EVALUATION OF RELATIVE EFFICACY OF SIMVASTATIN IN COMBINATION WITH HYDROXYAPATITE IN THE TREATMENT OF HUMAN PERIODONTAL INFRABONY DEFECTS-"A 6 MONTHS RANDOMIZED CONTROLLED CLINICAL STUDY"

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In Partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTICS

MAY 2020

CERTIFICATE - I

This is to certify that **Dr. SREELAKSHMI. P,** Post Graduate student in the Department of Periodontics, J.K.K. Nattraja Dental College and Hospital, Komarapalyam has done this dissertation titled **"EVALUATION OF RELATIVE EFFICACY OF SIMVASTATIN IN COMBINATION WITH HYDROXYAPATITE IN THE TREATMENT OF HUMAN PERIODONTAL INFRABONY DEFECTS - A 6 MONTHS RANDOMIZED CONTROLLED CLINICAL STUDY"** under my direct guidance during her post graduate study period 2017 - 2020.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY** in partial fulfillment of the degree of **MASTER OF DENTAL SURGREY, BRANCH II – PERIODONTICS.**

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CERTIFICATE - II

This is to certify that this dissertation work titled **"EVALUATION OF RELATIVE EFFICACY OF SIMVASTATIN IN COMBINATION WITH HYDROXYAPATITE IN THE TREATMENT OF HUMAN PERIODONTAL INFRABONY DEFECTS-"A 6 MONTHS RANDOMIZED CONTROLLED CLINICAL STUDY"** of the candidate **Dr. SREELAKSHMI. P** with the registration number **241713101** for the award of **MASTER OF DENTAL SURGERY** in the **Branch II - PERIODONTICS**. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the upload thesis file contains from introduction to conclusion pages and results shows 7% of plagiarism in the dissertation.

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Periodontitis is a multifactorial chronic inflammatory disease which results in the destruction of the tooth supporting structures.[1] The pathophysiology of periodontitis constitute key molecular pathways which ultimately leads to activation of host-derived immune responses that enable loss of marginal periodontal ligament fibers, apical migration of the junctional epithelium, and allows apical spread of the bacterial biofilm along the root surface.[2]

The primary etiological factor which plays a vital role in initiation and progression of periodontal diseases is the bacterial biofilm.[3] Contemporary periodontal therapy mainly aims to control the infection and regenerating lost supporting structures. The control of infection can be achieved by proper initial phase periodontal therapy including scaling and root planing, maintenance and anti-microbial therapy. Surgical treatment is essential in case of moderate to severe diseases which often show signs of inflammation after non-surgical therapy.[4]

The ultimate goal of any periodontal therapy is the regeneration of periodontal tissues affected by the disease to their original form, function and consistency. The current techniques aimed at periodontal regeneration include open flap debridement, the use of bone grafting material, guided tissue regeneration (GTR), by the use of biological modifiers like enamel matrix derivative and by the use of growth factors.[5]

Conventional techniques like scaling and root planning, open flap debridement may result in the formation of long junctional epithelium which is more susceptible to microbial invasion and results in less stable attachment.[6] The interest in bone grafting procedures has emerged from the desire to fill infra bony defects rather than radically resect surrounding intact bone tissues. The bone grafts have the

potential to manipulate the biological response into a regenerative pattern of periodontal healing. Thus bone grafting becomes more predictable in regenerative therapy. The regrowth of alveolar bone by the formation of new attachment can be achieved by the use of bone grafts by stimulating either by osteogenesis, osteoconduction or osteoinduction.[7]

Hydroxyapatite (HA) is one of the most widely used calcium phosphate graft biomaterial which has a similar composition and structure to natural bone. HA chemically bond directly to bone when implanted. It is biocompatible within the gingival tissue and serves as mineral reservoir to induce bone formation through osteoconductive mechanisms, resulting in improvement of clinical parameters.[8]

To achieve greater predictability with regenerative therapy, required an agent that can simultaneously eradicate infection and enhances new bone formation. Over the past two decades many pharmacological agents have been studied for their possible roles in management of periodontal diseases and its ability to regenerate the lost periodontal structures.[9]

Statins are the group of cholesterol lowering drugs gaining interest in the field of periodontology due to its pleotropic effects.[10] Beyond the cholesterol lowering action statins also inhibit the isoprenoid intermediates of the mevalonate biosynthetic pathway which favours its use in periodontal regenerative therapy. Potential pleotropic effects of statins include antioxidant, immunomodulatory, and endothelium stabilization, anti-thrombotic and enhance intracellular calcium mobilization. These effects of statins can modify the inflammatory cascades such as changing inflammatory mediators, inducing heme oxygenase, altering leukocyte-endothelial growth factor, which is known to stimulate bone formation.[11]

Simvastatin belongs to the family of statins which is widely used for the treatment of hypercholesterolemia. It has been shown to markedly increase new bone formation and osteoblast numbers in both *in vitro* and *in vivo* studies. The administration of simvastatin in the prodrug form is suitable for periodontal regenerative therapy as it is cost effective with long term safety profile and osteogenic effects.[12]

Thus the present study is performed to evaluate the efficacy of simvastatin used in combination with hydroxyapatite in the treatment of human periodontal infra bony defects.

To perform comparative evaluation of efficacy of simvastatin in combination with hydroxyapatite in the treatment of infrabony defects in patients with chronic periodontitis.

1. *In vitro* assay was done to evaluate the osteogenic potential of simvastatin by assessing the bone markers RUNX2 and osteopontin in MG-63 osteoblast cell lines after incorporation with simvastatin.
2. To compare changes in probing pocket depth and clinical attachment level following therapy of both the groups.
3. To compare and measure the amount of bone fill in both the groups following therapy.

PERIODONTAL REGENERATION

Melcher AH (1976)[13] postulated biological concepts at the base of periodontal regeneration. According to that periodontal structures are subdivided in to four compartments (gingival corium, periodontal ligament, cementum, and bone) and the nature of the new attachment following periodontal surgery was determined by the cells repopulating the root surface.

Caton JG *et al.,* (1993)[14] defined periodontal regeneration as healing after periodontal treatment that results in the restoration of the attachment apparatus, namely, cementum, alveolar bone and periodontal ligament.

Park JB (2009)[15] reported that bone regeneration is a complex repair process that involve osteoinduction, osteogenesis and the osteoconductive matrix which creates an appropriate environment and induces the differentiation of undifferentiated cells to make required structures.

Ivanovski S (2009)[5] explained the biological principle of periodontal regeneration that it is based on the concept that remaining healthy cells and cells attracted to the healing site have the potential to promote regeneration. To achieve periodontal regeneration progenitor cells of periodontal ligament and progenitor cells of bone must migrate, proliferate and mature in the healing site.

EFFECT OF OPEN FLAP DEBRIDEMENT ON PERIODONTAL REGENERATION

Froum *et al.,* (1983)[16] in a clinical and histological study demonstrated the healing response of the periodontium after periodontal flap and debridement

procedures found a significant positive correlation between gain in attachment, and osseous fill.

Heitz *et al.*, (2002)[17] in a systemic review evaluated the efficacy of scaling and root planing alone and scaling and root planning combined with flap procedure in which both the procedures showed effective in the treatment of chronic periodontitis in terms of attachment level gain and reduction in gingival inflammation. Open flap debridement results in greater PPD reduction and clinical attachment gain when compared with scaling and root planing alone.

EFFECT OF BONE GRAFT ON PERIODONTAL REGENERATION:

Reconstructing intraosseous defects produced by periodontal diseases with the use of bone grafts dates back to Hegedus in 1923.

Nabers *et al.*, (1965)[18] used shavings of cortical bone removed by hand chisels during osteoplasty and ostectomy was used to treat intra bony defects. This study showed successful treatment results with autogenous bone transplantation with significant coronal increase in bone height.

Robinson *et al.*, (1969)[19] used osseous coagulum and bone blend in the treatment of intra bony defects and compared to open flap debridement alone. The use of this auto graft in self-contained defects appear to result in improved levels of clinical attachment after healing.

Garret *et al*., (1994)[20] explained Bone grafts and other bony substitutes are the derivatives of bone or non-osseous materials and showed that the use of bone autografts and allografts in periodontal therapy found more bone formation in grafted site.

Camelo *et al.*, (1998)[21] demonstrated that autogeneous bone grafts supports the formation of new attachment apparatus.

Flemmig *et al.*, (1998)[22] in the meta analysis study revealed that superior gain in bone fill with demineralized bone allograft.

Giannoudis *et al.*, (2005)[23] in an overview of bone substitute explained hydroxyapatite as the main component of bone and the alloplastic HA ceramics have a stoichiometry similar to that of bone mineral.

HYDROXYAPATITE (HA) IN BONE REGENERATION:

Bagambisa *et al.*, (1993)[24] demonstrated the mechanisms and structure of the bond between bone and hydroxyapatite ceramics seems to involve dissolution/ reprecipitation phenomena which is believed to be the first morphological evidence of epitaxial growth involvement in the formation of this bond.

Okumura *et al.*, (1997)[25] analysed the bone-bonding property of hydroxyapatite ceramics (HA) in which de novo bone formation was observed primarily on the HA surface without fibrous tissue interposition after the subcutaneous implantation of marrow stromal stem cells.

Ogilvie A *et al.*, (1987)[26] found within 6 months of implantation of HA in the periodontal defect site resulted in osteoid formation resulting in firm bone bonding to the HA surface with the formation of small apatite crystals appeared in the centre of the aggregates between the relatively large crystals of synthetic hydroxyapatite.

Pradeep AR *et al.*, (2012)[27] in a randomized controlled clinical trial of 9 months investigation showed the superior regenerative effects observed with HA combined with an open flap debridement (OFD) group.

PHARMACOLOGICAL COMPOUNDS USED IN PERIODONTAL REGENERATION:

NSAIDs:

Tripton *et al.*, (2003)[28] described that Non-steroidal anti-inflammatory drugs (NSAIDs) in periodontal disease treatment accomplices the control of prostaglandin E2 (PGE2) by inhibiting cyclooxygenase-2 (COX-2) enzyme. Higher of the expression of PGE2 increases gingival inflammation and alveolar bone loss.

Cottrell *et al.*, (2010)[29] explained the effect of Non-Steroidal Anti-Inflammatory drugs on bone Healing. The levels of pro-inflammatory mediator's decrease, with the use of NSAIDs that may limit the host-mediated alveolar bone destruction observed in periodontitis and peri-implant disease. Non-specific and cyclooxegenase-2 (COX-2) selective non-steroidal anti-inflammatory drugs (NSAIDs) function by inhibiting the cyclooxygenase iso-enzymes and effectively reduce pain and inflammation attributed to acute or chronic musculoskeletal pathologies.

BISPHOSPHANATES:

Parfitt *et al.*, (1994)[30] demonstrated that the efficacy of Bisphosphanates (BPs) to inhibit the osteoclastic bone resorption has led to their application in periodontal diseases for preventing the alveolar bone loss.

Goziotis *et al.*, (1995)[31] in an *in vitro* study reported that Etidronate was capable of inhibiting mineralization reversibly which in turn stimulates osteoid formation.

Z Akram *et al.*, (2017)[32] assessed the efficacy of bisphosphonate therapy as an adjunct to scaling and root planning in the management of periodontitis and the meta analysis showed statistically significant probing depth reduction, clinical attachment level gain and bone defect fill for bisphosphonate group.

TETRACYCLINE:

Golub *et al.*, (1994)[33] demonstrated the ability of tetracycline to inhibit osteoclasts, neutrophils, and matrix metalloproteinases (MMPs) due to their anti-collagenase and anti proteolytic property. It has been shown to inhibit osteoblast-derived collagenases and have a modifying effect on osteoclast.

Caton *et al.*, (2011)[34] studied the therapeutic effect of doxycycline hyclate strongest collagenase inhibitor of tetracyclines as 20 mg taken twice daily for up to 9 months exhibited inhibition of osteoclast and MMP's, rather than by any antibiotic effect.

AZITHROMYCIN:

Hirsch R *et al.*, (2012)[35] reviewed the use of azithromycin in the treatment of advanced periodontal diseases has found better results and also it could found to have a triple role in the resolution and treatment of periodontal diseases such as anti-inflammatory activity, suppressing periodontopathogens and healing through persistence at low levels fibroblasts and macrophages in periodontal tissues.

Generally, the azithromycin lead to blocking the fibroblast proliferation and collagen synthesis MMP-2, which results in bone regeneration.

PARATHYROID HORMONE (PTH):

P Aggarwal *et al.*, (2012)[36] described the fundamental mechanism of PTH action at the molecular level, as well as in experimental animals and in humans. At doses tolerated by humans showed increased bone strength and able to prevent bone fractures, from continuous use that may lead to bone loss.

Valderrama *et al.*, (2010)[37] have demonstrated in an *in vitro* and *in vivo* studies that the intermittent administration of PTH induced the anabolic effects of cancellous and cortical bone, enhancing bone mass with increased mechanical strength of it.

ESTROGEN:

Ling L *et al.*, (2016)[38] investigated the effects of estrogen on the bone regeneration potential of periodontal ligament stem cells (PDLSCs) derived from osteoporotic rats and seeded on a collagen-based composite scaffold enhances the bone regeneration potential of PDLSCs derived from osteoporotic rats and seeded on nano-hydroxyapatite/collagen/poly Llactide (nHAC/PLA).

STATINS:

Horiuchi N *et al.*, (2006)[39] Statins are reported to have positive effects on bone metabolism by regulating various signaling pathways. The hypolipidemic effects of statins indirectly favour their osteogenic effects through the up-regulation of pro-osteogenic factors like bone morphogenic protein -2 (BMP-2) and vascular endothelial growth factor (VEGF).

Akira E (1970)[40] discovered Compactin first product of natural origin which could inhibit hydroxy methylglutaryl-CoA (HMG-CoA) reductase which is the rate limiting enzyme in the cholesterol synthesis pathway.

Mundy *et al.*, (1999)[41] reported stimulatory effects of statins on bone formation in rodents and increased new bone volume in mouse calvaria and this was brought about by inducing BMP-2 and Transforming growth factor-β (TGF-β) in osteoblasts.

Cunha CJ *et al.*, (2006)[42] were first explored the association between statin use and chronic periodontitis.

Goes P *et al.*, (2010)[43] evaluated the effect of atorvastatin on alveolar bone loss in rats and concluded atorvastatin was able to prevent alveolar bone loss seen on a ligature induced periodontitis model.

Lindy *et al.*, (2008)[44] investigated the pleotropic and anti-inflammatory effects of statins on periodontal tissue and concluded that patients on statins medication exhibit fewer signs of periodontal inflammatory injury than subjects without statin regimen.

Pleiotropic effects of statins

Blanco *et al.*, (2003)[45] studied about the anti-inflammatory and immunomodulatory effects of statins. In addition to the marked reduction in cardiovascular mortality statin therapy could be related endothelial dysfunction, a reduction in blood thrombogenicity, anti-inflammatory properties and immunomodulatory action which are attributed by the inhibition of isoprenoid synthesis.

Whitaker *et al.*, (2017)[46] analysed *in vitro* the antimicrobial effect of simvastatin on streptococci commonly found in the mouth. Simvastatin has efficacy against these specific strains of bacteria at concentrations slightly less than the observed MIC's of 15.6–7.8 μg/ml, which compares favorably with reported values for topical agents such as essential oil, chlorhexidine gluconate, and triclosan.

Zeiser *et al.*, (2018)[47] reviewed the immunomodulatory effects of statins. The synergistic action of statins is attributed mainly through inhibition of protein geranyl geranylation and protein farnesylation and the direct effects of statins on T cells is through calcium influx and IL-2 production.

Immunomodulatory effects of statins

Pahan K (2006)[48] reviewed the therapeutic effects of statins on periodontitis which are mediated through inhibition of signaling molecules involved in the expression of proinflammatory cytokines like interleukin-1 beta (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α).

Lazzerini *et al.*, (2013)[49] evaluated the effect of rosuvastatin on IL-6 production by human osteoblasts and revealed rosuvastatin decreases IL-6 production by osteoblasts and proved the inhibitory action of statins on osteoclast function which is beneficial in bone formation.

Cicek *et al.*, (2016)[50] evaluated the anti-inflammatory effects of statins in hyperlipidemic patients have also been demonstrated by increased IL-10 levels in gingival crevicular fluid (GCF).

Antioxidant effects of statins

Gazzerro *et al.*, (2012)[51] in a review explained the antioxidant effect of statins which is responsible for its various pleiotropic effects. Simvastatin has the potentiality to inhibit the major oxidant like Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase been reported to be most effective in this regard.

Swertz *et al.*, (2017)[52] demonstrated in experimental periodontal rats, simvastatin significantly reduced oxidative stress and increased bone fills.

EFFECTS OF SIMVASTATIN ON PERIODONTAL REGENERATION

***In vitro* studies**

Maeda *et al.*, (2001)[53] evaluated the effects of simvastatin on osteoblastic differentiation in non-transformed osteoblastic cells (MC3T3-E1). The mRNA expression of ALP, BMP-2 and Type 1 collagen throughout the culture period and markedly inhibited the gene expression of collagenase-1. Results indicated the anabolic effects on bone through the promotion of osteoblastic differentiation markers.

Baek *et al.*, (2004)[54] postulated the actions of simvastatin on bone metabolism in their experimental and epidemiological study which demonstrated the stimulatory effects of simvastatin on bone formation through osteoblastic differentiation. 10^{-6} M of simvastatin increased the ALP activity and enhanced the osteocalcin mRNA expression level in human bone marrow stromal cells.

Yasawa *et al.*, (2005)[55] analysed the effects of simvastatin on cell proliferation and osteoblastic differentiation of human periodontal ligament cells. Cultured periodontal ligament (PDL) cells with 10^{-8} M of simvastatin for 7, 14 and

21 days stimulated alkaline phosphatase (ALP) activity and osteopontin content after 7 days. Simvastatin also enhanced cell proliferation and metabolism of PDL cells dose dependently after 24 hours.

Ruiz-Gazpa *et al.*, (2007)[56] assessed the gene expression of collagen type 1, osteocalcin in primary human osteoblasts and MG-63 cultures. The quantification of mRNA expression using real time polymerisation chain reaction (PCR) after incubation for 24hs with simvastatin showed statistically significant increase in Collagen 1, Osteocalcin and BMP. These findings supports the bone forming action of simvastatin through BMP 2 pathway.

Chen *et al.*, (2010)[57] investigated about the events of osteoblastic differentiation induced by statins and hypothesised that simvastatin promotes viability of osteoblast and differentiation induced through Ras/Smad/Erk bone morphogenic protein (BMP-2) signalling pathway. Results showed a positive effects on the metabolism of osteoblast via membrane bound Ras/Smad/Erk/BMP-2 pathway.

Lui *et al.*, (2012)[58] evaluated the effects of simvastatin on the osteogenic behaviour of alveolar osteoblast and PDL cells. MTT assay was used to assess viability of cells after 24, 48 and 72 h after incubation with simvastatin. The mRNA expression of these markers are induced at a concentration of 1nM of simvastatin in alveolar osteoblast and the expression was induced in PDLs at higher concentration of 100nM of simvastatin.

Suthanthiran TK *et al.*, (2012)[59] showed that simvastatin enhanced cell metabolism dose dependently at 24-h time and the maximum effect was obtained at a concentration of 1.5 mg of simvastatin. These results indicate that collagen with

1.5 mg simvastatin exhibits positive effect on cell metabolism of human osteoblast-like SaOS-2 cells.

Niu *et al.*, (2015)[60] investigated the effects of simvastatin on the bone differentiation and immunological characteristics of bone marrow mesenchymal stem cells. 1.0 µm/ml of simvastatin enhanced alkaline phosphatase activity, mRNA expression levels of osteocalcin, bone sialoprotein signified the capacity of simvastatin to promote osteogenic differentiation of bone-marrow mesenchymal stem cells significantly without causing immunosuppression.

Kamada *et al.*, (2017)[61] examined the effects of statins on gene expression in human dental pulp cells. On day 3 of culturing with simvastatin significantly increased the gene expression of BMP-2 and RUNX2 and suppressed the expression of RUNX2 on day 16. Hence showed the effects of simvastatin on the initial osteogenic differentiation and also on final differentiation through the regulation of the transcription factor.

***In vivo* studies:**

Bradley JD *et al.*, (2007)[62] confirmed the action of simvastatin which stimulates bone formation in an experimental study using a rat model. Simvastatin was found to enhance local bone morphogenetic protein 2, nitric oxide and regional bone formation rate at a concentration of 0.5 mg and showed the negative effect of cyclooxygenase inhibitor on bone growth.

Nyan *et al.*, (2009)[63] assessed the optimum dose of simvastatin that can stimulate bone formation without causing inflammation after combining with α-Tri calcium phosphate (α-TCP). Different doses of simvastatin (0, 0.01, 0.1, 0.25 and

0.5 mg) was combined with α-TCP and applied in 5 mm diameter calvarial defects in adult wistar rats. The study revealed that 0.1 mg of simvastatin when combined with α-TCP is the optimal dose that can stimulate maximum bone regeneration without inducing inflammation.

Rutledge *et al.*, (2011)[64] evaluated the effects of locally injected simvastatin in several human-like clinical situations in a beagle dog model. Demonstrated the ability of simvastatin to induce modest amounts of new bone formation in closed injection sites over a periosteal surface.

Dalcico *et al.*, (2013)[65] investigated the effects of simvastatin on rats subjected to experimental periodontal disease. Suggested that simvastatin prevents inflammatory bone resorption in experimental periodontitis, which may be mediated by its anti-inflammatory and antioxidant properties.

Human studies:

Kinra P *et al.*, (2010)[66] evaluated the effects of simvastatin drug when combined with demineralised freeze dried bone allograft (DFDBA) in the treatment of human periodontal defects. 10^{-8} M of simvastatin was combined with DFDBA and treated 15 patients with infrabony defects and assessed probing pocket depth, clinical attachment level and infrabony defect depth. Combination of DFDBA with simvastatin results significantly greater reduction in probing pocket depth, gain in clinical attachment level and linear defect fill than when the graft is used alone in the treatment of human periodontal infrabony defects.

NS Rao *et al.*, (2013)[67] investigated the effectiveness of 1.2% of simvastatin gel as an adjunct to scaling and root planing (SRP) in the treatment of smokers with

chronic periodontitis. Mean probing depth reduction and mean clinical attachment level gain was greater in the simvastatin group than the control group. Percentage of bone fill in the simvastatin group also significantly improved.

Surve *et al.*, (2015)[68] assessed the efficacy of simvastatin and atorvastatin as an adjunct to scaling and root planing and delivered sub-gingivally in the treatment of chronic periodontitis. 1.2% of simvastatin application significantly reduced the clinical parameters and interleukin α in the GCF.

Martande *et al.*, (2016)[69] compared the efficacy of subgingivally delivered 1.2% Atorvastatin and 1.2% Simvastatin in the treatment of intrabony defects in chronic periodontitis. Simvastatin resulted in greater improvements in clinical parameters with higher percentage of radiographic defect depth reduction.

Zhu *et al.*, (2017)[70] analysed and compared the efficacy of alendronate sodium gel and simvastatin gel in the treatment of mandibular molar furcation involvement. Clinical effect of topical administration of simvastatin gel in the treatment of mandibular molar furcation involvements is more significant than comparing with alendronate sodium gel.

Priyanka N *et al.*, (2017)[71] in a randomised controlled clinical trial investigated the efficacy of 1.2 mg of simvastatin as an adjunct to scaling and root planing in 24 patients with aggressive periodontitis. Clinical parameters included are plaque index, probing pocket depth, clinical attachment level and bleeding index and were recorded at baseline, 3 and 6 months. The results showed significant improvement in clinical parameters in the group treated with simvastatin and this proved the ability of simvastatin in bone formation.

R Ranjan *et al.*, (2017)[72] investigated the effects of in situ application of 1.2 mg simvastatin gel in the surgical management of Intrabony defects in chronic periodontitis patients. Higher amount of decrease in gingival index and pocket depth along with more amount of CAL gain was observed in treatment group than control group. Radiological assessment confirmed that significant intrabony defect fill and percentage fill of original defect in treatment group than controlled group.

CARRIERS USED IN PERIODONTAL REGENERATION:

FIBERS:

Lindhe *et al.*, (1979)[73] first proposed the concept of controlled delivery in the treatment of periodontitis performed to assess the effect of tetracycline, locally administered via hollow fiber devices, Fibers, or thread-like devices, are reservoir-type systems, placed circumferentially into the pockets with an applicator and secured with cyanoacrylate adhesive for the sustained release of trapped drug into the periodontal pocket. Tetracycline filled hollow fibers in the gingival sulcus effect on both microbial count and clinical manifestation of the disease.

Goodson *et al.*, (1979)[74] showed Tetracycline filled hollow fibers in the gingival sulcus has effect on both microbial count and clinical manifestation of the disease.

Addy *et al.*, (1982)[75] demonstrated that Chlorhexidine was released from reservoir fibres over 4 day's *in-vitro* and more than 95% of the drug release occurred in the first 24 hours.

Higashi *et al.*, (1990)[76] developed a controlled release insert for topical chemotherapy in periodontal disease. The effect of ofloxacin release profile can be

used as a controlled release insert and found that it was a suitable pharmaceutical preparation for periodontal chemotherapy.

Sadaf *et al.*, (2013)[77] showed in periodontal therapy, statistically significant reduction in all the clinical parameters when tetracycline fibers were used.

GEL FORM:

Stoltze *et al.*, (1992)[78] demonstrated that the bioavailability of drugs administered in gel form exceeds than drugs administered in tablet form.

Jeong *et al.*, (1994)[79] reported that tetracycline gel results in reduction in probing pocket depth, which are not significantly different from the results obtained from scaling and root planing. Single application was not sufficient to provide suitable results.

MICROCAPSULES:

Baker *et al.*, (1998)[80] suspended tetracycline containing microcapsules in a Pluronic F 127 gel. This material forms a gel at body temperature to hold the microcapsules in periodontal pockets for the duration of the treatment. They showed that after administration of the gel containing microcapsules to periodontal pockets in monkeys, the concentrations in the GCF was maintained at effective levels for 3-4 days.

STRIPS/FILMS:

Golomb *et al.*, (1984)[81] developed a sustained release device containing metronidazole for insertion within periodontal pockets and to examine the release kinetics *in vitro* and *in vivo.*

Strips are thin and elongated matrix bands in which drugs are distributed throughout the polymer. Embedding metronidazole in ethyl cellulose provide sustained release of the drug within the periodontal pocket for three days.

Films are most widely used intra pocket drug delivery device prepared either by solvent casting or direct milling. Films are matrix type of drug delivery device in which drug were distributed throughout matrix and drug release occurs by erosion, matrix dissolution or drug diffusion.

Kyun *et al.*, (1990)[82] demonstrated by embedding minocycline in monolithic film prepared from polycaprolactone (PCL) is feasible to obtain sustained release of the drug within the periodontal pocket.

CALCIUM PHOSPHATE CEMENTS:

Guo *et al.*, (2009)[83] explained about the most recent advances in tissue engineering is the use of calcium phosphate cements (CPC). It is a potential bone substitute. This biomaterial has a structure and composition similar to the mineral portion of the bone. CPC has been considered as a suitable material to develop scaffolds for bone tissue engineering.

COLLAGEN MEMBRANE:

Albu *et al.*, (2011)[84] proved collagen biomaterials as matrices, hydrogels, and composites to be effective in tissue repairing, in guiding functional angiogenesis and in controlling stem cell differentiation.

Sahithi *et al.*, (2013)[85] explained that collagen possess properties such as biocompatibility, absorbability on biological membranes, no antigenicity, low toxicity, synergism with other bioactive compounds etc.

HYDROXYAPATITE:

Deligianni *et al.*, (2001)[86] studied the response of human bone marrow cells when cultured on HA surface and evaluated the effect of surface roughness of HA on the cell adhesion, proliferation, differentiation and detachment strength. It was shown that with increased surface roughness there was increased cell adhesion, proliferation and detachment strength.

Wang *et al.*, (2007)[87] prepared nano hydroxyapatite composite scaffold to investigate its efficacy in bone tissue engineering. The results of this study concluded that nano-hydroxyapatite (n-HA) scaffold exhibit good biocompatibility and extensive osteoconductivity with host bone which indicates that the n-HA fulfil the basic requirements of bone tissue engineering scaffold and have the potential to be applied in orthopaedic, reconstructive and maxilla facial surgery.

Li *et al.*, (2012)[88] reviewed that hydroxyapatite (HA) exhibits excellent biocompatibility with soft tissues such as skin, muscle and in gingiva. Synthetic hydroxyapatite has been used widely in bone repair, bone augmentation. Nano

hydroxyapatite may be a potential biomaterial due to its good biocompatibility and bone integration ability.

AR Pradeep *et al.*, (2012)[27] in a randomized controlled clinical trial, evaluated the effectiveness of autologous platelet rich fibrin (PRF) with PRF+HA in treatment of intrabony defects in chronic periodontitis subjects. Clinical and radiological parameters such as probing depth (PD), clinical attachment level (CAL), infrabony defect depth and % defect fill were recorded at baseline and 9 months postoperatively. It was concluded that HA when added to PRF increases the regenerative effects observed with PRF in the treatment of human three wall intrabony defects.

IN VITRO STUDY

CHEMICALS

DMEM (Sigma-Aldrich, USA), FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin, Streptomycin and Amphotericin B were purchased from Biochrome, Germany. Ethanol and all other chemicals were purchased from SRL, India and were of analytical grade. Simvastatin was purchased from Sigma-Aldrich (Fig: 1).

CELL LINE

Human osteosarcoma MG-63 cells were procured from National Centre for Cell Sciences (NCCS), Pune, India.

CULTURE REAGENTS

1. **DMEM (pH 7.4):** 10 g of DMEM was dissolved in 800 ml of sterile distilled water. To this solution, 32.5 ml of 7.5% sodium bicarbonate solution was added followed by addition of 10 ml penicillin/streptomycin-amphotericin B solution. The pH was adjusted to 7.4. The final volume was made up to 1 L with distilled water. Then the medium was sterile filtered (0.22) and stored at 4°C.
2. **DMEM with 10% FBS:** 10 ml of FBS was made up to100 ml using sterile DMEM. It was stored in a sterile container in cool and aseptic condition.
3. **BSA (100x):** 10 g of bovine serum albumin (BSA) was dissolved in 100 ml sterile distilled water. It was sterile filtered (0.22) and freezed. Freeze-thaw cycle does not harm the carrier protein. 0.1% BSA: 1 ml of 100x BSA was made up to 100 ml with plain DMEM.

4. **Phosphate buffered saline (PBS; pH 7.4):** 0.63 g of sodium phosphate monobasic (NaH_2PO_4), 0.17 g of sodium phosphate dibasic (Na_2HPO_4) and 4.5 g of sodium chloride (NaCl) were 55 dissolved in 500 ml of sterile distilled water. The pH was then adjusted to 7.4 using 1 N HCl and 1 N NaOH, sterile filtered (0.22 µ) and then stored in a sterile container.

5. **Trypsin-EDTA solution:** Commercially available trypsin (0.25% trypsin in 0.05% EDTA) was procured in ready to use form.

CULTURE OF MG-63 CELLS

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphoteracin B (2.5 µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

ISOLATION OF TOTAL RNA (TRIzol method)

Total RNA was isolated using the total RNA isolation kit according to the manufacture instruction (Invitrogen- Product code 10296010). Addition of TRIzol solution causes the disruption of cells and the release of RNA. Chloroform extraction following centrifugation, exclusively in the aqueous phase whereas proteins are in the interphase and organic phase. On mixing with isopropanol, RNA gets precipitated as a white pellet on the side and the bottom of the tube.

After attaining 70% confluency of cells in 6 well plate (approximately 4×10^5 cells), the cells were treated with simvastatin and incubated for 24 hours and 14 days. A set of untreated control were also incubated at 37^0C for 24 hours in a CO_2

incubator. After incubation DMEM media was removed aseptically and 200 µL of TRIzol reagent was added to culture well plate and incubated for 5 minutes. The contents were then transferred to a fresh sterile eppendorf tube. 200 µL of chloroform was added and shaking was done vigorously for 15 seconds and incubated for 2-3 minutes at room temperature, followed by centrifugation at 14000 rpm for 15 minutes at 40°C. The aqueous layer was collected and 500 µL of 100% isopropanol was added. It was incubated for 10 minutes at room temperature and then centrifuged at 14000 rpm for 15 minutes at 40^{o}C. The supernatant was discarded and pellet thus obtained was washed with 200 µL of 75% ethanol (Merck). It was then centrifuged at 14000 rpm for 5 minutes at 40^{o}C in a cooling centrifuge (Remi CM12). The RNA pellet was dried and suspended in TE buffer.

GENE EXPRESSION ANALYSIS BY RT-qPCR

Real time quantitative PCR assay is a development of the invasive signal amplification assay that combines two signal amplification reactions in series to generate and amplify a fluorescent signal in the presence of the correct target sequence (Light cycler 96, Roche, Switzerland) (Fig: 2). The assay is sensitive down to sub-attomole levels and may well become a method of choice. In qPCR assay quantify target nucleic acids accurately.

Total RNA was extracted using TRI Reagent (Sigma). The purity and the concentration of total RNA was determined. Template complementary DNA was synthesized using the cDNA preparation kit (Thermoscientific, Product code-AB1453A, Verso cDNA Synthesis kit) (Fig: 3). Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (Applied Biosystem, Life technologies) (Fig: 4).

The various steps involved, the time taken and temperature of each step is given in the table.

Steps	Time required	Temperature
Initial activation step	2 minutes	95°C
3 step cycling		
Denaturation	10 seconds	94°C
Annealing	1 minutes	55°C
Extension	1 min/kb	72°C
Number of cycles	40 cycles	68°C
End of PCR cycling	indefinite	4°C

The primer sequences used were summarized.

Oligo Name	Forward		Reverse	
	Sequence (5' ->3')	Tm	Sequence (5' ->3')	Tm
H-GAPDH	ACTCAGAAGACTGTGGATGG	57.3	GTCATCATACTTGGCAGGTT	55.3
H-RUN X2	TCTTAGAACAATTCTGCCCTTT	55.3	TGCTTTGGTCTTGAAATCACA	54.0
H-Osteopontin	TTGCAGTGATTTGCTTTTGC	53.2	GTCATGGCTTTCGTTGGACT	57.3

LOADING OF HYDROXYAPATITE WITH SIMVASTATIN [89]

Materials Required:

- Calcium Chloride-1.5M
- Trisodium citrate-0.2M
- Simvastatin-1.5 mg/mL[59]
- Disodium hydrogen phosphate
- Distilled water

Procedure:

Hydroxyapatite was prepared by co-precipitation method. 50 mL of Calcium chloride (1.5 M) solution was prepared with distilled water. 10 mL of fresh conjugate base prepared with 0.2 M Trisodium citrate with then introduced. The solution was stirred for 15 minutes. After stirring, 0.1 mL of Simvastatin (for 100 mL) and 50 mL of Disodium hydrogen phosphate was added drop wise from a burette. The reaction was allowed to proceed under stirring for 24 hour. The resulting suspension obtained were washed with distilled water, centrifuged and lyophilized. The hydroxyapatite thus obtained was collected and stored for future use.

CLINICAL STUDY

The participants for this study were selected from the outpatient section of the Department of Periodontics, J.K.K. Nattraja Dental College and Hospital, Komarapalayam, Tamilnadu, India. Ten patients, aged 20 to 50 years, diagnosed with chronic periodontitis with the probing depth of ≥ 5 mm were enrolled in this study. Patients were instructed about the utility and design of this clinical trial and informed signed consent were obtained.

INCLUSION CRITERIA

1. Patients age limit of 20-50 years of both genders.

2. Probing depth of ≥ 5 mm as assessed by William's graduated probe.

3. Clinical attachment level (CAL) ≥ 4 mm.

4. Patients with minimum of two contralateral infra bony defects.

EXCLUSION CRITERIA

1. Patients with known systemic diseases, short and long term therapies.

2. Previous periodontal therapy.

3. Known drug allergy.

4. Teeth with traumatic occlusion.

5. Smokers.

6. Pregnancy and lactating women.

STUDY DESIGN

The study was designed as a single blinded randomized controlled split mouth clinical trial for a period of 6 months. The study population comprised of 10 subjects and a total of 20 infrabony defects.

GROUP CRITERIA

Group 1 : Infrabony defects treated with hydroxyapatite (Control sites).

Group 2 : Infrabony defects treated with simvastatin in combination with hydroxyapatite (Test sites).

CLINCAL PARAMETERS

The following variables were measured at baseline, 3 months and 6 months post-operative period.

1. Gingival Index (GI) - (Loe and Silness 1963)

2. Plaque Index (PI) - (Silness and Loe 1964)

3. Oral Hygiene Index - Simplified (OHI-S) - (JC Green and JR Vermillion 1964)

4. Probing pocket depth (PPD) – deepest probing depth was measured

5. Clinical Attachment Level (CAL)

Gingival Index (GI) - (Loe and Silness 1963)

The tissues surrounding each tooth are divided into four gingival scoring units: Distal facial papillae; Facial margin; mesial facial papillae; Entire lingual gingival margin.

0	No inflammation
1	Mild inflammation, no bleeding elicited on probing.
2	Moderate inflammation, bleeding on probing
3	Severe inflammation

The scores of the four areas of the tooth can be summed and divided by 4 to get the GI for the tooth.

0.1-1.0	Mild inflammation
1.1-2.0	Moderate inflammation
2.1-3.0	Severe inflammation

Plaque Index (PI) - (Silness and Loe 1964)

The surfaces examined are the four gingival areas of the tooth: Disto-facial, Facial, Mesio-facial and Lingual.

Scoring criteria

0	No plaque in the gingival area.
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be recognized only by running a probe across the tooth surface.
2	Moderate accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surface that can be seen by the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.

The scores of the four areas of the tooth can be summed and divided by four to get the PI for the tooth. A score from

Good	0.1-0.9
Fair	1.0-1.9
Poor	2.0-3.0

Oral Hygiene Index - Simplified (OHI-S) - (JC Green and JR Vermillion 1964)

Teeth selection

In the posterior portion of the dentition, the first fully erupted tooth distal to the second bicuspid (15), usually the first molar (16) but sometimes the second (17) or third molar (18), is examined. The buccal surfaces of the selected upper molars and the lingual surfaces of the selected lower molars are inspected.

In the anterior portion of the mouth, the labial surfaces of the upper right (11) and the lower left central incisors (31) are scored. In the absence of either of these anterior teeth, the central incisor (21 or 41 respectively) on the opposite side of the midline is substituted.

Debris Index – Simplified (DI-S)

Scores	Criteria
0	No debris or stain present
1	Soft debris covering not more than one third of the tooth surface, or presence of extrinsic stains without other debris regardless of surface area covered
2	Soft debris covering more than one third, but not more than two thirds, of the exposed tooth surface.
3	Soft debris covering more than two thirds of the exposed tooth surface.

Calculus Index – Simplified (CI-S)

Scores	Criteria
0	No calculus present
1	Supragingival calculus covering not more than third of the exposed tooth surface.
2	Supragingival calculus covering more than one third but not more than two thirds of the exposed tooth surface or the presence of individual flecks of subgingival calculus around the cervical portion of the tooth or both.
3	Supragingival calculus covering more than two third of the exposed tooth surface or a continuous heavy band of subgingival calculus around the cervical portion of the tooth or both.

DI-S = (The buccal-scores) + (The lingual-scores) / (Total number of examined buccal and lingual surfaces).

CI-S= (The buccal-scores) + (The lingual-scores) / (Total number of examined buccal and lingual surfaces).

OHI-S = DI-S + CI-S

Interpretation:

Good	0.0-1.2
Fair	1.3-3.0
Poor	3.0-6.0

Probing Pocket Depth (PPD)

Probing pocket depth was measured at desired sites with William's periodontal probe. The probe was inserted parallel to the tooth surface until resistance was felt and the readings were recorded to the nearest millimeter marking from the gingival margin to the base of the pocket. Acrylic stents were used to standardize the path of insertion and angulations of the probe.

Clinical Attachment Level (CAL)

The level of attachment is the distance between the base of the pocket and cemento-enamel junction (CEJ). The distance from the CEJ (if CEJ is not clinically detected, the coronal border of the stent was used) to the base of the pocket was measured. The readings were noted.

Occlusal stents were fabricated with cold cure resin on patient model cast for positioning and measuring probe markings were fabricated with cold cured acrylic resin on patient cast model. Notch was made on the stent to permit and standardize the entry of periodontal probe into the pocket. The occlusal stent was made to cover the occlusal surfaces of the tooth being treated and occlusal surface of one tooth in mesial and distal directions. The stents were also extended apically on the buccal and lingual surfaces to cover the coronal third of teeth involved.

Radiographic parameters

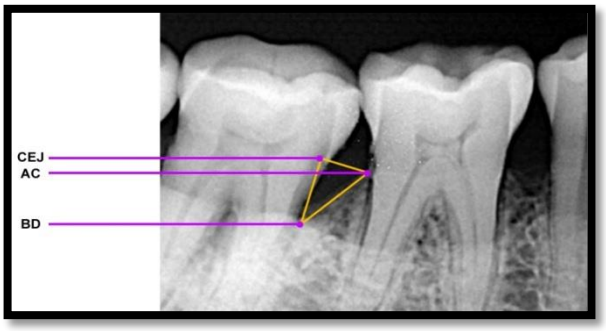
Radiographs were taken using the (Kodak) RVG 5200 System by the standardized paralleling cone technique with the digital Radiovisiography (RVG).

The following anatomical landmarks of the infrabony defect were identified on the radiograph images based on criteria by **Bjorn *et al.*,(1969)**[90] and **Schei *et al.*, (1959).**[91]

1. CEJ: The cemento-enamel junction of the tooth with the infrabony defect.

2. AC: The most coronal position of the alveolar bone crest of the infrabony defect when it touches the root surface of the adjacent tooth before treatment, the top of the crest.

3. BD: The most apical extension of the intrabony destruction where the periodontal ligament space still retained its normal width before treatment, the bottom of the defect.



Pre surgical therapy

For all the selected patients, routine blood investigations were taken. Initial therapies consisted of scaling and root planing, oral hygiene instructions, diet counselling and medications. Three weeks following phase I therapy, re-evaluation was performed.

Surgical procedures

Following pre surgical phase periodontal surgical procedures were performed. The patient as anaesthetized using lignocaine 2% with 1:1,00,000 epinephrine. Using Bard parker blade number 15, buccal and lingual sulcular incisions were made to elevate the mucoperiosteal flaps. Pocket epithelium and degranulation tissue from the inner surface were removed gently. Thorough soft tissue debridement and root planing were accomplished with Hu-Friedy curettes and washed with saline.

Surgical Procedure (Group 1)

Hydroxyapatite was placed in the infrabony defect. Then the flaps were repositioned to accomplish complete inter proximal closure. Then the flaps were approximated with simple interrupted suture using 3-0 non absorbable silk thread. Periodontal dressing (Coe pak) was given. Post-surgical instructions were given to the patient and recalled after one week for suture removal and further follow up.

Surgical Procedure (Group 2)

Simvastatin in combination with hydroxyapatite was placed in the infrabony defect. Then the flaps were repositioned to accomplish complete inter proximal closure. Then the flaps were approximated with simple interrupted suture using 3-0 non absorbable silk thread. Periodontal dressing (Coe pak) was given. Post-surgical instructions were given to the patient and recalled after one week for suture removal and further follow up.

APPENDIX – 1

POST THERAPY INSTRUCTIONS

1. Report immediately on development of any untoward reactions like pain, swelling, hypersensitivity, drug allergy.
2. Avoid intake of any hot and hard foods, not to disturb the operated area with tongue.
3. Report if dressing is dislodged.
4. Avoid brushing the area with periodontal dressing, 1 week from the day of therapy; Use a cotton tip applicator to gently clean the area.
5. Not to use dental floss and toothpicks at the site.
6. Follow up visits have to be done in 1 week, 3 months and 6 months.
7. The patients were asked to perform regular oral hygiene habits by appropriate brushing technique using toothpaste and toothbrush.
8. The patients were instructed to report on the subsequent appointment.

APPENDIX - 2

PROFORMA

OP NO: **CASE NO:**

NAME: **DATE:**

AGE: **SEX:**

OCCUPATION:

ADDRESS:

CHIEF COMPLAINT:

DENTAL HISTORY:

MEDICAL HISTORY:

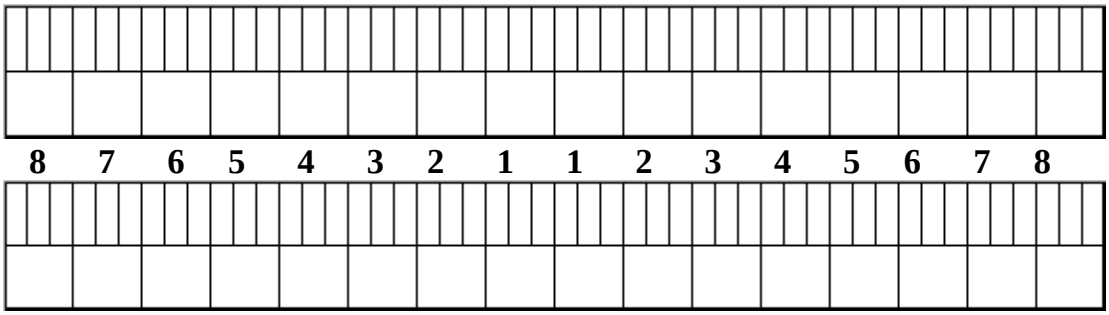
PERSONAL HISTORY:

TEST SITE:

CONTROL SITE:

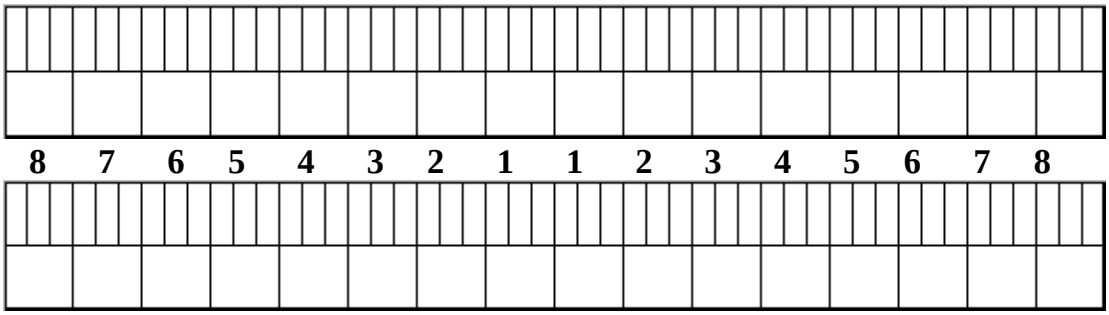
AT BASELINE LEVEL

PLAQUE INDEX (PI) - (SILNESS AND LOE 1964)



SCORE

GINGIVAL INDEX (GI) - (LOE AND SILNESS 1963)

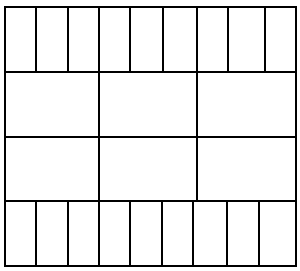


SCORE

ORAL HYGIENE INDEX - SIMPLIFIED (OHI-S) - (JC GREEN AND JR VERMILLION 1964)

DEBRIS INDEX – SIMPLIFIED (DI-S)

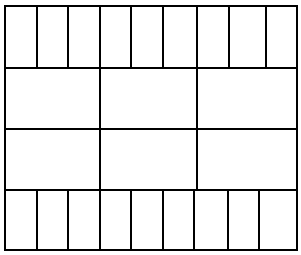
16 11 26



36 31 46

CALCULUS INDEX – SIMPLIFIED (CI-S)

16 11 26



36 31 46

OHI-S SCORE = (DI-S +CI-S):

INTERPRETATION:

AT BASELINE LEVEL

GROUP 1

PROBING POCKET DEPTH – PPD (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CLINICAL ATTACHMENT LEVEL -CAL (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

GROUP 2

PROBING POCKET DEPTH – PPD (mm)

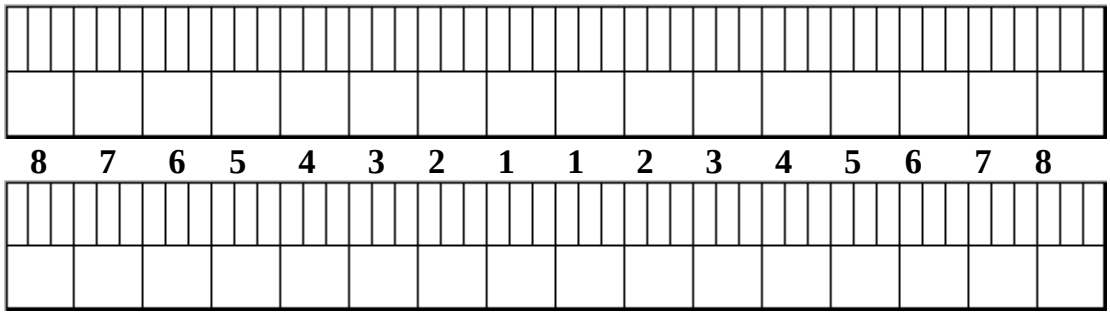
8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CLINICAL ATTACHMENT LEVEL - CAL (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

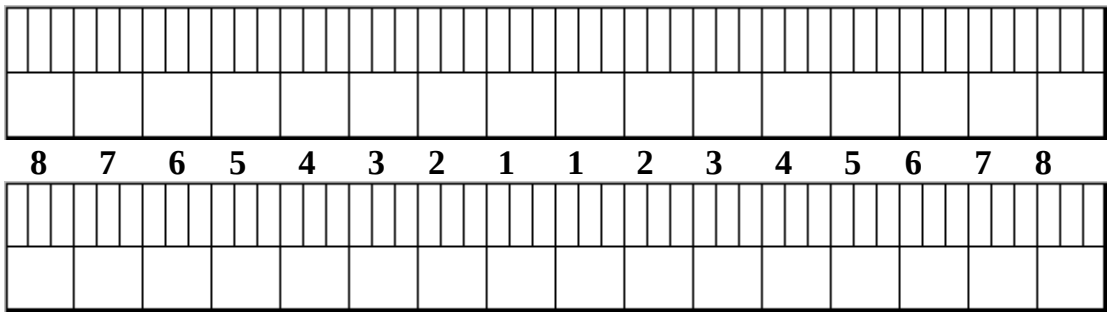
3 MONTHS AFTER TREATMENT

PLAQUE INDEX (PI) - (SILNESS AND LOE 1964)



SCORE ☐

GINGIVAL INDEX (GI) - (LOE AND SILNESS 1963)

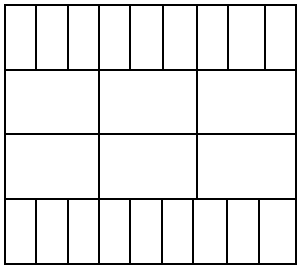


SCORE ☐

ORAL HYGIENE INDEX - SIMPLIFIED (OHI-S) - (JC GREEN AND JR VERMILLION 1964)

DEBRIS INDEX-SIMPLIFIED (DI-S)

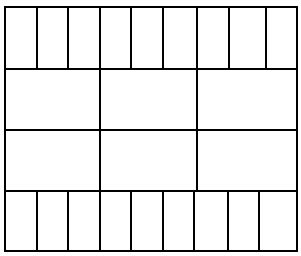
16 11 26



36 31 46

CALCULUS INDEX - SIMPLIFIED (CI-S)

16 11 26



36 31 46

OHI-S SCORE= (DI-S +CI-S):

INTERPRETATION:

3 MONTHS AFTER TREATMENT

GROUP 1

PROBING POCKET DEPTH – PPD (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CLINICAL ATTACHMENT LEVEL - CAL (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

GROUP 2

PROBING POCKET DEPTH – PPD (mm)

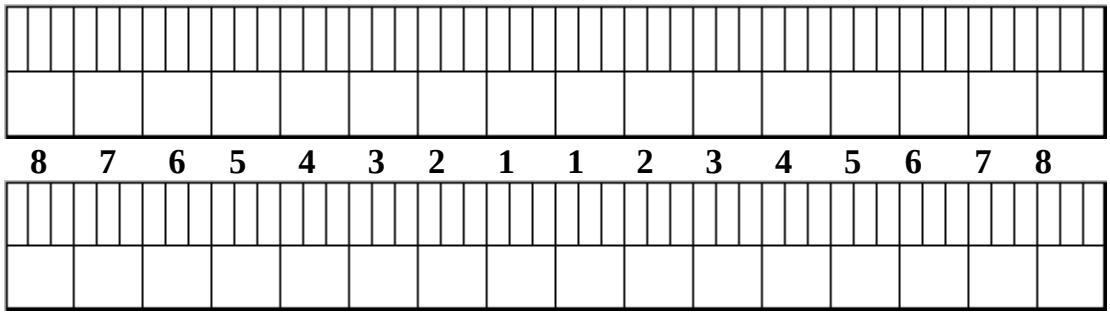
8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CLINICAL ATTACHMENT LEVEL - CAL (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

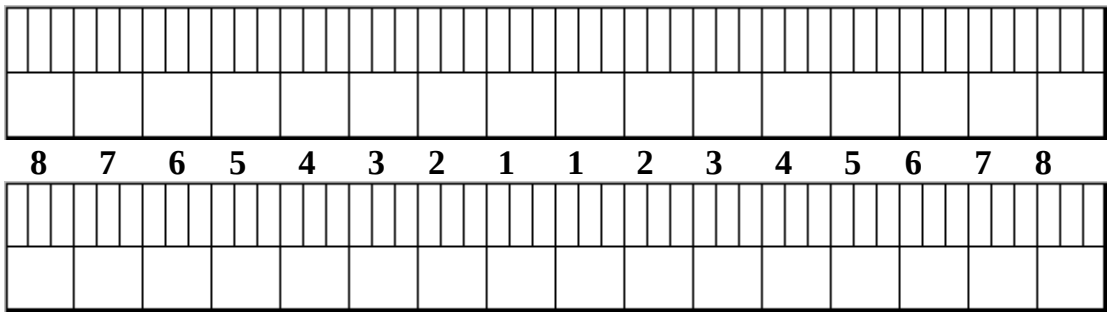
6 MONTHS AFTER TREATMENT

PLAQUE INDEX (PI) - (SILNESS AND LOE 1964)



SCORE ☐

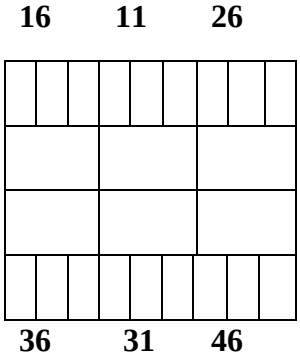
GINGIVAL INDEX (GI) - (LOE AND SILNESS 1963)



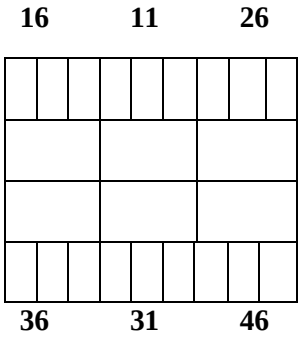
SCORE ☐

ORAL HYGIENE INDEX - SIMPLIFIED (OHI-S) - (JC GREEN AND JR VERMILLION 1964)

DEBRIS INDEX – SIMPLIFIED (DI-S)



CALCULUS INDEX - SIMPLIFIED (CI-S)



OHI-S SCORE = (DI-S+CI-S):

INTERPRETATION:

6 MONTHS AFTER TREATMENT

GROUP 1

PROBING POCKET DEPTH – PPD (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CLINICAL ATTACHMENT LEVEL - CAL (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

GROUP 2

PROBING POCKET DEPTH – PPD (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

CLINICAL ATTACHMENT LEVEL - CAL (mm)

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

J.K.K. NATTRAJA DENTAL COLLEGE & HOSPITAL,

KOMARAPALYAM, DEPARTMENT OF PERIODONTICS

INFORMED CONSENT OBTAINED FROM THE PATIENT

PATIENT NAME:

I have been explained about the nature and purpose of this study in which, I have been asked to participate. I understand that I am free to withdraw my consent and discontinue at any time without prejudice to me or effect on my treatment.

I have been given the opportunity to question about the material and study. I have also given the consent for photographs to be taken at the beginning, during and end of the study. I agree to participate in this study.

I hereby have given the consent to be included in "Evaluation of relative efficacy of simvastatin in combination with hydroxyapatite in the treatment of periodontal infrabony defects."

Place:

Date: **Signature of patient**

APPENDIX – 3

ARAMAMENTARIUM

MATERIALS AND INSTRUMENTS USED FOR PERIODONTAL FLAP SUREGRY

- Gloves
- Mask
- Patient apron
- Chair apron
- Head cap
- Sterile cotton rolls
- Sterile gauze
- Saline
- Kidney tray
- Betadine solution
- Lignocaine
- Injection syringe

DIAGNOSTIC INSTRUMENTS

- Mouth mirror.
- Straight probe.
- Explorer.
- Tweezer.

SURGICAL INSTRUMENTS

- Bard parker handle.
- Bard – parker blade number 11 and 15.
- Periosteal elevator.
- Hu – Friedy Gracey curettes.
- Hu – Friedy universal scaler.
- Hu – Friedy cumin scaler.
- Tissue holding forceps.
- Schugler bone file.
- Scissors.
- 3 - 0 non - absorbable silk suture.
- Simvastatin combined hydroxyapatite powder.
- Hydroxyapatite.
- Plastic spatula.
- Zinc oxide eugenol coe pak.

IN VITRO ANALYSIS

FIG 1: SIMVASTATIN

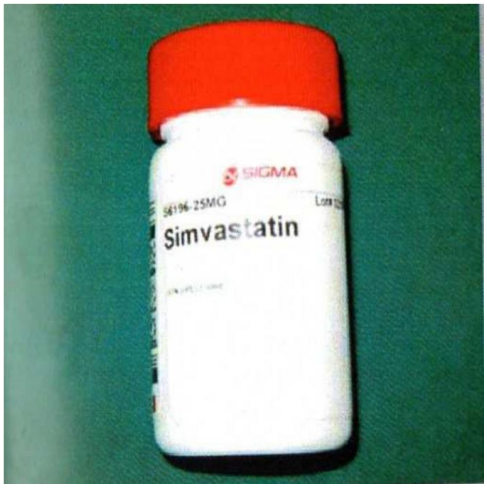


FIG 2: LIGHTCYCLER 96 (ROCHE)

FIG 3: PCR THERMAL CYCLER

FIG 4: SYBR GREEN MIX

FIG 7: SURGICAL INSTRUMENTS USED FOR PERIODONTAL SURGERY

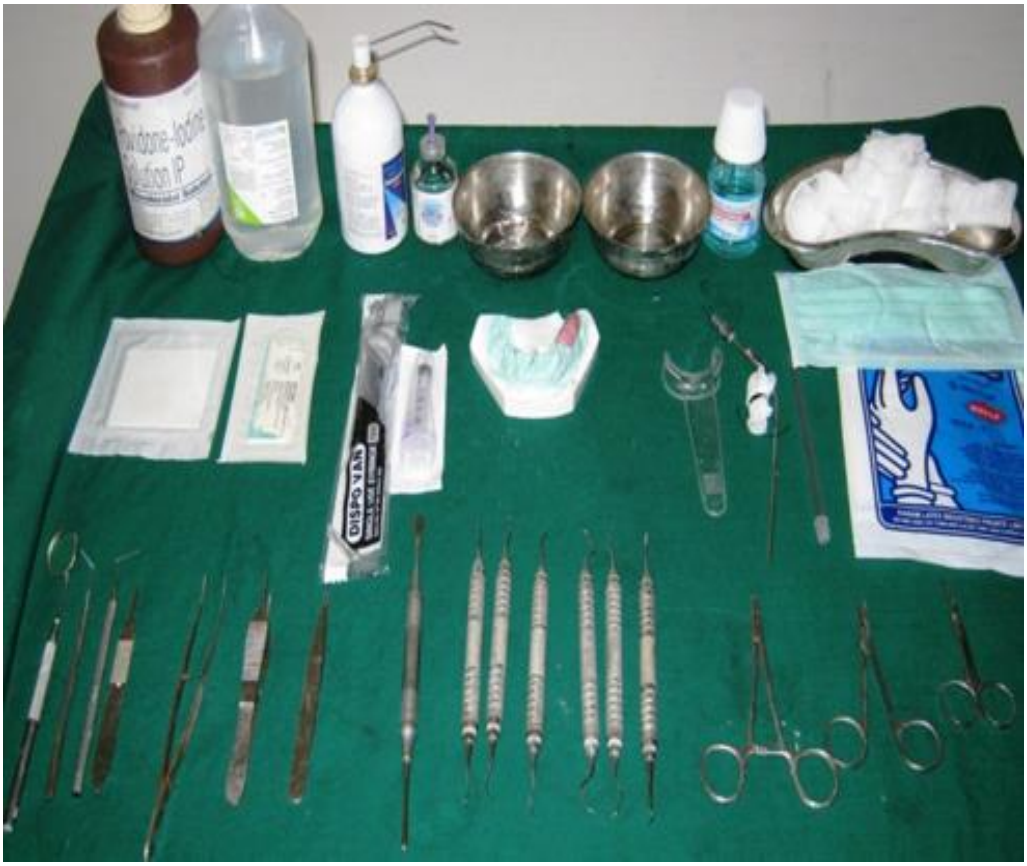


FIG 8: (A) HYDROXYAPATITE,

(B) SIMVASTATIN + HYDROXYAPATITE

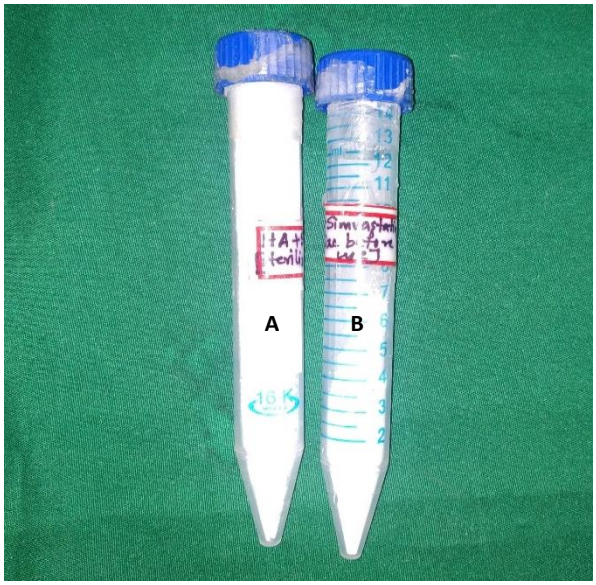
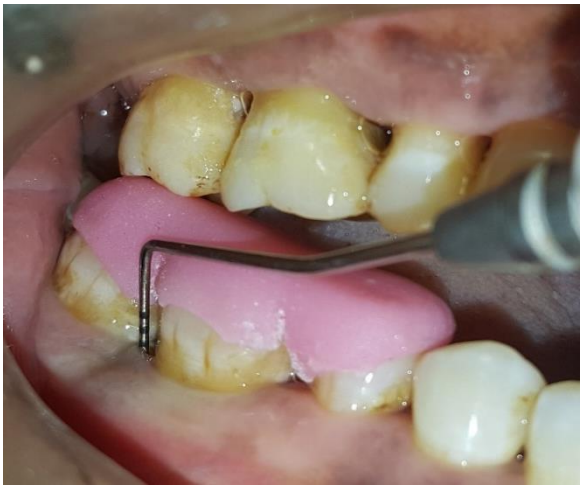


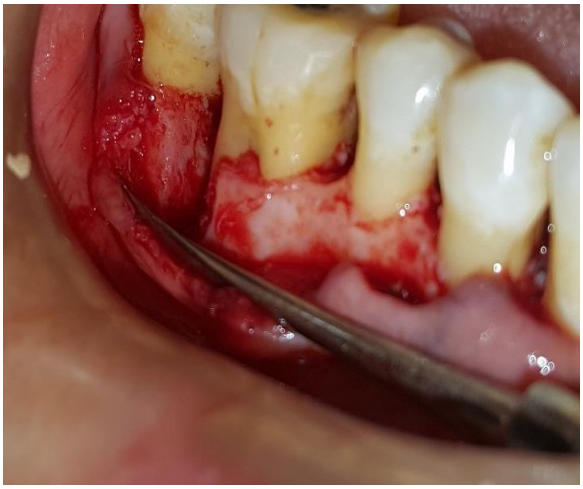
FIG 9: CLINICAL CASES

GROUP-1

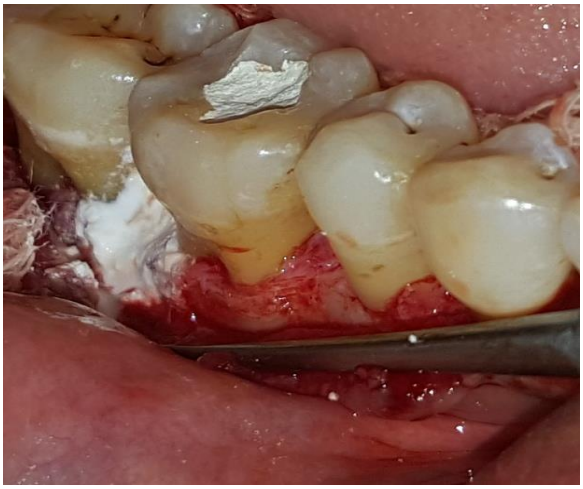
PRE-OPERATIVE VIEW



OPERATIVE VIEW

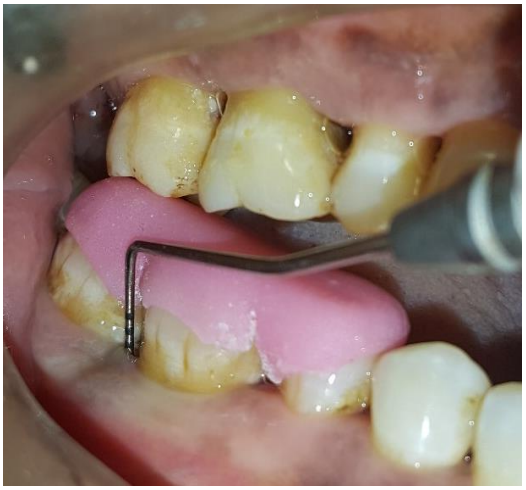


HA PLACED IN IBD

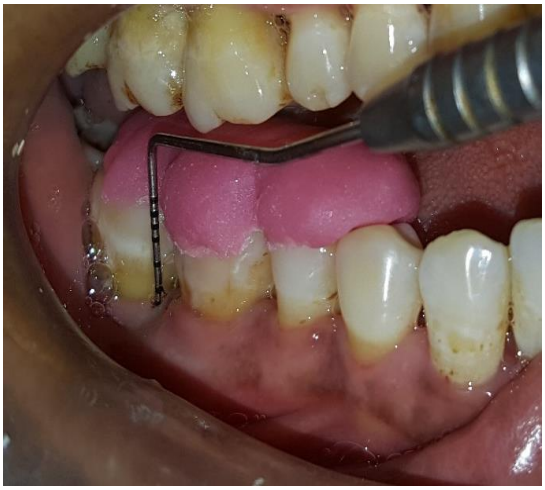


GROUP 1

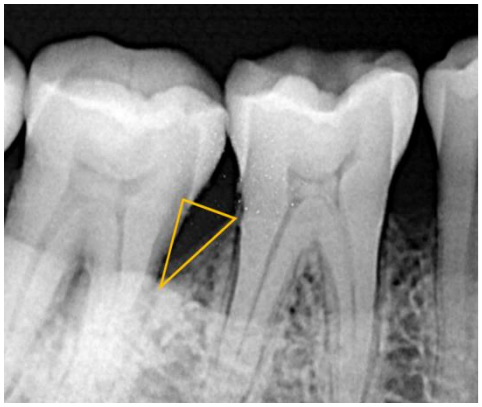
PRE-OPERATIVE



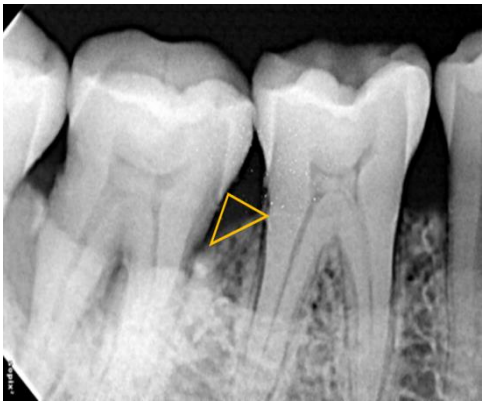
6 MONTHS POST OPERATIVE



PRE-OPERATIVE

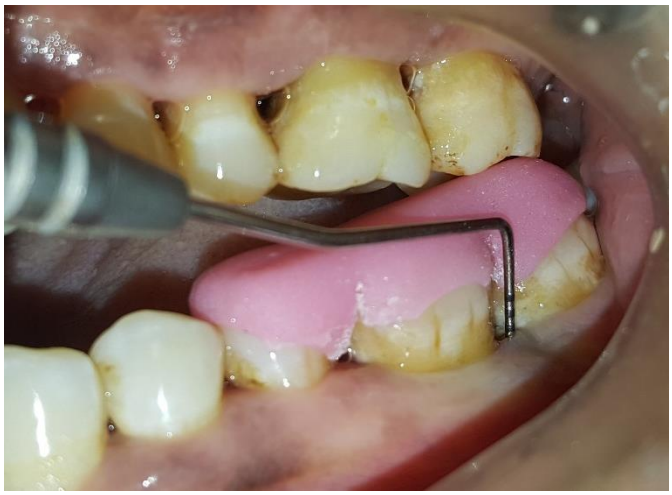


6 MONTHS POST OPERATIVE

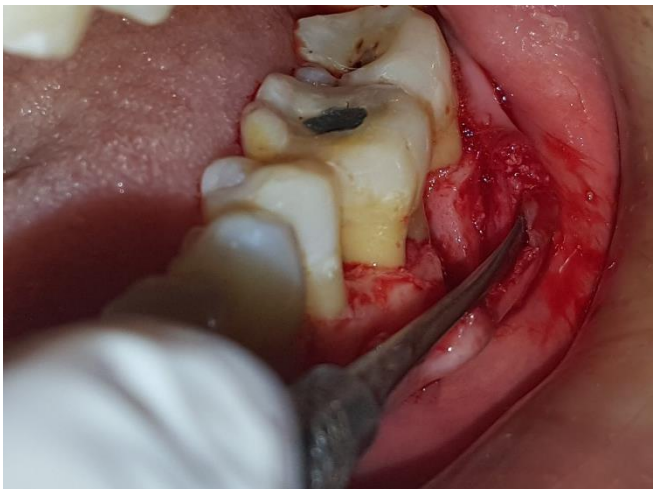


GROUP-2

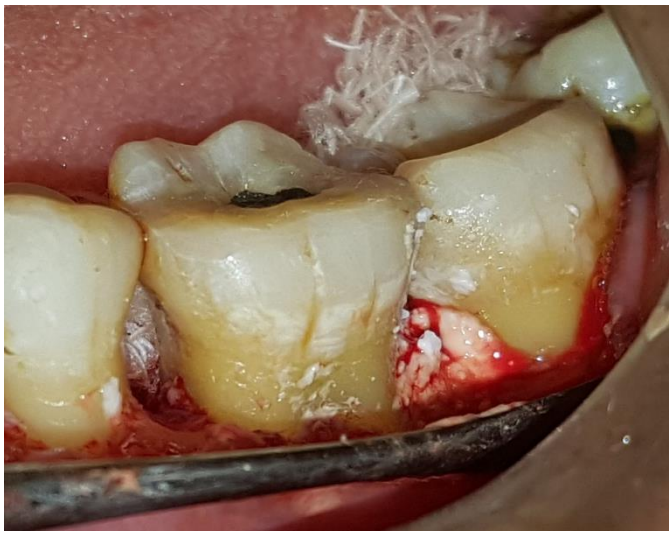
PRE-OPERATIVE VIEW



OPERATIVE VIEW

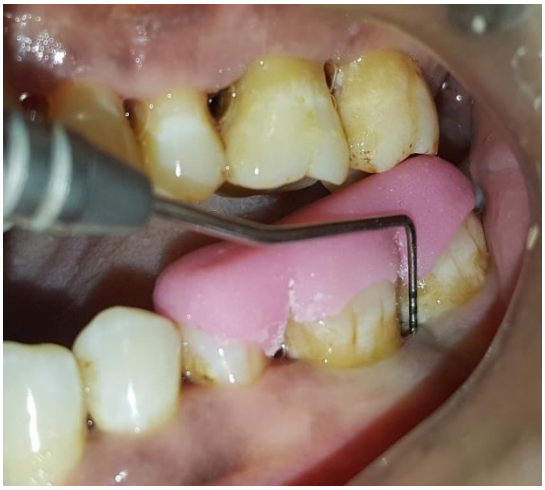
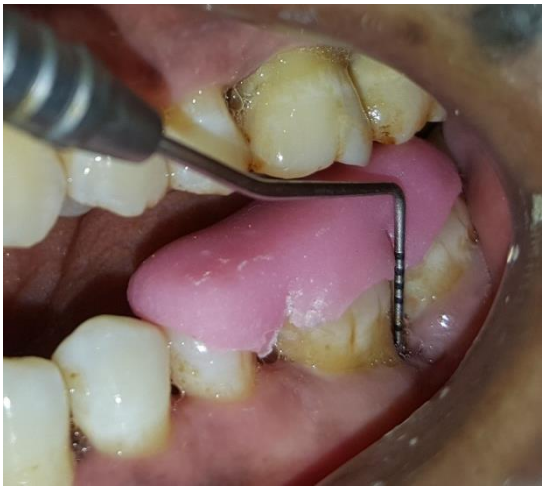


HA + SMV PLACED IN IBD

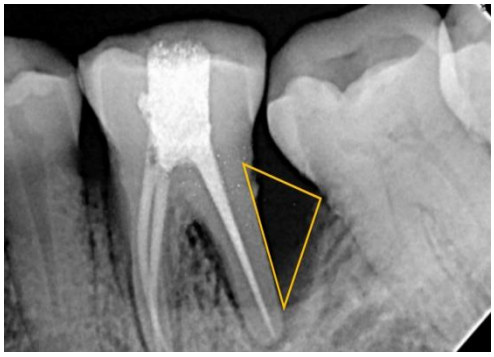
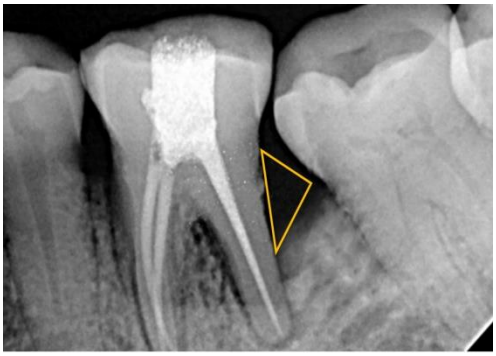


GROUP 2

PRE-OPERATIVE **6 MONTHS POST OPERATIVE**

PRE-OPERATIVE **6 MONTHS POST OPERATIVE**

STATISTICAL ANALYSIS

The results obtained were analyzed statistically and comparisons were made within each group using Students paired 't' test. 'p value' between baseline, 3 months and 6 months post-operative were evaluated. $p < 0.001$ denoted statistically significant at 1% level, $p < 0.05$ denoted statistically significant at 5% level and $p > 0.05$ denoted statistically insignificant at 5% level. The statistical analysis was done using SPSS software Version 19.0.

IN VITRO RESULTS

Effect of simvastatin on RUNX2 gene expression

The effect of simvastatin on the RUNX2 gene expression on MG-63 cells after 24 hours and 14 days of stimulation is shown in Table and Graph 1. The gene expression was significantly increased compared to control ($p < 0.001$) after 24 hours and 14 days in simvastatin treated MG-63 cells.

Effect of simvastatin on osteopontin gene expression

The effect of simvastatin on the osteopontin gene expression on MG-63 cells after 24 hours and 14 days of stimulation is shown in Table and Graph 2. The gene expression was significantly increased compared to control ($p < 0.001$) after 24 hours and 14 days in simvastatin treated MG-63 cells.

CLINICAL RESULTS

A randomized controlled clinical trial was conducted to evaluate the clinical and radiographic efficacy of simvastatin combined with hydroxyapatite in infrabony defects. The present study comprised of 10 patients with 20 infra bony defects that were randomly selected and divided into two groups (Group 1 and Group 2). Group 1 patients received hydroxyapatite and Group 2 patients received simvastatin combined with hydroxyapatite in infrabony defects. Clinical parameters such as probing pocket depth, clinical attachment level, and radiographic measurements were recorded.

Plaque Index (PI)

Statistically significant reduction in the plaque score was seen from the baseline value 2.45 ± 0.35 which reduces to 1.35 ± 0.28 at 3 months and 1.12 ± 0.27 at 6 months. Highly significant p - value < 0.001 was obtained. (Table and Graph 3).

Gingival Index (GI)

The mean gingival index score at baseline was 2.78 ± 0.29 reduced to 1.35 ± 0.42 at 3 months and further reduced to 1.19 ± 0.42 at 6 months post-operative period. The values at 3 month and at 6 months were statistically significant when compared to baseline, with p - value < 0.001 (Table and Graph 3).

Oral Hygiene Index-Simplified (OHI-S)

The mean Oral Hygiene Index–Simplified score at baseline was 2.6 ± 0.31, reduced to 1.48 ± 0.32 at 3 months and further reduced to 1.23 ± 0.27 at 6 months post-operative period. Compared to baseline, the values at 3 months and at 6 months were statistically significant with p - value < 0.001. (Table and Graph 3).

Probing Pocket Depth (PPD)

In Group 1, at baseline the mean probing pocket depth was 7.20 ± 0.42 mm, reduced to 3.97 ± 0.43 mm at 3 months and 3.85 ± 0.20 mm at 6 months post-operative period. In Group 2, at baseline it was 7.10 ± 0.87 mm, reduced to 3.40 ± 0.46 mm at 3 months and 3.07 ± 0.28 mm at 6 months post-operative period. The reduction in the probing pocket depth was found to be greater in Group 2 when compared to Group 1 which is statistically significant with p - value < 0.05 (Table and Graph 4). The mean % reduction of PPD in Group 1 was 45.9 ± 0.05 % after 3 months and 46.23 ± 0.07 % after 6 months. The mean % reduction of PPD in Group 2 was 53.21 ± 0.09 % after 3 months and 57.01 ± 0.07 % after 6 months. Group 2 showed more significant reduction in PPD at 3 months and 6 months post-therapy, compared to Group 1 (p – value < 0.05). (Table and Graph 5).

Clinical Attachment Level (CAL)

In Group 1, at baseline the mean clinical attachment level was 7.0 ± 0.67 mm, reduced to 3.93 ± 0.33 mm at 3 months and further reduced to 3.57 ± 0.37 mm at 6 months postoperative period. In Group 2, at baseline it was 6.90 ± 0.73 mm, reduced to 3.23 ± 0.41 mm at 3 months and further reduced to 3.04 ± 0.27 mm at 6 months. The gain in the clinical attachment level was found to be greater in Group 2 when compared to Group 1 which is statistically significant with p – value < 0.05 (Table and Graph 6). Mean % gain in CAL in Group 1 was 43.2 ± 0.02 % after 3 months and 49.12 ± 0.02 % after 6 months. Mean % gain in CAL in Group 2 was 53.23 ± 0.54% after 3 months and 56.0 ± 0.52% after 6 months post-op. When comparing both the groups, more significant gain in CAL was seen in Group 2

compared to Group 1, after 3 months (p – value < 0.05) and 6 months (p – value < 0.05) post therapy. (Table and Graph 7).

Infra Bony Defect depth (IBD)

In Group 1, at baseline the mean defect was 7.21 ± 0.15 mm and the defect depth reduction measured was 5.22 ± 0.51 mm at 3 months and 4.33 ± 0.29 mm at 6 months post-operative period. In Group 2, at baseline it was 7.20 ± 0.07 mm, and the measured defect depth reduction was 4.27 ± 0.89 mm at 3 months and 3.86 ± 0.31 mm at 6 months. When compared both the groups, the infrabony defect depth reduction was greater in Group 2 when compared to Group 1 and found to be statistically significant with p – value < 0.05 (Table and Graph 8). Mean % gain in IBD reduction in Group 1 was 27.10 ± 0.04 % after 3 months and 51.0 ± 0.32 % after 6 months. Mean % gain in CAL in Group 2 was 33.42 ± 0.25 % after 3 months and 54.25 ± 0.54 % after 6 months post-op. When comparing both the groups, more significant % reduction in IBD was seen in Group 2 compared to Group 1, after 3 months (p – value < 0.05) and 6 months (p – value < 0.05) post therapy. (Table and Graph 9).

TABLE 1: Effect of simvastatin on RUNX2 mRNA expression in 24 hours and 14 days in MG-63 cells using student 't' test.

RUNX2	Group 1	Group 2	Mean difference	95% C.I.		t	p value
	Mean±SD	Mean±SD		lower	upper		
24 hours	1±0	1.19±0.09	0.19	0.03	0.1	35	0.001**
14 days	1±0	1.55±0.04	0.78	0.78	0.87	39	0.001**

p – value < 0.001**denotes highly significant at 1% level.

TABLE 2: Effect of simvastatin on osteopontin mRNA expression in 24 hours and 14 days in MG-63 cells using student 't' test.

Osteopontin	Group 1	Group 2	Mean difference	95% C.I.		t	p value
	Mean±SD	Mean±SD		lower	upper		
24 hours	1±0	1.25±0.10	0.25	0.1	0.15	32	0.001**
14 days	1±0	1.55±0.04	0.8	0.4	0.9	36	0.001**

p –value < 0.001**denotes highly significant at 1% level.

TABLE 3: **Mean Plaque index, Gingival Index and Oral Hygiene Index-S at baseline, 3 months and 6 months post-therapy.**

Indices	Baseline	3 months	6 months	p – value
Gingival index	2.78 ± 0.29	1.35 ± 0.42	1.19 ± 0.42	< 0.001**
Plaque index	2.45 ± 0.35	1.35 ± 0.28	1.12 ± 0.27	< 0.001**
Oral hygiene index-S	2.65 ± 0.31	1.48 ± 0.32	1.23 ± 0.27	< 0.001**

p – value < 0.001** denotes highly significant at 1% level.

TABLE 4: Inter group difference in mean Probing Pocket Depth (PPD) at baseline, 3 months and 6 months.

Probing pocket depth	Group 1	Group 2	p - value
	Mean±SD	Mean±SD	
Baseline values	7.20 ± 0.42	7.10 ± 0.87	> 0.05
At 3 months	3.97 ± 0.43	3.34 ± 0.46	< 0.05**
At 6 months	3.85 ± 0.20	3.07 ± 0.28	< 0.05**

p – value > 0.05 denotes statistically insignificant at 5% level.

p – value < 0.05** denotes statistically significant at 5% level.

TABLE5: Intergroup difference in % of Probing Pocket Depth (PPD) reduction at 3 months and 6 months

% PPD reduction	GROUP 1	GROUP 2	p-value
3 months	45.90 ± 0.05	53.21 ± 0.09	<0.05**
6 months	46.23 ± 0.07	57.01 ± 0.07	

p –value < 0.05** denotes statistically significant at 5% level.

Table 6: Inter group difference in mean Clinical Attachment Level (CAL) at baseline, 3 months and 6 months

Clinical attachment level	Group 1	Group 2	p - value
	Mean±SD	Mean±SD	
Baseline values	7.00 ± 0.66	6.90 ± 0.73	> 0.05
At 3 months	3.93 ± 0.33	3.23 ± 0.41	< 0.05**
At 6 months	3.57 ± 0.37	3.04 ± 0.27	< 0.05**

p- value > 0.05 denotes statistically insignificant at 5% level.

p- value < 0.05** denotes statistically significant at 5% level.

TABLE 7: Intergroup difference in % of Clinical Attachment Level (CAL) gain at 3 months and 6 months

% of CAL gain	GROUP 1	GROUP 2	p-value
3 months	43.2 ± 0.02	53.23 ± 0.54	<0.05**
6 months	49.12 ± 0.02	56.0 ± 0.52	

p- value < 0.05** denotes statistically significant at 5% level.

Table 8: Inter group difference in mean Infra Bony Defect depth reduction at baseline, 3 months and 6 months

Infra bony defect depth	Group 1	Group 2	p - value
	Mean±SD	Mean±SD	
Baseline values	7.21± 0.15	7.20 ± 0.07	> 0.05
At 3 months	5.22± 0.51	4.27± 0.89	< 0.05**
At 6 months	4.33± 0.29	3.86± 0.31	< 0.05**

p - value > 0.05 denotes statistically insignificant at 5% level.

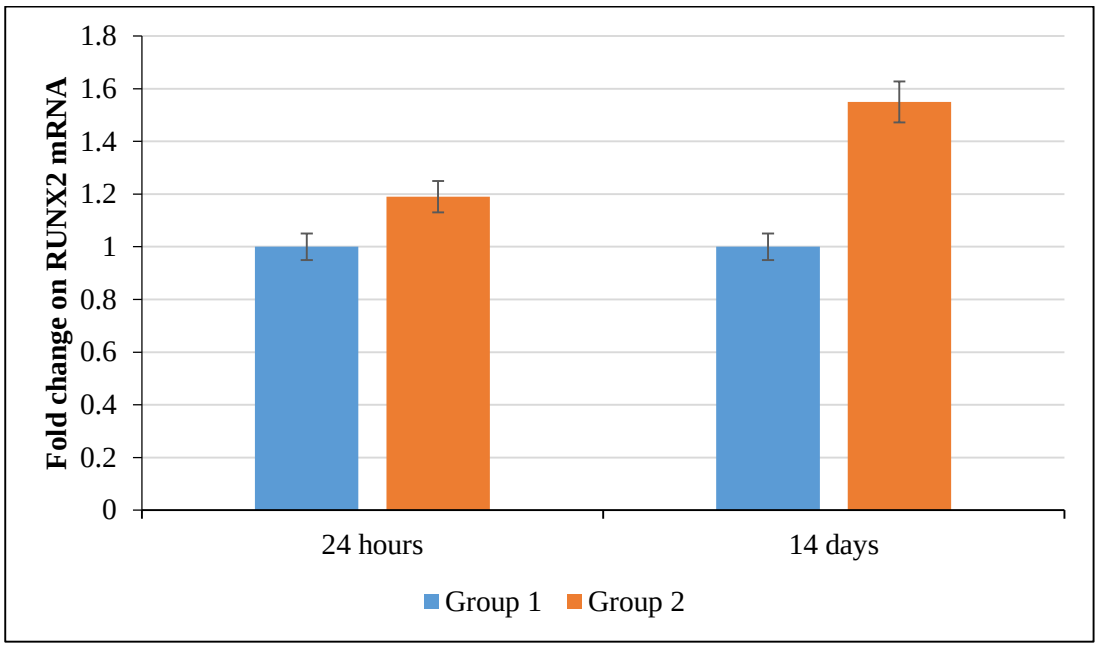
p- value < 0.05** denotes statistically significant at 5% level.

Table 9: Intergroup difference in % of Infra Bony Defect depth reduction at 3 months and 6 months

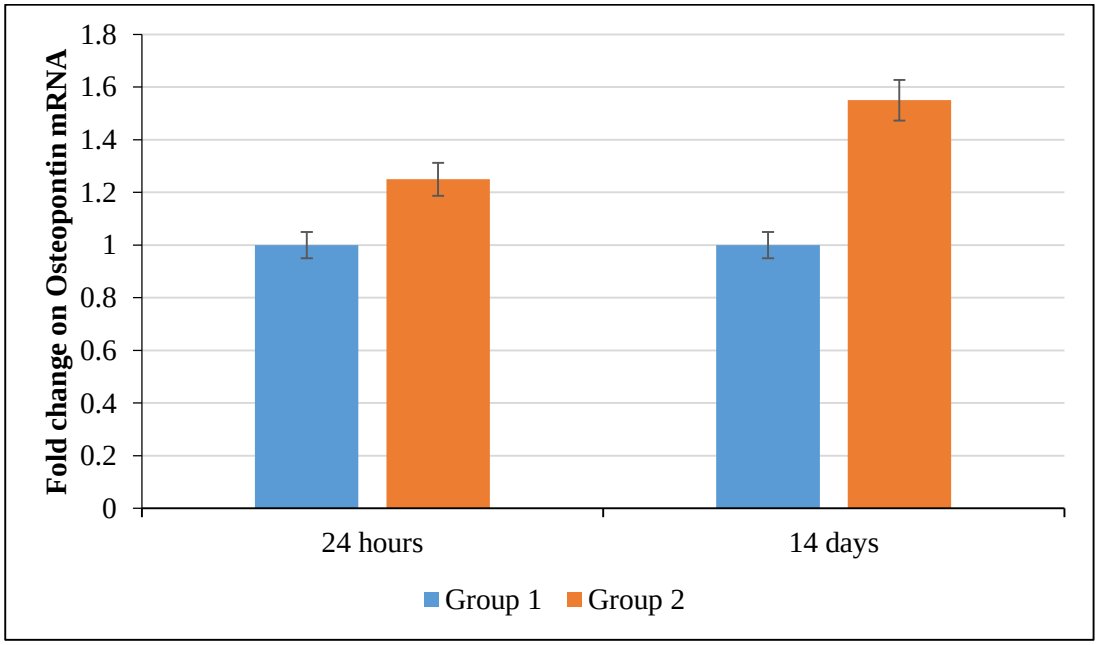
% of Infra bony defect depth reduction	GROUP 1	GROUP 2	P - value
3 months	27.10 ± 0.42	33.42 ± 0.25	< 0.05**
6 months	51.0 ± 0.32	54.23 ± 0.54	

p – value < 0.05** denotes statistically significant at 5% level.

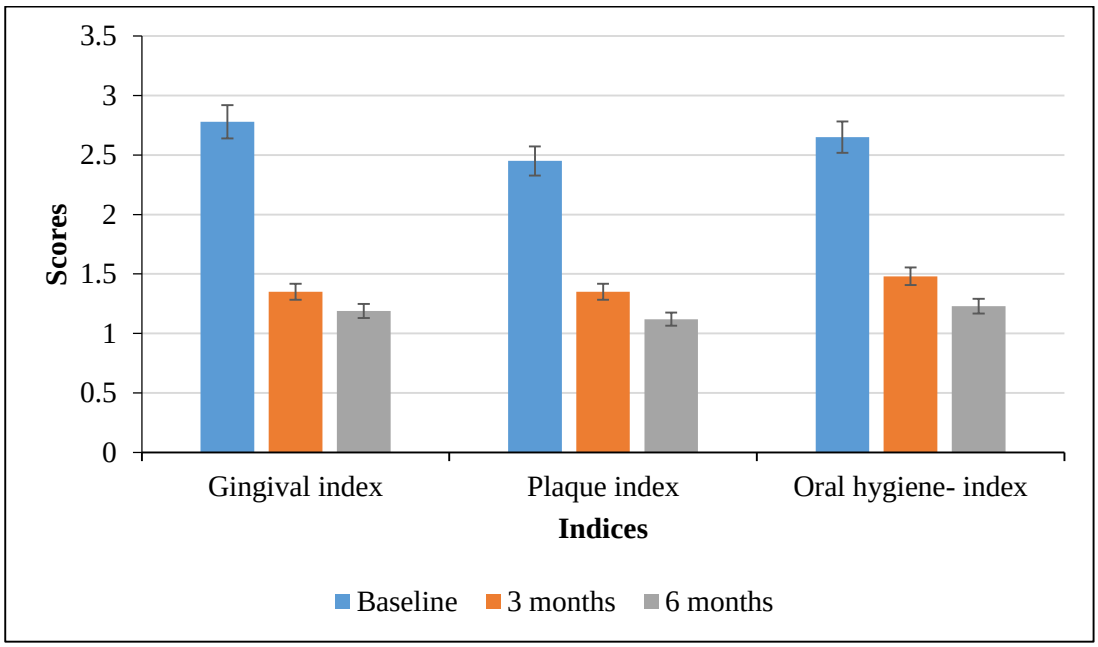
GRAPH 1: Effect of simvastatin on RUNX2 mRNA expression in 24 hours and 14 days in MG-63 cells using student 't' test.



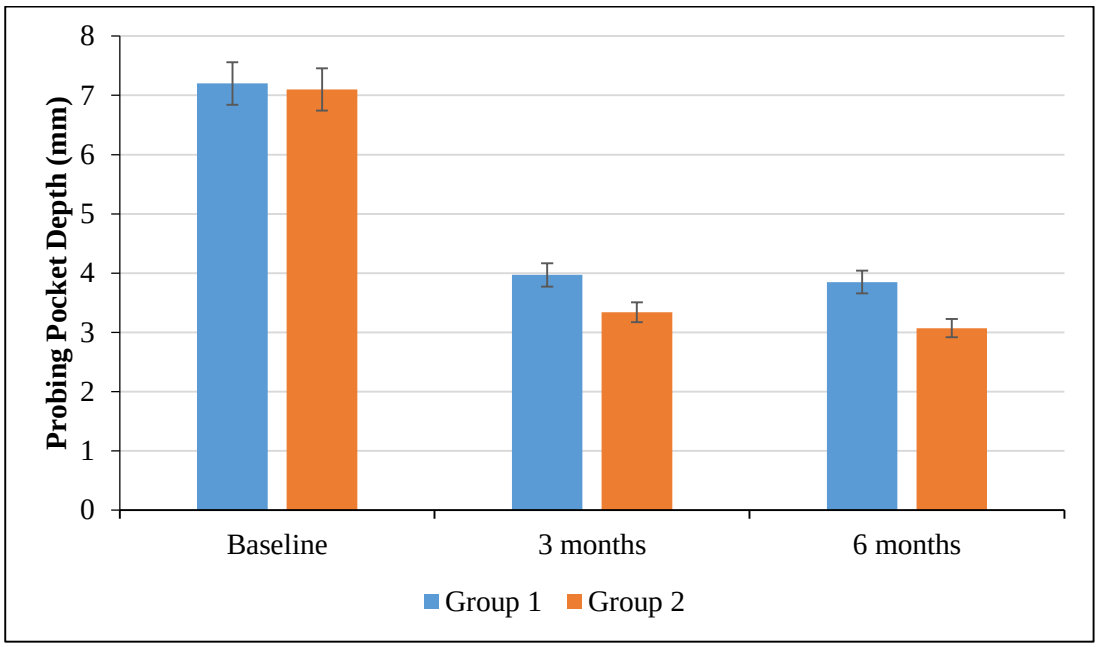
GRAPH 2: Effect of simvastatin on osteopontin mRNA expression in 24 hours and 14 days in MG-63 cells using student 't' test.



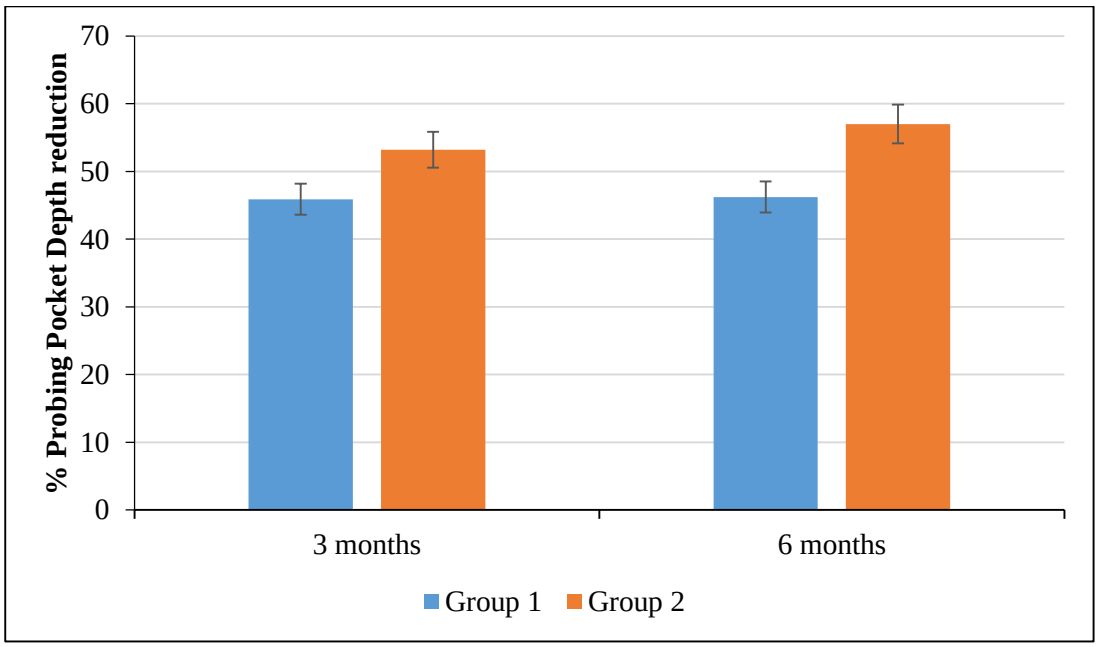
GRAPH 3: Mean gingival index, Plaque index, Oral hygiene index at baseline, 3 months, 6 months post therapy.



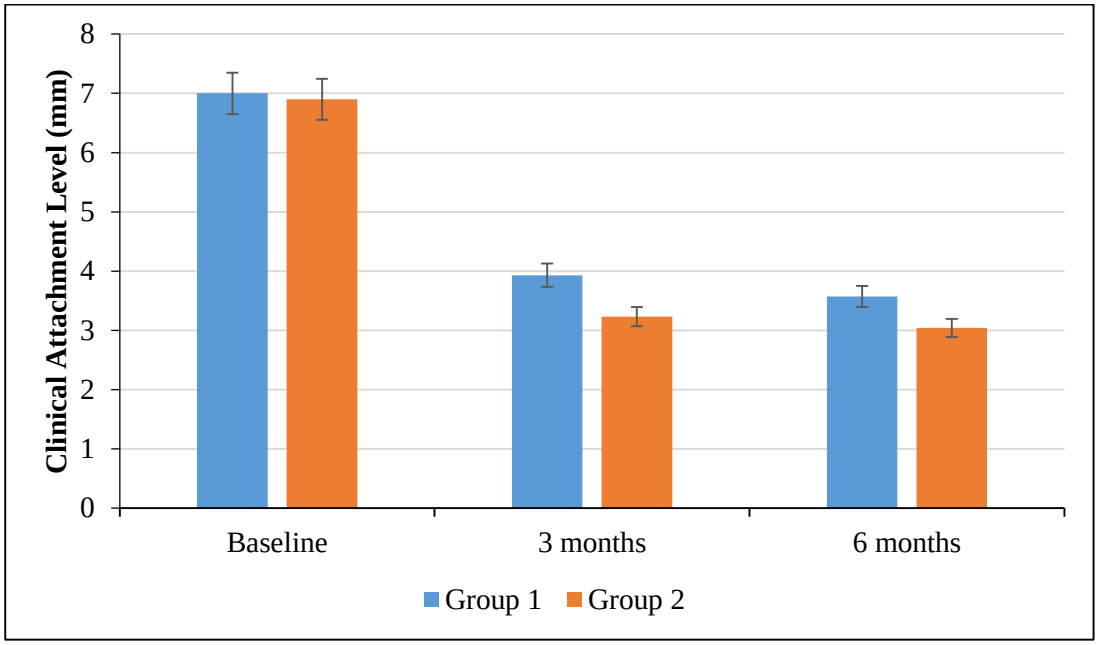
GRAPH 4: Probing Pocket Depth in both groups at baseline, 3 and 6 months post-therapy.



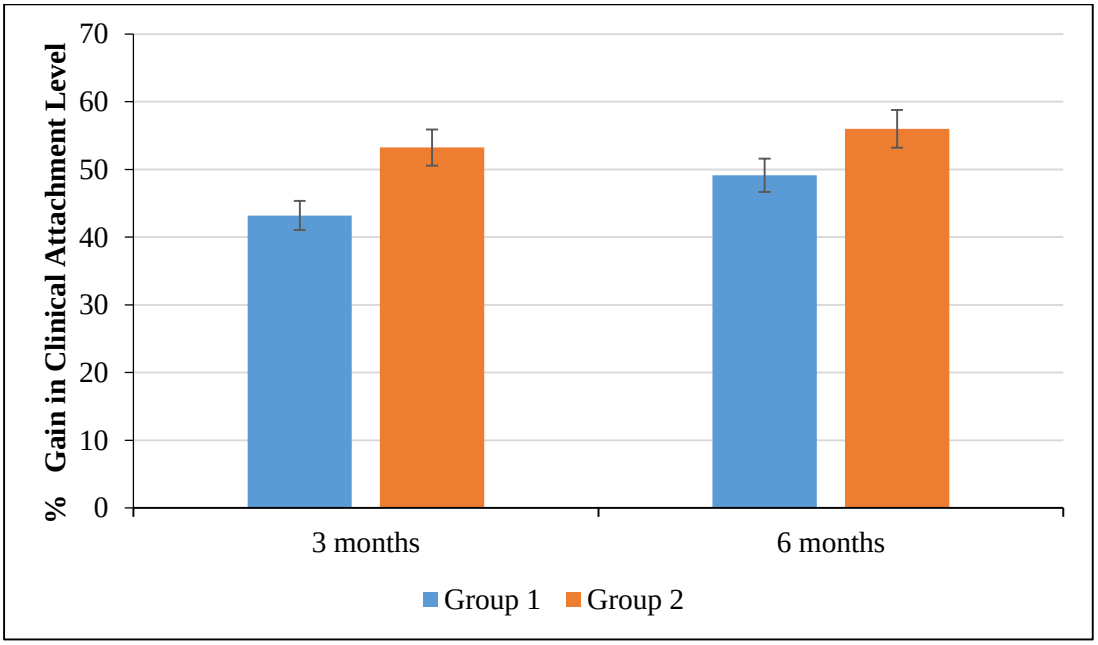
GRAPH 5: Intergroup difference in % of Probing Pocket Depth (PPD) reduction at 3 months and 6 months.



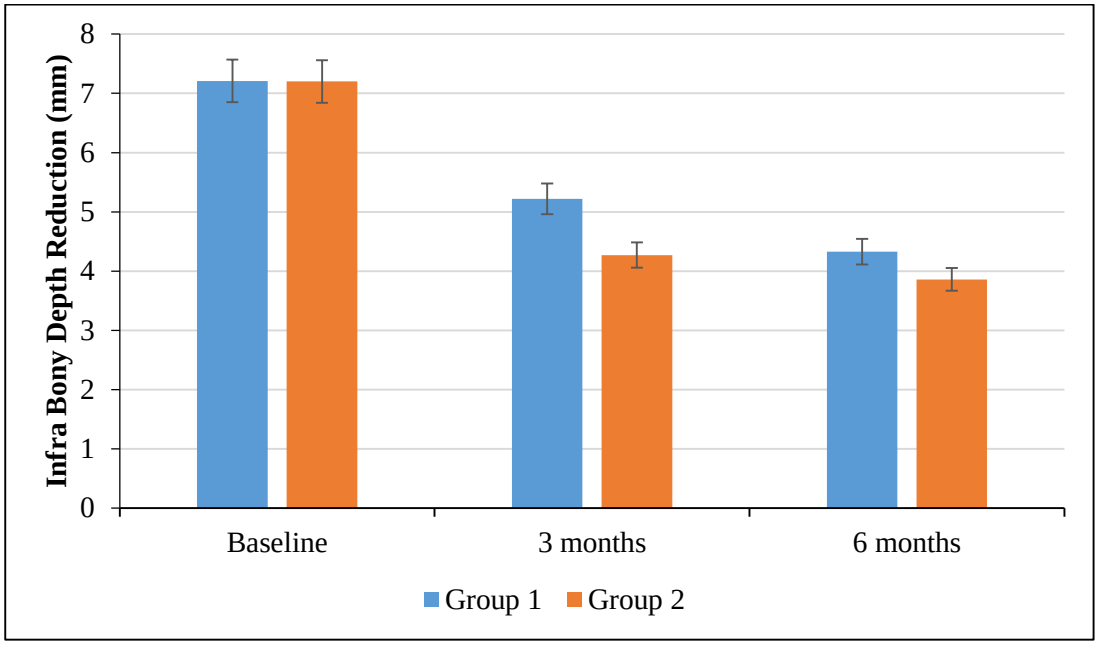
GRAPH 6: Clinical Attachment Level (CAL) in both groups at baseline, 3 and 6 months post-therapy.



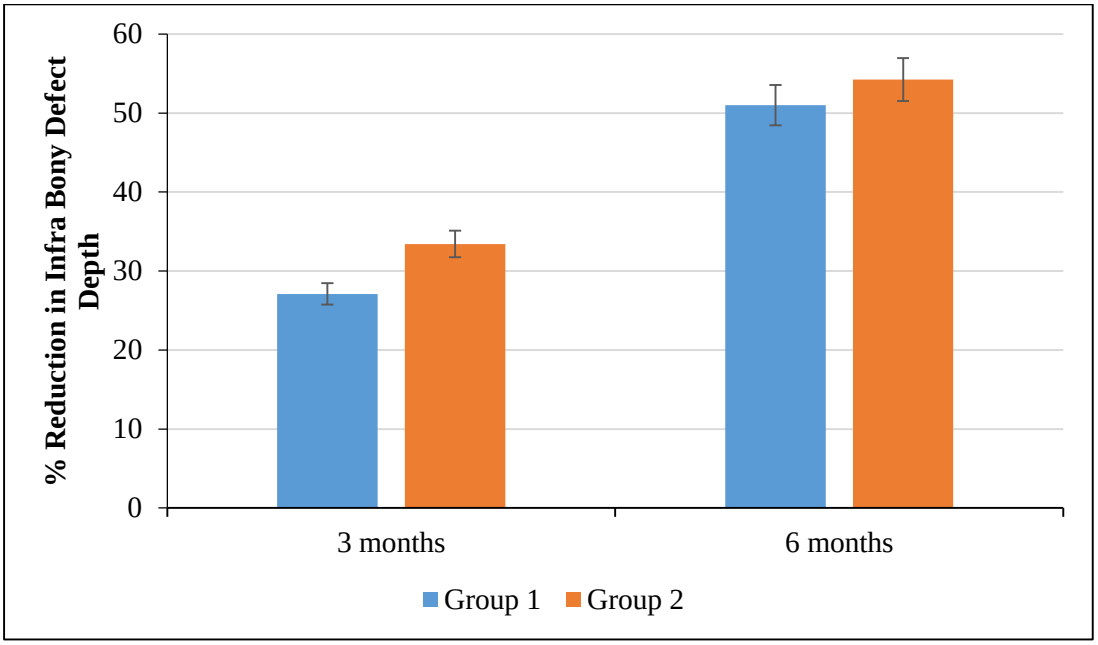
GRAPH 7: Intergroup difference in % of Clinical Attachment Level (CAL) gain at 3 months and 6 months.



GRAPH 8: Infra Bony Defect depth reduction in both groups at baseline, 3 and 6 months.



GRAPH 9: Intergroup difference in % of Infra Bony Defect depth reduction at 3 months and 6 months.



Periodontal therapy mainly aims to reconstruct the lost periodontal structures destroyed by periodontal diseases. Various bone replacement grafts have been used in the past two decades to achieve regeneration. Pharmacological agents like statins were added to the graft material to enhance the regeneration as statins not only inhibits bone resorption but also enhances bone formation in a cost effective manner.[9]

In the present study the osteogenic action of simvastatin was evaluated by assessing the bone markers RUNX2 and osteopontin on cultured MG-63 cells using real time PCR technique after 24 hours and 14 days of culturing. The results of present study showed that simvastatin has stimulatory effects on the expression of the osteoblast specific genes RUNX2 and osteopontin in MG-63 cultures after 24 hours (p – value < 0.001) and 14 days (p – value < 0.001). This was in conjunction with the study done on mouse embryonic stem cells by Ling *et al.*, (2011)[38], where simvastatin upregulates the mRNA expression of RUNX2, osterix, osteocalcin, osteopontin. Yasawa *et al.*, (2005)[55] also demonstrated the bone stimulatory effects of simvastatin on human periodontal ligament cells, in which simvastatin enhanced the ALP activity and osteopontin content significantly and these effects may be caused by the inhibition of the mevalonate pathway.

In the present study, simvastatin in combination with hydroxyapatite was compared with hydroxyapatite alone in the treatment of human infrabony periodontal defects. In our study, statistically significant reduction in the plaque index score was seen from the baseline value 2.45 ± 0.35 which reduces to 1.35 ± 0.28 at 3 months and 1.12 ± 0.27 at 6 months with p - value < 0.001. Similar results were demonstrated by Priyanka N *et al.*, (2017)[69] showed significant reduction in

plaque index indicates better plaque control by the patients. Tonneti M *et al.*, (1996)[92] also showed that clinical outcome of regenerative therapy is strongly associated with control of patients oral hygiene and residual periodontal infection in the oral cavity.

In the present study, the mean Oral Hygiene Index-Simplified score at baseline was 2.6 ± 0.31, reduced to 1.48 ± 0.32 at 3 months and further reduced to 1.23 ± 0.27 at 6 months post-operative period. Compared to baseline, the values at 3 months and at 6 months were statistically significant with p - value < 0.001. This was in accordance with the study done by Trombelli *et al.*, (2010)[93] showed marked improvement in plaque scores after periodontal therapy and explained that patients maintained optimal oral hygiene throughout the study period.

In this study, the mean Gingival index (GI) score at baseline was 2.78 ± 0.29 reduced to 1.35 ± 0.42 at 3 months and further reduced to 1.19 ± 0.42 at 6 months post-operative period. The values at 3 months and at 6 months were statistically significant when compared to baseline, with p - value < 0.001. Sakoda *et al.*, (2006)[94] demonstrated in human oral epithelial cells that the anti-inflammatory effects of simvastatin was due to the inhibition of Rac 1 guanosine triphosphatase and impedes IL-6 and IL-8 production.

In the present study significant reduction in probing pocket depth with adjunctive use of simvastatin was obtained (p – value < 0.05). In Group 1, at baseline the mean probing pocket depth was 7.20 ± 0.42 mm, reduced to 3.97 ± 0.43 mm at 3 months and 3.85 ± 0.20 mm at 6 months post-operative period. In Group 2, at baseline it was 7.10 ± 0.87 mm, reduced to 3.40 ± 0.46 mm at 3 months and 3.07 ± 0.28 mm at 6 months post-operative period. This can be explained based on the

findings of Davignon *et al.,* (1999)[95] reported that simvastatin reduces the plasma levels of inflammatory markers like C- reactive protein due to its anti- inflammatory and anti-oxidant properties and these properties may have contributed to a greater reduction in probing pocket depth by continued facilitation of tissue healing and shrinkage.

In our study, in Group 1, at baseline the mean clinical attachment level was 7.0 ± 0.67 mm, reduced to 3.93 ± 0.33 mm at 3 months and further reduced to 3.57 ± 0.37 mm at 6 months postoperative period. In Group 2, at baseline it was 6.90 ± 0.73 mm, reduced to 3.23 ± 0.41 mm at 3 months and further reduced to 3.04 ± 0.27 mm at 6 months. The gain in the clinical attachment level was found to be greater in Group 2 when compared to Group 1 which was statistically significant with p – value < 0.05. Rutledge J *et al.,* (2011)[64] described simvastatin reduces osteoclast numbers by enhancement of mineralization by stimulating alkaline phosphatase activity, sialoprotein, osteocalcin, and vascular endothelial growth factors, which all together resulted in marked gain in clinical attachment level in simvastatin treated group. The results of our study was in accordance with the study done by Pradeep *et al*., (2012)[71] demonstrated simvastatin treated patients exhibited greater CAL gain.

In the present study, in Group 1, at baseline the mean infra bony defect depth was 7.21 ± 0.15 mm and the defect depth reduction measured was 5.22 ± 0.51 mm at 3 months and 4.33 ± 0.29 mm at 6 months post-operative period. In Group 2, at baseline it was 7.20 ± 0.07 mm, and the measured defect depth reduction was 4.27 ± 0.89 mm at 3 months and 3.86 ± 0.31 mm at 6 months. When compared both the groups, the infrabony defect depth reduction was greater in Group 2 when compared to Group 1 and found to be statistically significant with p – value < 0.05. The result

was in parallel with the study done by Mundy G *et al*., (1999)[41] who found that simvastatin promotes bone formation by upregulating the gene expression of BMP - 2. Thylin *et al.*,(2002)[96] showed single dose of simvastatin application stimulate bone apposition in murine calvaria. Ayukawa *et al*., (2009)[97]showed that simvastatin inhibit small GTPases mediated osteoclast function and bone resorption. Bradley *et al*., (2016)[98]demonstrated that simvastatin injected into the rat mandibles in methyl cellulose gel stimulate BMP-2 production and bone formation at the site of injection. Luan *et al*., 2003[99] showed statin induced inhibition of MMPs from macrophages and Park *et al*., 2009[15] also demonstrated the protective features on periodontal attachment apparatus.

The results of the present study showed that simvastatin combined with hydroxyapatite enhanced the regenerative potential in the treatment of periodontal infrabony defects.

However, limitations of this study include a small sample size and a study design of larger sample size with longer follow up is needed. Surgical reentry and immuno histological assay of treated sites would provide accurate data.

The *in vitro* study was conducted to evaluate the osteogenic potential of simvastatin by assessing the bone markers RUNX2 and osteopontin mRNA expression in MG-63 cell lines after incorporation with simvastatin using Real time PCR technique.

The *in vivo* study was designed as a single blinded randomized controlled split mouth clinical trial for a period of 6 months. The study population comprised of 10 subjects and a total of 20 infrabony defects were treated. Group 1 consist of 10 sites, in which hydroxyapatite was placed (Control site) and Group 2 consist of 10 sites, in which simvastatin combined with hydroxyapatite was placed (Test site).

Clinical parameters such as Plaque index (PI), Gingival index (GI), Oral hygiene index- Simplified (OHI-S), Probing Pocket Depth (PPD), Clinical Attachment Level (CAL) Infra Bony Defect depth (IBD) were evaluated.

Within the framework of this study, the following conclusions have been elucidated,

The results of present study indicates simvastatin has stimulatory effects on the expression of the osteoblast specific genes RUNX2 and Osteopontin in MG-63 cell cultures and thus this study supports & confirms the action of simvastatin and its efficacy as bone regenerative material.

1. Both Test and Control group yielded favorable clinical results in periodontal infrabony defects.

2. Probing pocket depth and gain in attachment level were significant in both the groups when compared to pre-operative level. But Probing Pocket Depth and Clinical Attachment Level gain was greater in Test group with a

statistically significant p - value of < 0.05 than control group at 3 and 6 months post operatively.

3. Both the groups exhibited significant amount of bone fill than the pre-operative levels and the mean bone fill was higher in simvastatin treated group. Infra Bony Depth reduction was greater in Test group with a statistically significant p – value of < 0.05.

In conclusion, simvastatin combined with hydroxyapatite showed a potential role in periodontal regeneration. As simvastatin can fulfill the dual action by inhibiting bone resorption as well as enhancing bone formation, it promises to be a viable alternative for periodontal regeneration in a cost-effective manner.

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