context and Objective: Statin treatment, apart from its hypolipidemic action has proven its antimicrobial activity by improving the survival rate of patients with severe systemic bacterial infections. Periodontitis is an inflammatory disorder of tooth supporting structures caused by a group of specific microorganisms. The objective of the present study was to determine the antimicrobial activity of pure simvastatin drug against the primary periodontal pathogens. **Materials and Methods:** Minimum inhibitory concentration (MIC) was determined against *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* using serial dilution method. **Results:** MIC of simvastatin against *P. gingivalis* was 2 μg/ml and *A. actinomycetemcomitans* was found to be <1 μg/ml which requires further dilutions to determine the exact value. **Conclusions:** Data suggests a potent antimicrobial activity of simvastatin against both *A. actinomycetemcomitans* and *P gingivalis*. Hence simvastatin can be prescribed as a dual action drug in patients with both hyperlipidemia and periodontal disease.

Keywords: Actinobacillus actinomycetemcomitans, minimum inhibitory concentration, Porphyromonas gingivalis, simvastatin

Introduction

Periodontal diseases are among the most widespread oral bacterial diseases of mankind that affects 10-15% of the world's population eventually leads to tooth loss, if left untreated. Although bacteria belonging to more than 630 different taxa exist in the oral cavity, only 10-15 bacterial species are recognized as potential periodontal pathogens. Of them, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* are recognized as the major pathogens for initiation and progression of destruction of tooth supporting structures. *A. actinomycetemcomitans* is a nonmotile, capnophilic, Gram-negative, coccobacillus, and a facultative anaerobe. It produces a number of potentially damaging metabolites including leukotoxin, cytolethal distending toxin. *P. gingivalis* is a nonmotile, asaccharolytic, Gram-negative coccobacillus, and an obligate anaerobe that produces large array of virulence factors such as leukotoxin and lipopolysaccharides. Longitudinal and retrospective studies have demonstrated an increased risk of periodontal breakdown in *A. actinomycetemcomitans* and *P. gingivalis* positive sites and better posttreatment results in their absence.[1]

The logical initiation to control these organisms by mechanical means resulted in minimal long-term effects due to their ability to invade gingival epithelial cells *in vitro* and buccal epithelial cells *in vivo*.[2,3] Hence, adjunctive antimicrobial therapy along with appropriate mechanical therapy helped in a significant reduction of both *A. actinomycetemcomitans* and *P. gingivalis*.[4] Table 1 lists the characteristics of few important antimicrobials that are commonly used to treat periodontitis.[5] Each antimicrobial agent has its own characteristic, which plays an important role in its action against specific bacteria.

Minimum inhibitory concentration (MIC) is the lowest concentration of a drug that inhibits the visible growth of test organism. *In vitro* detection of MIC of a drug against pathogens acts as a guideline for its *in vivo* application. Clinicians use MIC scores to choose appropriate antibiotics to patients with specific infections and to identify an effective dose of the drug.[6]

Considering the pathogenic potential of *A. actinomycetemcomitans* and *P. gingivalis*, the MIC of various commonly used antimicrobials has been investigated. Table 2 represents few reports with variable results, which can be attributed to change in temperature, inoculum size, pH, growth medium, and specific strain of microorganism during *in vitro* evaluation.[7]

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Now-a-days cardiovascular diseases (CVDs) rank among the leading causes of death. Nearly, a two-fold increased risk of coronary heart disease was observed in individuals with periodontal disease.[8] On examination of 50 carotid endarterectomized human specimens, 26% were positive for *P. gingivalis* and 18% were positive for *A. actinomycetemcomitans*.[9] These findings supports the therapeutic end points to eliminate or lower the number of these specific pathogenic microbes to prevent the initiation, progression and recurrence of periodontitis and in turn CVD.

Table 1: Characteristics of commonly used antimicrobials to treat periodontitis

Antibiotic	Dose (mg)	C serum (µg/ml)	C crevicular fluid (µg/ml)	t max serum (h)	Half-life (h)
Penicillin	500	3	ND	1	0.5
Amoxicillin	500	8	3-4	1.5-2	0.8-2
Doxycycline	200	2-3	2-8	2	12-22
Tetracycline	500	3-4	5-12	2-3	2-3
Clindamycin	150	2-3	1-2	1	2-4
Metronidazole	500	6-12	8-10	1-2	6-12
Ciprofloxacin	500	1.9-2.9	ND	1-2	3-6

C: Concentration; t max: Hours to reach peak serum concentration; ND: Not determined

Table 2: MIC values of *A. actinomycetemcomitans* and *P. gingivalis* to selected antimicrobial agent

Antimicrobial	MIC (µg/ml)	
	A. actinomycetemcomitans	*P. gingivalis*
Penicillin	4.0 6.25	0.016 0.29
Amoxicillin	1.0 1.6	0.023<1
Doxycycline	1.0 3.1	0.047
Metronidazole	32 12.5	0.023 2.1

MIC: Minimum inhibitory concentration; *A. A. actinomycetemcomitans: Actinobacillus actinomycetemcomitans; P. gingivalis: Porphyromonas gingivalis*

These days' statins are one of the most commonly prescribed drugs to control the serum cholesterol levels to reduce the risk of CVD. Apart from this, statins have been ascribed for showing various additional pleiotropic effects such as anti-inflammatory, immune modulatory, antioxidant, and anti-carcinogenic properties.[10] Systemic antimicrobial effects of statins have also been proved.[11] MIC values of different statins were evaluated against various nonperiodontal pathogens.[12,13] The results showed a better antibacterial activity of simvastatin on the majority of tested bacteria [Table 3].

Simvastatin, a type I statin is obtained by natural fermentation of fungus, *Aspergillus terrus*.[14] It is marketed in the form of tablets (10-80 mg) under the trade name Zocor®. Present scientific literature on simvastatin revealed its good applicability in the field of periodontics as a local drug delivery agent due to its anti-inflammatory and bone regenerative properties.[15]

Although simvastatin's antimicrobial activity was proven on many systemic pathogenic bacteria,[11] until date no studies have been carried out on its activity against specific periodontal pathogens. Hence, the present *in vitro* study was aimed to find out the MIC of pure simvastatin drug that can be safely and effectively administered as an antimicrobial agent on specific periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans*.

Materials and Methods

Pure simvastatin drug (powder form) was obtained from Dr. Reddy's laboratories, Hyderabad, India. It was certified to be free from any form of bacteria, yeast or mold by the manufacturer after microbial analysis.

Preparation of bacterial suspension

From the maintained frozen stock cultures of *A. actinomycetemcomitans* (ATCC No-25586) and *P. gingivalis* (ATCC No-33277) (American type culture collection, Manassas, VA, USA), small quantity of cells were recovered and subcultured. Brain heart infusion (BHI) broth was used as the culture medium to support the growth of bacteria.

Table 3: MICs (µg/ml) of different statins against few systemic bacteria

Bacterial strain	Rosuvastatin (µg/ml)	Atorvastatin (µg/ml)	Simvastatin (µg/ml)
Escherichia coli ATTC 35218	104.17±36.08	26.04±9.02	52.08±18.04
Staphylococcus aureus ATTC 25213	208.33±72.16	41.67±18.04	26.04±9.02
Streptococcus epidermidis ATTC 12228	166.67±72.16	20.83±9.02	26.04±9.02
Klebsiella pneumonia ATTC 13883	333.33±144.33	166.67±72.16	166.67±72.16
Strepococcus pyogenes ATTC 19615	166.67±72.16	83.33±36.08	62.5±0.00
Hemophilus influenza ATTC 29247	166.67±72.16	83.33±36.084	52.08±18.04

MIC: Minimum inhibitory concentration

This culture was transferred into the tubes containing 2 ml of the BHI medium to get culture suspension of *A. actinomycetemcomitans* and *P. gingivalis* respectively. The selected test bacterial strains were adjusted for 0.5 McFarland turbidity standards (10^8 colony forming units/ml) to check the MIC of simvastatin against them.[16]

Determination of minimum inhibitory concentrations

In the present study, serial tube dilution technique was followed based on the guidelines of the Clinical and Laboratory Standards Institute due to its ability to determine antimicrobial activity of the drug along with its MIC values.[17] Stock solution was prepared by dissolving the pure drug in dimethyl sulfoxide at a concentration of 10 mg/ml. In the initial tube 100 µl of stock solution was added into the 300 µl of BHI broth to make a volume of 400 µl, from which nine serial dilutions were prepared in separate test tubes containing 200 µl of BHI broth. To each serially diluted tube, 200 µl of the previously prepared bacterial suspension was added and incubated for 24 h in an anaerobic jar at 37°C and observed for turbidity which indicates the growth of the organisms. The turbidity in each tube was compared with a positive control, which contained only the pure bacterial culture. The least concentration of the drug in the tube, which does not show any turbidity, was considered as MIC of the drug for that particular test organism.

Results

In vitro antibacterial activity

In the present study, both *A. actinomycetemcomitans* and *P. gingivalis* were sensitive to pure simvastatin drug. *P. gingivalis* was sensitive until 2 µg/ml dilution and showed resistance to further dilution by illuminating its MIC. But for *A. actinomycetemcomitans*, the performed dilutions could not show any visible growth of the organism; hence, MIC value was considered to be <1 µg/ml [Table 4]. *A. actinomycetemcomitans* was found to be more sensitive than *P. gingivalis* showing its susceptibility to the last dilution (1 µg/ml) set for the study. To determine the exact value of MIC for *A. actinomycetemcomitans,* further dilutions of <1 µg/ml are required.

Discussion

Severe periodontitis acts as an inflammatory focus in the oral cavity, that potentiates the atherosclerotic process by stimulation of humoral and cell-mediated inflammatory pathways.[18] The bacteria detected in atheromatic plaque lesions included many oral microbiota which principally comprised of anaerobic *A. actinomycetemcomitans* and *P. gingivalis*.[19] As per the regression model, a linear dose response gradient was observed between increasing numbers of sites with >20% of alveolar bone loss and coronary heart disease independent of other known risk factors.[20] This was further supported by National Health and Nutrition Examination Survey III, where a positive correlation was proved between periodontal disease and CVD.[21]

P. gingivalis secretes toxic products which contribute to inactivation of the effector molecules of the host immune response leading to tissue destruction. It can invade aortic and heart endothelial cells through fimbriae.[22] It also expresses a virulence factor called collagen like platelet aggregation associated protein, that induces platelet aggregation and plays role in atheroma formation.[23] Periodic *P. gingivalis* bacteremia induced aortic and coronary lesions consistent with atherosclerosis even in normocholesterolemic pigs.[24] This suggests their role in exerting atherogenic stimulus independent of high cholesterol levels. *A. actinomycetemcomitans* secretes leukotoxin, which was suggested to be in association between periodontitis and CVD.[25]

Limitations such as inaccessible areas, residual sub-gingival pathogens and inability to eliminate the bacteria that penetrated the host tissues, led to the initiation of antimicrobial therapy along with mechanical debridement. Widespread use of common antimicrobials independently for different infectious conditions disturbs the delicate ecologic equilibrium of the body, allowing the proliferation of resistant bacteria, development of super infections and systemic toxic effects.[26] Hence, it would be of great advantage to the patient, if a single drug could simultaneously take care of more than a single infection like periodontitis and other systemic disease.

Though presently age is not a primary determinant to classify periodontitis, most of the existing literature shows that periodontal disease affects the majority of the adult population above the age of 35-40 years.[27] 50% of the population older than 60 years are known to be the sufferers of periodontitis and of them, approximately 55% had either the diagnosis of atherosclerosis, or a history of stroke or acute coronary syndrome.[28] Although multiple causes have been recognized for initiation of CVD, the most common

Table 4: MIC values of simvastatin against *A. actinomycetemcomitans* and *P. gingivalis*

Drug concentrations (µg/ml)	500	250	125	62.5	31.25	16	8	4	2	1
A. actinomycetemcomitans	S	S	S	S	S	S	S	S	S	S
P. gingivalis	S	S	S	S	S	S	S	S	S	R

S: Sensitive (no visible growth of the microorganism); R: Resistant (visible growth of the microorganism); *A. A. actinomycetemcomitans: Actinobacillus actinomycetemcomitans; P. gingivalis: Porphyromonas gingivalis*; MIC: Minimum inhibitory concentration

and modifiable risk factor associated is hyperlipidemia. In this modern era, due to the changing lifestyle, though hyperlipidemia is observed in the younger generations, it is predominantly detected in the age range of 50-70 years.[29] Hence, age range seems to be a common factor for the occurrence of both periodontitis and hyperlipidemia.

Statins are inhibitors of 3-hydroxy-3-methylglutarylcoenzymeA (HMG-CoA) reductase enzyme and prevents the formation of an intermediate product, mevalonate. This compound is also a precursor for other nonsteroidal isoprenoid compounds, farnesyl pyrophosphate and geranylgeranyl pyrophosphate which take part in protein prenylation, an important post translational modification, required for the biological functions of the cell.[30] Blocking of mevalonate formation inhibits protein prenylation that affects several signal transduction steps causing various pleiotropic effects such as improving endothelial function, immunomodulation, antioxidant activity, and treatment of malignancies.[10]

Simvastatin, a semi-synthetic statin on systemic administration improved bone mineral density in older women and reduced the risk of tooth loss in diabetes mellitus patients with chronic periodontitis.[31,32] It showed many beneficial effects to improve the health of periodontium through mechanisms like:

Periodontal regeneration; by augmenting bone morphogenetic proteins-Smad signaling that enhances bone formation, and antagonizing tumor necrosis factor-α (TNF-α) to Ras/Rho/mitogen activated protein kinase that causes osteoclastic differentiation. It also causes significant increase in levels of osteoblast differentiation factors such as alkaline phosphatase, osteopontin, osteocalcin and vascular endothelial growth factor.

Anti-inflammatory action; by reducing the proinflammatory cytokines like Interleukin-6 (IL-6) and IL-8 and down regulation of nuclear factor kappa β and activator protein 1 that are essential for IL-1α stimulated IL-6 and IL-8 expression.[33]

Minimum inhibitory concentration is important in diagnostic laboratories to confirm the resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents.[6] MIC is a measure of the potency of an antimicrobial drug. Different bacterial species have varying MICs. Sensitive strains have relatively low MICs and resistant strains have relatively high MICs.

Most frequently employed antimicrobial drugs to treat periodontal disease are used at dosages ranging from 100 to 500 mg, twice or thrice a day [Table 1]. In contrast, the recommended usual dose of simvastatin is 10-80 mg once a day for its hypolipidemic activity.[34] In an *in vitro* study, a relatively low concentration of simvastatin (10^{-8} and 10^{-7}M) promoted periodontal ligament cell proliferation and osteoblastic differentiation with increase in osteopontin, calcium, and alkaline phosphatase activity.[33] As a general rule of thumb, the concentration of antimicrobial drug in the blood should exceed the MIC by a factor of 2-8 times to offset the tissue barriers that restrict access to the infected site.[35] This higher concentration may sometimes lead to systemic toxic effects. In the present study, the MIC values of simvastatin obtained for *P. gingivalis* is higher and for *A. actinomycetemcomitans*, it is very much lower than the other antimicrobials [Table 4].

Clinically, periodontitis patients under statin therapy had a 37% less number of pathological periodontal pockets compared with those without under statin medication.[36] Improvement in the clinical parameters and infrabony defects fill was observed following scaling and root planing (SRP) with locally delivered simvastatin in periodontitis patients, compared with SRP plus placebo.[15] In a retrospective analysis with 7 years follow-up, systemic administration of simvastatin showed reduced risk of tooth loss in patients with chronic periodontitis.[37] Hence in this context, simvastatin may play a dual role in treating both hyperlipidemia to prevent CVD and periodontitis, which might prevent additional prescription of antimicrobials for periodontitis patients who were already under statin therapy. Statins were beneficial even in patients suffering from sepsis through immunomodulation by altering the function of both T cells and antigen-presenting cells.[38] They reduced the release of the proinflammatory cytokines such as TNF-α, IL-1, and IL-6 in rat models.[39]

It was found that bacterial HMG-CoA is 10,000 times weaker than the enzyme found in humans. Hence, it is unlikely to attribute hypolipidemic mechanism of action (i.e., inhibition of HMG CoA reductase) of statins to their antibacterial activity.[13] Previous studies have attributed the antimicrobial action of statins to increased bacterial clearance from the infected site[40] or by promoting the apoptosis of microbial cells.[12] In addition, hydrophobic nature of simvastatin also explained its antibacterial action, where it distresses the bacterial membrane in a "soap like" manner causing its death.[11] However, the exact mechanism of action needs further research.

The present *in vitro* MIC study helps us to focus on an intervention approach to design and conduct a clinical trial to detect the beneficial effect of simvastatin on patients at risk for periodontitis. However, *in vitro* values of MIC may not hold good for *in vivo* studies due to their inherent limitations. The growth of microorganisms *in vitro* is exponential, whereas the growth *in vivo* can be very slow to none.[41] Though MIC does not indicate the true activity of the drug at the locus of infection, the *in vitro* MIC serves as a surrogate marker attempting to quantify the drug activity.[42]

Scientifically based understanding of the etiopathogenesis of periodontal disease has laid a new responsibility on dentists to care for present and future periodontitis

patients not only for their dental health, but also for the systemic health. Within limitations of the present study, lowest concentration of simvastatin was proven to be effective for both *A. actinomycetemcomitans* and *P. gingivalis*. However, since periodontitis is a polymicrobial disease, the susceptibility of various other periodontal pathogens to this drug must to be evaluated. Though the long-term safety profiles of statins are well-documented, further studies are required to: (a) Investigate the safety of using simvastatin in nonhyperlipidemic patients to treat periodontitis. (b) Assess the *in vivo* efficacy of simvastatin with other traditionally prescribed antimicrobials used for periodontal therapy. (c) Evaluate the *in vivo* effect of simvastatin in different formulations (gel, chips, strips, fibers, etc.,) with variable concentrations. The *in vitro* determination of their concentration in gingival crevicular fluid and serum samples might help us to know the ideal dosage and formulation required for antimicrobial and regenerative activity of simvastatin to treat periodontitis.

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