INFLUENCE OF SALIVARY PYROPHOSPHATE LEVELS ON CALCULUS FORMATION AND PERIODONTAL DISEASE PROGRESSION

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"INFLUENCE OF SALIVARY PYROPHOSPHATE LEVELS ON CALCULUS FORMATION AND PERIODONTAL DISEASE PROGRESSION"** is a bonafide and genuine research work carried out by me under the guidance of **Dr.K.V.ARUN, M.D.S.,** Professor and Head, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

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This dissertation is submitted to **THE TAMILNADU DR.MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

PSD	-	Polymicrobial synergy and dysbiosis
GCF	-	Gingival Crevicular Fluid
PDGF	-	Platelet Derived Growth Factor
EGF	-	Epidermal Growth Factor
MMP	-	Matrix MetalloProteinases
PMNs	-	Polymorphonuclear Leukocytes
ICTP	-	Collagen Telopeptide
DMP	-	Dentin Matrix Protein
PTH	-	Parathyroid Hormone
FGF	-	Fibroblast Growth Factor
ENPP	-	Ectonucleotide Pyrophosphate
ELISA	-	Enzyme Linked ImmunoSorbent Assay
Ig	-	Immunoglobulin
ANK	-	Ankylosis Protein Homolog

EHDP	-	Ethane-1-Hydroxy-1,1-diphosphonate
TRK	-	Disodium dihydrogen methane bisphosphonate
CPPD	-	Calcium pyrophosphate dehydrate deposition
PBTA	-	2-Phosphonobutane tricarboxylate

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Introduction

INTRODUCTION

Periodontal diseases are thought to be as a result of host bacterial imbalance. It is thought that much of the destruction that occurs in periodontal tissues is a result of an exaggerated nonprotective host response. It is also equally well accepted that this host response is initiated by microorganisms present in plaque.**(Calkins et al, 1998)**[11] Plaque is therefore, considered the primary etiologic factor in periodontal disease.**(Lang et al, 2009)**[45] There are other predisposing factors that potentiate the role of plaque on the host tissues. Dental calculus is defined as the mineralized form of bacterial plaque. Its contribution as a primary plaque retentive factor in pathogenesis of periodontal disease has been well documented.**(Schroeder et al, 1965)**[69]

Mandel et al[50], have indicated that a large amount of calculus may hamper the efficacy of daily oral hygiene and thereby accelerate plaque formation, the accumulation of which initiates the inflammatory reaction in the gingiva that leads to periodontitis[1]. Several theories have been proposed to describe the process of mineralization of calculus. All of them have suggested that the source of mineralization of supragingival calculus is from saliva and subgingival calculus is from the GCF.

Several constituents in saliva have been demonstrated as playing an regulatory role in mineralization of plaque. Salivary pyrophosphate levels play an important role in inhibition of calculus formation. Enzyme alkaline phosphatase present in saliva and in plaque, releases inorganic orthophosphate

from organic phosphate, increasing the concentration of orthophosphate locally, which reacts with calcium ions leading to precipitation of insoluble calcium apatite crystals (**Jenkins et al**, 1978)[40]. Pyrophosphate, a byproduct of many biosynthetic reactions (**Alcock et al,**1969)[2] present in saliva inhibits crystallization and competes with orthophosphate (**Vogel et al,** 1967)[83] for minerals[4], thus having a strong inhibitory effect on plaque mineralization. Apart from its activity in saliva, there have been various reports in literature wherein pyrophosphate plays other important roles. **Fleisch et al**,[19] reported its role in inhibition of calcification of cartilage and collagen and its activity on enamel and dentin. **(Bisaz et al,** 1968**)**[20]

The role of poor oral hygiene in the development of plaque and calculus has been well documented. Several socioeconomic and demographic factors have been documented to contribute to the general lack of awareness and poor oral hygiene practices in underdeveloped countries.**(Petersen et al, 2003)**[63] Conversely, it has also been reported that individual disease susceptibility is crucial for development and progression of periodontal disease even in patients with equal amount of plaque.**(Genco et al, 2013)**[24] It is not yet fully understood if individual susceptibility could play a role in calculus formation, given that each individual has been documented to have a distinct microbiome.**(Dewhirst et al, 2010)**[14]

We hypothesize that salivary constituents may play an important role in determining calculus formation, in patients with similar oral hygiene

practices. Considering the paucity in literature, a large scale study was planned to determine the various salivary constituents that promote/inhibit mineralization. In this study, the mineralization inhibitor pyrophosphate was examined for its role in formation of supragingival calculus.

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Aim and Objectives

AIM AND OBJECTIVES

AIM:

The aim of this study was to identify the influence of salivary pyrophosphate levels on supragingival calculus formation and to correlate it to periodontal disease activity.

OBJECTIVES:

1. To identify the pyrophosphate levels in saliva in patients with local factors and periodontal disease.
2. To compare the levels of salivary pyrophosphate in three groups of patients exhibiting mild/ no calculus, moderate and severe calculus formation.

Review of Literature

REVIEW OF LITERATURE

Calculus

Dental calculus can be defined as a hard concretion that forms on the surfaces of natural teeth and dental prosthesis through calcification of bacterial plaque.

Classification

Calculus is classified according to its relation to the gingival margin as

1. Supragingival calculus
2. Subgingival calculus

Supragingival calculus

The calculus deposited on the teeth coronal to the gingival margin is designated as supragingival calculus. It is usually lighter in color and is less dense than subgingival calculus. Supragingival calculus occurs predominantly on the lingual surface of lower anterior teeth, with lesser amounts on the buccal surface of the upper molars.It has a hard clay-like consistency and can be easily detached from the tooth surface. It is also called as salivary calculus as most of its mineral component is derived from the saliva.

Subgingival calculus

The calculus deposited on the tooth structure and found apical to the gingival margin within the periodontal pocket is designated as subgingival calculus. It is usually dense, dark brown or greenish black in color has a hard or flint like consistency and it is firmly attached to the tooth surface. It is also known as seruminal calculus based upon the assumption that most of the mineral content of subgingival calculus is derived from the GCF.

Composition of calculus

Dental calculus is primarily composed of calcium phosphate mineral salts deposited between and within the remnants of formerly viable microorganisms. The plaque formation serves as an organic matrix for the subsequent mineralization of the deposit. Calculus is composed of inorganic and organic components. Supragingival calculus consists of 70-90% of inorganic content and remaining organic content. The organic content of calculus consist of mixture of protein-polysaccharide complexes, desquamated epithelial cells, leukocytes and microorganisms. Inorganic content is composed of minerals, two-thirds of which is crystalline in structure.

Organic component

The organic contents of supra and subgingival calculus contain amino acids, lipids, carbohydrates and other macromolecules. Seventeen amino acids were detected with glutamic, aspartic, glycine, alanine, valine and leucine

forming the largest proportion. The lipid content was 15.3% of the dry weight of the decalcified calculus and included phospholipids, cholesterol esters, diglycerides, triglycerides and free fatty acids. Carbohydrate content consist of glucose, galactose, galactosamine, glucuronic acid, glucosamine galactosamine and rhamnose. A variety of macromolecules have been identified in dental calculus. Sulphate glycopeptides, sulphated glycosaminoglycans and hyaluronic acid were found in supragingival calculus. In addition to these chondroitin sulphate and dermatan sulphate were found in subgingival calculus.

Inorganic component

Inorganic component consists of calcium phosphate, calcium carbonate, traces of magnesium phosphate and calcite. The principal components are calcium, phosphorus, carbondioxide and magnesium. Trace elements found are sodium, zinc, strontium, bromine, copper, manganese, tungsten, gold, aluminium, silicon, iron and fluoride Crystalline structure consists of hydroxyapatite, magnesium whitlockite, octacalcium phosphate and brucite.

Calculus formation

Mineralization of dental plaque results in calculus formation. Precipitation of mineral salts starts between first and fourteenth days of plaque formation. (**Tibbetts et al, 1970**)[80] Inorganic material increases as plaque

mineralizes to calculus. Mineral content reaches its maximum within 2 days.(**Schroeder et al, 1965)**[69] Plaque may become 50% mineralized in 2 days and 60% to 90% mineralized in 12 days. (**Muhlemann et al, 1964**)[55] Supragingival calculus gets its source of mineralization from saliva. Subgingival calculus mineralizes from GCF source (**Stewart et al, 1966**).[75] The calcium concentration content in plaque is 2 to 20 times that found in saliva. (**Birkeland et al, 1974**)[10]

Phosphorus content is more in plaque of heavy calculus formers indicating that phosphorus plays a critical role in plaque mineralization. (**Mandel et al, 1969**)[50] Calcium binds to carbohydrate protein complexes of organic matrix resulting in precipitation of calcium phosphate salts leading to calcification. Crystals form in the intercellular matrix initially and then on the surface of the bacteria and within them (**Zander et al, 1960**)[87] Calcification progresses along the inner surface adjacent to the tooth. It is accompanied by alterations in bacterial content. The number of filamentous bacteria increases. Foci of calcification increases in size and coalesce to form calculus mass formed in layers (**Manly et al, 1973**)[53]. There is variation between individuals in initiation of calcification and rate of calculus accumulation. (**Muhler et al, 1962**)[56]

Calculus formers may be classified as heavy, moderate or slight/non calculus formers. The average daily increment in calculus formers is from 0.10% to 0.15% of dry weight calculus. (**Sharawy et al, 1966**)[73]. Calculus

formation reaches maximum between 10 weeks and 6 months (**Volpe et al, 1969**)[84]. The decline from maximal calculus accumulation, referred to as reversal phenomenon, may be explained by vulnerability of bulky calculus to mechanical wear from tooth and from the cheeks, lips and tongue

THEORIES OF MINERALIZATION OF CALCULUS

Mineralization theory

According to this theory, saliva acts as a source for crystal growth by precipitation of inorganic ions.The mechanisms by which plaque becomes mineralized can be divided into two categories. Degree of saturation of calcium and phosphate ions rises locally resulting in precipitation of minerals. Precipitation of calcium phosphate salts occurs as precipitation constant lowers due to rise in pH of saliva. Calcium and phosphate ions bind to colloidal proteins in saliva and maintain a supersaturated solution in calcium phosphate salts with stagnation of saliva colloids leading to loss of the supersaturated state resulting in precipitation of calcium and phosphate salts. (**Prinz et al, 1921**)

Bacterial theory

Multiple mechanisms have been suggested by which bacteria may facilitate calculus formation. It has been proposed that bacteria may, form phosphatases, which may increase the local concentration of phosphates leading to calcification. Affect the Ph of plaque and saliva and destroy

protective colloidal action of saliva. Helps in the attachment of calculus to the tooth and provide chemicals that induce mineralization. It has been shown that initial deposition of apatite in calcifying bacteria is associated with a membrane or acidic membrane associated components. Calcifiability of bacteria is positively correlated with increasing concentration of phospholipids.

Mineralization of plaque starts extracellularly around both gram positive and gram negative organism and can also start intracellularlly. (**Leach et al, 1966**)[46]. Filamentous organisms, diptheroids, bacterionema and Veillonellaspecies have the ability to form intracellular apatite crystals. Mineralization spreads until the matrix and bacteria are calcified. (**Gonzales et al, 1960**)[26] Phosphatases formed by plaque bacteria changes the pH of plaque resulting in mineralization. (**Ennever et al, 1983**)

Phosphatase liberated from dental plaque desquamated epithelial cells or bacteria precipitates calcium phosphate by hydrolyzing organic phosphates in saliva this increasing the concentration of free phosphate ions. (**Wasserman et al, 1958**)[85]. Esterase is another enzyme that is present in the cocci and filamentous organisms, leukocytes, macrophages and desquamated epithelial cells of dental plaque (**Baer et al, 1959**)[5]. Esterase may initiate calcification by hydrolyzing fatty esters into free fatty acids.

Carbondioxide theory

Differences in the CO_2 tension in saliva and the atmosphere may facilitate calculus formation. The freshly secreted saliva, leaving the opening of salivary ducts has a CO_2 tension of about 60mmHg, in the expired air it is about 29mmHg and in atmosphere, it is about 0.3mmHg.

Ammonia theory

The abundant supply of urea from the major salivary gland secretions tends to increase the Ph of plaque. The increase in ph of plaque is primarily due to proteolytic activity in plaque which results in the formation of amines, urea and ammonia.The pH may be elevated by the loss of CO_2 and formation of ammonia by dental plaque bacteria or by dental plaque bacteria or by protein degradation during stagnation. (**Hodge et al, 1950**)[38]

Booster concept

According to this concept, one or more changes in the oral environment may promote calculus formation, which include mechanisms facilitating an increase in ph of saliva, formation of supersaturated solutions of colloidal proteins in saliva. Enzymatic mechanisms which facilitate the precipitation of calcium and phosphate ions.

Epitactic theory

Epistaxis refers to crystal formation through seeding by another compound. Calcified masses are formed when small foci of calcification is induced by seeding agents. (**Neuman et al, 1958**)[58]. This concept has been referred to as epitactic concept or more appropriately, heterogenous nucleation.The carbohydrate-protein complexes may initiate calcification by removing calcium from the saliva and binding with it to form nuclei that induce subsequent deposition of minerals.

Inhibition theory

Calcification occurs at specific sites due to the existence of an inhibiting mechanism at non-calcifying sites. During calcification the inhibitor is apparently altered or removed. One possible inhibiting substance is thought to be pyrophosphate and among the controlling mechanisms is the enzyme alkaline phosphatase. Pyrophosphate inhibits calcification by competing with orthophosphate for minerals and thereby preventing initial nucleus from growing.

Transformation theory

According to this theory, amorphous non-crystalline deposits and brushite can be transformed to octacalcium phosphate and then to hydroxyapatite. Early amorphous deposits, could be transformed into a more crystalline material. Nucleating substances arising from bacterial activity or

salivary proteins and lipids could also initiate calcification and account for the hydroxyapatite in early deposits. It has been suggested that the controlling mechanism in the transformation process may be pyrophosphate

.**Role of plaque and calculus**

Calculus is always covered with aunmineralized layer of plaque (**Schroeder et al, 1965**)[67]. Plaque accumulation is closely related to the initiation of periodontal diseases in young people, whereas calculus accumulation is moreprevalent in chronic periodontitis found in older adults. (**Greene et al, 1963**)[28]The incidence of calculus, gingivitis and periodontal disease increases with age. It is very rare to find periodontal pockets in adults without at least some subgingival calculus being present. Calculus does not contribute directly to gingival inflammation but provides a fixed nidus for the continued accumulation of plaque and its retention in close proximity of gingiva.

Plaque initiates gingival inflammation which leads to pocket formation and the pocket inturn provides a sheltered area for plaque and bacterial accumulation. The increased flow of gingival fluid associated with gingival inflammation provides the minerals that mineralize the continually accumulating plaque resulting in formation of subgingival calculus.

Microbial Dysbiosis

The most prominent organisms involved in etiology of periodontitis are group of bacteria known as the "red complex," namely, Porphyromonasgingivalis, Treponemadenticola and Tannerella forsythia. **(Socransky et al, 1998)**[74] However, culture-independent molecular methods used in recent metagenomic studies have revealed a more heterogeneous and diverse periodontitis-associated microbiota than previously known from cultural studies.**(Griffen et al, 2011)**[29] Many of the newly recognized organisms (e.g., certain gram-positive bacteria and other species from the gram-negative genera Prevotella, Selenomonas, Desulfobulbus, Dialister and Synergistes) show as good or better a correlation with disease than the red complex bacteria .**(Dewhirst et al, 2010)**[13]

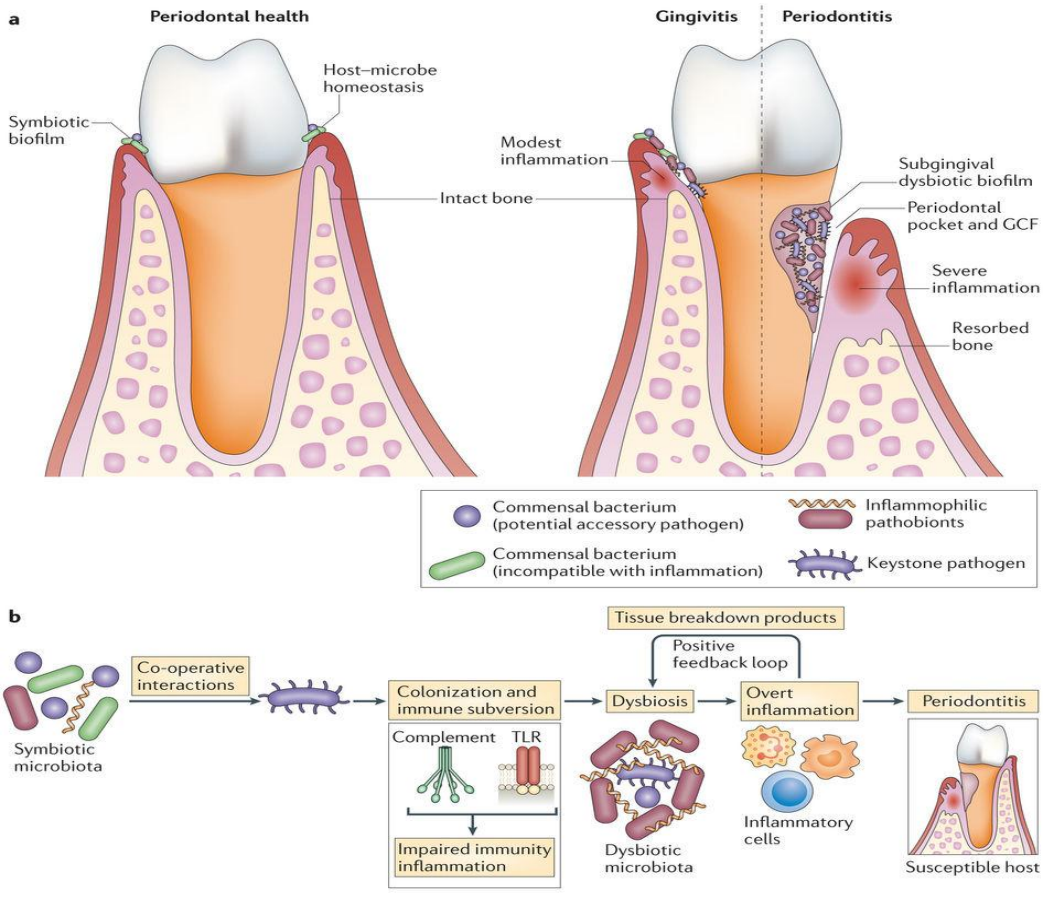
A recent metatranscriptomic study revealed that the majority of virulence factors that are upregulated in the microbiome of periodontitis patients is primarily derived from the previously underappreciated species that were not traditionally associated with periodontitis .**(Chen et al, 2014)**[14] These recent human microbiome analyses and animal model- based mechanistic studies collectively suggest that the pathogenesis of periodontitis involves polymicrobial synergy and dysbiosis.**(Lamont et al, 2012)**[44] The dysbiosis of the periodontal microbiota represents an alteration in the relative abundance or influence of individual components of the bacterial community (relative to their abundance or influence in health) leading to dysregulated host- microbial

crosstalk sufficient to induce destructive inflammation and bone loss. **(Hajishengallis et al, 2013)[32].**

Dysbiotic communities exhibit synergistic interactions that can enhance colonization, persistence, or virulence; bacteria known as keystone pathogens are involved in the breakdown of periodontal tissue homeostasis, whereas other, known as pathobionts, can trigger destructive inflammation once homeostasis is disrupted. Certaincommensals, though non-pathogenic by themselves intheoral environment, can promote keystone pathogen colonization and, as such, are implicated as accessory pathogens. P. gingivalis acts as a keystone pathogen at low colonization levels. Specifically, P. gingivalis induces the conversion from a symbiotic community structure to a dysbiotic one capable of causing destructive inflammation and periodontal bone loss. **(Darveau et al, 2012)[12]**

According to the polymicrobial synergy and dysbiosis (PSD) model, the host immune response is initially subverted by keystone pathogens with the help of accessory pathogens and is subsequently over-activated by pathobionts, leading to destructive inflammation in susceptible hosts. Therefore, according to the PSD model, periodontitis is not a bacterial infection in the classical sense (i.e., not caused by a single or a select few pathogens) but rather represents a polymicrobial community-induced perturbation of host homeostasis that leads to destructive inflammation in susceptible individuals.

MICROBIAL DYSBIOSIS



Periodontitis is induced by a polymicrobial bacterial community, wherein different members have distinct roles that synergize to cause destructive inflammation. Keystone pathogens, the colonization of which is facilitated by accessory pathogens, manipulate the host response leading from a symbiotic to a dysbioticmicrobiota, in which pathobionts over-activate the inflammatory response and cause destructive resorption of the supporting bone. Inflammation and dysbiosis reinforce each other by engaging in a positive feedback loop. Inflammatory tissue breakdown products are used as nutrients by the dysbioticmicrobiota, which further exacerbates inflammation. Periodontal pockets serve as a niche that can harbordysbiotic bacterial communities feeding on the inflammatory tissue breakdown products (e.g., degraded collagen peptides, haem-containing compounds) transferred with the gingival crevicular fluid (GCF).

Saliva

Saliva secretion are protective in nature because it maintains the oral tissues in physiologic state. Saliva exerts a major influence on plaque by mechanically cleansing the exposed oral surface, by buffering acids produced by bacteria and by controlling bacterial activity. Most of the salivary secretion is contributed by the major salivary glands (parotid, submandibular and sublingual) while some of it is by minor salivary glands.

Composition of saliva

Saliva is an exocrine secretion comprising of 99% water and 1% inorganic and organic substances, saliva is produced in human body in quantity of 1000 to 1500ml per day. PH of saliva is 6.35 to 6.85. Saliva that is expressed at rest is called unstimulated saliva, which covers, moisturizes and lubricates oral cavity. But 90% of daily salivary secretion is produced on stimulation in response to gustatory, olfactory, mechanical or pharmacological stimulus.

Organic substances present in saliva are protein in the form of glycoproteins- gamma globulins, histatins, albumin and enzymes. Inorganic substances are calcium, phosphate, sodium, potassium and magnesium. Electrolytes found are bicarbonate, calcium, chloride, phosphate.Enzymes found are x-amylase, invertase. Mucine, Immunoglobulins IgA,IgG,IgM,IgA_2, Lipids such as neutral lipids, Glycolipids, Phospholipids. Proteins- Protein rich proteins, salivary agglutinins, stathexin, histidine rich proteins, lactoferrin, lysozyme.

Functions of saliva

Saliva helps in digestion of food by aiding in providing taste and bolus formation. Plays a protective role by remineralization of enamel-using calcium and phosphorus ions, protection and lubrication of oral tissues.Bicarbonates and phosphates present in saliva buffers bacterial acids and foodthereby helps

in cleansing of oral cavity. IgA prevents attachment of micro-organisms to tooth and oral mucosa. Leucocytes – migrate in large number in saliva.Lysozyme acts as a scavenger as it splits bacterial cell wall. Sialo peroxidase enzyme acts as antibacterial against streptococci.

Saliva as a diagnostic tool

Collection of saliva is simple and non-invasive. The physiological state of periodontal health and disease can be determined by specific biomarkers present in saliva. Patients with susceptibility to disease and with active disease sites can be identified by changes in these biomarkers. For monitoring the effectiveness of therapy, salivary biomarker detectors can be used for point- of -care disease screening and detection.

Biomarkers in saliva

Biomarkers in saliva are alkaline phosphatase, amino peptidase, trypsin, gelatinase, esterase, collagenase. Immunoglobulins namely IgA, IgG, IgM and IgA. PDGF, EGF and VEGF. Lactoferrin, fibronectin and cystatin. A.Actinomycetemcornitans, P. Gingivalis, P. intermedia, C. rectus, T. denticola, B. forsythus, P. micros.Volatile compounds such as hydrogen sulphide, methyl mercaptan, picolines, pyridines. Prostaglandin, interleukins, MMP – 8, 9 and 13 in saliva are markers of soft tissue loss. MMP 8,9 and 13, alkaline phosphatase, osteonectin, collagen telo-peptides (ICTP) are markers of alveolar bone loss.

Factors influencing salivary flow rate and composition

The degree of hydration is potentially the most important factor. **Holmes et al 1964,** reported that when body water content was reduced by 8%, salivary flow decreased virtually to zero. In contrast, hyperhydration causes an increased salivary flow rate **(Shannon et al, 1972)**[72]**,** and also reported that subjects maintained in a standing or lying position had higher and lower flow rates, respectively. Salivary flow rate was increased in subjects who were smoking or subjected to olfactory stimulation. **Dawes and Chebib et al, 1972** found that flow rate was increased when preceded by gustatory stimulation. Salivary flow rate shows a circadian rhythm of high amplitude with peak value in the afternoon.

The circannual rhythm in flow rate of parotid saliva shows an acrophase in winter months and reduced flow rate in summer months. **(Shannon et al, 1966)**[71] Many classes of drugs, particularly those having anticholinergic action (antidepressants, anxiolytics, antipsychotics, antihistaminics and antihypertensives) cause reduced salivary flow as a side effect. **(Schubert et al, 1987)**[70] Unstimulated salivary flow rate increases with age upto age 15, thereafter it appears to be independent of age. **(Andersson et al, 1974)**[4] The unstimulated salivary flow rate is independent of both body weight and gland size. **(Ericson et al, 1968)** and is relatively unaffected by various psychic stimuli such as thought of food. **(Enfors et al, 1962)**[18]

Salivary flow index is the main factor affecting salivary composition and it varies in accordance with type, intensity and duration of the stimulus. As the salivary flow increases, the concentrations of total protein, sodium, calcium, chloride and bicarbonate as well as the pH increases to various levels, whereas the concentrations of inorganic phosphate and magnesium diminish.

Mechanical or chemical stimulus is associated with increased salivary secretion. Acid substances such as citric acid are considered potent gustatory stimuli.Other factors that influence total salivary composition are the relative contribution of the different salivary glands and the type of secretion. The percentage of contribution by the glands during unstimulated SF is 20% by the parotid glands, 65%-70% submandibular glands, 7% to 8% sublingual glands,<10% by the minor salivary glands.When salivary flow is stimulated, there is an alteration in the percentage of contribution of each gland withthe parotids contributing over 50% of the total salivary secretion.

The salivary secretions may be serous, mucous, or mixed. Serous secretions, produced mainly by the parotids, are rich in ions and enzymes. Mucous secretions are rich in mucins (glycoproteins) and present little or no enzymatic activity. They are produced mainly by the smaller glands. In the mixed glands, such as the submandibular and sublingual glands, the salivary content depends on the proportion between the serous and mucous cells.Physical exercise can alter secretion and induces changes in various salivary components, such as: immunoglobulins, hormones, lactate, proteins,

and electrolytes. In addition to the determined intensity of the exercise, there is a clear rise in salivary levels of α-amylase and electrolytes mainly sodium. During physical activities sympathetic stimulation appears to be strong enough to diminish or inhibit salivary secretion.

The intake of alcohol causes a significant reduction of stimulated salivary flow. This diminishment results from the altered release of total proteins and amylase as well as in diminished release of electrolytes.In some chronic diseases such as: pancreatitis, diabetes mellitus, renal insufficiency, anorexia, bulimia, and celiac disease, the amylase level is high.Alterations in the psycho-emotional state may alter the biochemical composition of saliva. Depression is accompanied by diminished salivary proteins.

Nutritional deficiencies may also influence salivary function and composition.Although short-term fasting reduces salivary flow, it does not lead to hyposalivation, and the flow is restored to normal values immediately after the fasting period ends. Stimulated salivary flow increases when preceded by gustatory stimulation in less than one hour before saliva collection

Navazesh et al. found the total unstimulated salivary flow is significantly lower in healthy patients between the ages of 65 and 83 years, in comparison with patients between the ages of 18 and 35 years. However, total stimulated salivary flow was significantly higher in the elderly in comparison with the younger persons. Histologic analyses have demonstrated with

advancing age the parenchyma of the salivary glands is gradually replaced by adipose and fibrovascular tissue, and the volume of the acini is reduced. However, functional studies among healthy individuals indicate aging itself does not necessarily lead to diminished glandular capacity to produce saliva.

Phosphate Metabolism

Phosphate plays several essential roles in our body. Phosphate isessential for proper mineralization of bone as a constituent of hydroxyapatite crystal. In addition, several phosphorylated proteins like osteopontin and dentin matrix protein 1 (DMP1) have been shown to regulate bone mineralization. Phosphate is also a constituent of biomembranes and nucleic acids. Furthermore, many phosphorylated metabolites such as adenosine triphosphate, 2,3-diphosphoglycerate, glucose-6phosphate and phosphorylated proteins are necessary for diverse actions of all cells such as energy metabolism, differentiation, proliferation and specific function of differentiated cells. In order to accomplish at least some of these functions, it seems to be necessary that concentration of extracellular phosphate is maintained in a certain range.

Hypophosphatemia can cause several abnormalities like muscle weakness, rhabdomyolysis, consciousness disturbance and rickets/osteomalacia characterized by impaired mineralization of bone matrix. Hyperphosphatemia can result in ectopic calcification. Although it is not entirely clear how intracellular phosphate level is regulated,

extracellularphosphateseemstoaffectittoacertaindegreeas hypophosphatemia is known to induce tissue hypoxia by lowering 2,3-diphosphoglycerate level in red blood cells. Serum phosphate level is regulated by several hormones including parathyroid hormone (PTH), 1, 25-dihydroxyvitamin D (1, 25(OH) 2D) and fibroblast growth factor 23 (FGF23).

There are several hundred grams of phosphate in adult human body. Approximately 85% of phosphate is present in bone or teeth as hydroxyapatite and 15% is present within cells. Therefore, extracellular inorganic phosphate is <1% of total phosphate. **(Penido et al, 2012)**[61] Serum phosphate is maintained by intestinal phosphate absorption, renal phosphate handling **(Craig et al, 2007)**[8] and equilibrium of extracellular phosphate with that in bone or intracellular fluid. In healthy adults, several hundred milligrams of phosphate are daily absorbed in intestine and nearly the same amount is excreted into urine, thereby maintainingphosphate balance. **(Bemdt et al, 2007)**[7]

Although intestinal phosphate was reported to rapidly modulate renal phosphate handling, the responsible signals for this regulation of renal phosphate handling are unidentified. Inaddition, there is a movement of several hundred milligrams of phosphate perday between extracellular fluid and intracellular pool or bone. Phosphate shift into cells is enhanced by insulin and respiratory alkalosis and occurs within minutes to hours. Respiratory

alkalosis is considered to enhance glycolysis by increasing intracellular pH and cause uptake of phosphate by cells.

This shift of phosphate into cells is less evident in metabolic alkalosis. In contrast, serum phosphate levels mainly regulated by renal handling of phosphate in a chronic state. In renal proximal tubules, 80–90%ofphosphate filtered through glomeruli is reabsorbed by type 2a and 2c sodium–phosphate cotransporters. **(Haller et al, 2010)** These cotransporters are expressed in brush border membrane of proximal tubular cells. The expression levels rather than the activity of the expressed transporters are considered to regulate proximal tubular phosphate reabsorption.

Pyrophosphate

Pyrophosphate anion is an acid anhydride of phosphate. It is stable in aqueous solution and hydrolyzes into inorganic phosphate. Pyrophosphate occurs in synovial fluid, saliva, blood plasma and urine at levels sufficient to block calcification and may be natural inhibitor of hydroxyapatite formation in extracellular fluid. ANK gene and Ectonucleotide pyrophosphatase (ENPP) may function to raise extracellular pyrophosphate. The plasma concentration of inorganic pyrophosphate has a reference range of 0.58 to 3.78 µM.

Pyrophosphatase is an enzyme that catalyzes the conversion of one molecule of pyrophosphate to two phosphate ions. By promoting the rapid hydrolysis of pyrophosphate, inorganic pyrophosphatase provides the driving

force for the activation of fatty acids destined for oxidation. When all three H+ ions are lost from orthophosphoric acid, an orthophosphate ion is formed. Orthophosphate is the simplest in a series of phosphates.

Salivary Pyrophosphate

Pyrophosphate, a byproduct of many biosynthetic reactions, present in saliva inhibits crystallization and competes with orthophosphate for minerals, thus having a strong inhibitory effect on plaque mineralization. Various studies in this regard have proved that severity of calculus formation is inversely proportional to pyrophosphate concentration in saliva. (**Sawinski et al, 1967**)[66] Enzyme alkaline phosphatase present in saliva and in plaque releases inorganic orthophosphate from organic phosphate, increasing concentration of orthophosphate locally, which reacts with calcium ions leading to precipitation of insoluble calcium apatite crystals.

Enzyme pyrophosphatase plays an exciting role in formation of calculus by hydrolyzing, pyrophosphate to orthophosphate, thus removing its inhibitory power and simultaneously converting into booster by increasing the concentration of orthophosphate. (**Jenkins et al, 1978**)[40].Pyrophosphate at a concentration above 0.125 mM caused precipitation of calcium pyrophosphate and decreased the rate of orthophosphate precipitation. Pyrophosphate at a higher concentration of 0.4 mM caused a reduction in precipitation rate. (**Mukherjee et al, 1968**)[57].

The production of pyrophosphate in saliva was reported by **Rapp et al**, 1960, who found its accumulation, when pyrophosphatase activity was inhibited by fluoride. The first quantitative determination was done by **Sawinski and Cole**[66] in 1965. The result was 0.17 to 1.03 mg of P/100 ml of saliva. The mean value is 0.47 ± 0.16. **Vogel and Amdur**[83] (1967) applied the calorimetric PPi assay method of **Flynn et al** (1954) and got slight higher value 400 µM. **Hausmann et al (1970)**[35] found much less PPi in saliva. They separated it by ion exchange chromatography and after acid hydrolysis by **Russel et al (1971)**. According to their results saliva contained only 0.92 µM.

Materials and Methods

MATERIALS AND METHODS

The material for this study consisted of 80 patients (45 males and 35 females) with mean age 32.61years . The subjects were selected from patients who reported to the out patient section, Department of Periodontics, Ragas Dental College, Chennai in the year 2017. The patients were informed that this research work is no way directly related to the therapy or cure of the disease. Full mouth was examined and **Greene and Vermillion** Oral hygiene index was used for calculus assessment. Depending on calculus scores, patients were divided into three groups, mild/ no calculus, moderate and severe calculus formers. Bleeding scores were recorded for all the patients using gingival bleeding index. Patients with mild/ no calculus were treated as control group. The patients with mild/ no calculus were clubbed into one group and served as controls as there was no patient examined in this study who did not report with at least a fleck of calculus somewhere in the oral cavity.

Inclusion criteria

- The patients were selected with same demographic and socioeconomic status and similar oral hygiene habits (brushing teeth using tooth paste and tooth brush once a day without using any other oral hygiene aids such as floss or interdental brush). This was done to ensure that oral hygiene practices do not act as confounders.
- Patient were advised to do their routine oral hygiene measures and restrict carbohydrate diet from one hour prior to sample collection, so

that the various other factors influencing the salivary composition of pyrophosphate were maintained constant in all the patients.

- Patients with deposits covering only apical 1/3rd of the crown were classified as mild, upto middle 1/3rd as moderate and upto coronal 1/3rd as severe group.
- Patients included had no history of scaling procedure done in past1 year.

Exclusion criteria

Patients having systemic diseases such as

- Diabetes mellitus
- Immune compromised patients
- Illness affecting salivary gland and salivary flow rate.
- Under any medication affecting salivary flow rate.
- Adverse oral habits tobacco chewing, smoking and alcohol consumption.

Saliva collection

Salivary collection was done according to the technique by **Navazesh et al 2008**. The subjects were advised to refrain from intake of any food or beverage one hour before sampling. The subjects were advised to relax for five minutes after rinsing his or her mouth several times with distilled water. The patient was asked to lean the head forward over the container with the

mouth slightly open and allow the saliva to drain into the container with the eyes open. The time lasted for saliva collection was 5 min. The saliva was collected in a sterile disposable plastic container and were centrifuged at 2800 rpm for 15 min and were stored at – 20^0C and used for further analysis.

Armamentarium

1. Autoclavable containers for saliva collection
2. Ice pack (for transfer)
3. Centrifuge tubes
4. Eppendorf tubes
5. Micro pipette
6. Laboratory centrifuge
7. Deep freezer
8. Test tubes
9. Elisa plate
10. Pyrophosphate assay kit

Procedure

Full mouth plaque score, bleeding score and calculus score, probing depth and clinical attachment loss were recorded. Unstimulated whole saliva

was collected from all the patients between 10 am and 12 pm using the method described by **Navazesh**, and stored in -20^{0}C until further use. Centrifugation of saliva at 2800 rpm was done for 15min at room temperature to remove salivary proteins. The clear supernatant saliva was analyzed for pyrophosphate levels by Elisa method using pyrophosphate assay kit.

ELISA method

Antigen from the sample was attached to a surface. Then, specific antibody was applied over the surface so that it can bind to the antigen. This antibody was linked to an enzyme, and in the final step, a substance containing the enzymes substrate was added. The subsequent reaction produced a detectable signal, a color change in the substrate.

ELISA was performed with at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen was immobilized on a polystyrene microtiter plate by adsorption to the surface. After the antigen was immobilized, the detection antibody was added, forming a complex with the antigen. The detection antibody was covalently linked to an enzyme. Between each step, the plate was washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate was developed by adding an enzymatic substrate to produce a visible signal, which indicated the quantity of antigen in the sample.

ELISA involved detection of an "analyte" the specific substance whose presence was quantitatively analyzed in saliva sample by a method that continues to use liquid reagents during the "analysis" controlled sequence of biochemical reactions that generated a signal which was quantified and interpreted as a measure of the amount of analyte in the sample that stays liquid and remains inside a reaction chamber or well needed to keep the reactants contained. The quantitative reading was based on detection of intensity of transmitted light by spectrophotometry, which involved quantitation of transmission of specific wavelength of light through the liquid as well as the transparent bottom of the well in the multiple-well plate format.

The sensitivity of detection was dependent on amplification of the signal during the analytic reactions. The signal was generated by enzymes which were linked to the detection reagents in fixed proportions to allow accurate quantification. The ligand-specific binding reagent was immobilized, coated and dried onto the transparent bottom and also side wall of a well, which was constructed as a multiple-well plate known as the "ELISA plate".

PYROPHOSPHATE ASSAY

Preparation of Assay Solutions

All the four components were thawed at room temperature before use 200X PPi Sensor Stock Solution was prepared by adding 50 µL of DMSO (Component D) into the vial of PPi Sensor (Component B) to make 200X PPi

Sensor Stock Solution. 25 µL of the PPi Sensor Stock Solution was enough for one 96-well plate. The unused PPi Sensor Stock Solution was divided into single-use aliquots and stored at -20 °C and protected from light. Assay Solution was prepared by adding 25 µL of 200X PPi Sensor Stock Solution to 5 mL of Assay Buffer (Component A), and mixed well.

Preparation of Pyrophosphate Standards and Test Samples

Pyrophosphate 1mM Standard Solution was prepared by adding 10 µL of 50 mM Pyrophosphate Standard (Component C) into 490 µL of Assay Buffer (Component A), to make 1 mM pyrophosphate standard solution. 50 µL of 1 mM pyrophosphate standard solution was added into 450 µL of Assay buffer (Component A) to get 100 µM pyrophosphate standard solution, and then 200 µL of 100 µM pyrophosphate standard solution was taken to perform 1:3 serial dilutions to get 30, 10, 3, 1, 0.3, 0.1 and 0 µM serially diluted pyrophosphate standards.Serially diluted pyrophosphate standards and pyrophosphate-containing test samples were added into a solid black 96-well microplate.

Pyrophosphate Assay:

50 µL/well of Assay Solution was added to the wells of pyrophosphate standards, blank control, and test samples and the reagents were mixed thoroughly. Incubated at room temperature for 10 to 30 minutes. Fluorescence in a microplate reader was measured at Ex/Em 316/456 nm.

Statistical Analysis

The mean calculus scores and pyrophosphate concentration between mild, moderate and severe groups were compared using oneway Anova test. Kruskal-Wallis test and Mann-Whitney test were used for grouping the variables in three groups. Correlation analysis between calculus and pyrophosphate concentration in mild, moderate and severe groups were done using Pearson correlation values and Kendall's tau test.

Photographs

FIG.1. MILD CALCULUS

FIG.2. MODERATE CALCULUS

FIG.3. SEVERE CALCULUS

FIG .4.DISPOSABLE SALIVA CONTAINERS

FIG.5. EPPENDORF TUBES

FIG.6. SAMPLE COLLECTION

FIG.7. CENTRIFUGE

FIG.8. AUTO WASHER

FIG.9. MICRO PIPETTE AND TIPS

FIG. 10 .MICRO PLATE READER

FIG.11. REFRIGERATOR

FIG.12.COMPUTER

FIG.13. IMMUNOANALYZER

PROCEDURE

FIG.14. MICROPLATE WELL DURING PROCEDURE

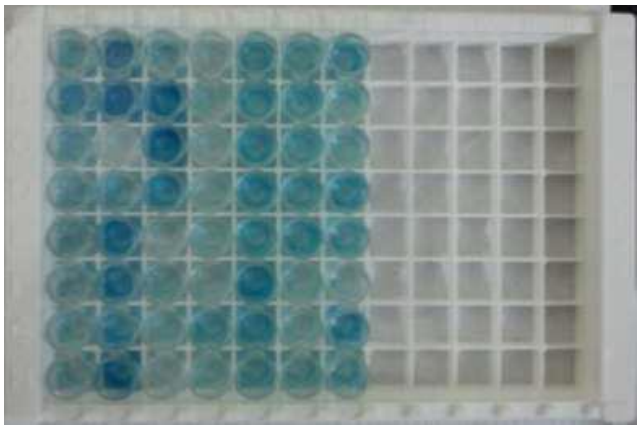


FIG.15. MICROPLATE WELL AFTER STOP REAGENT

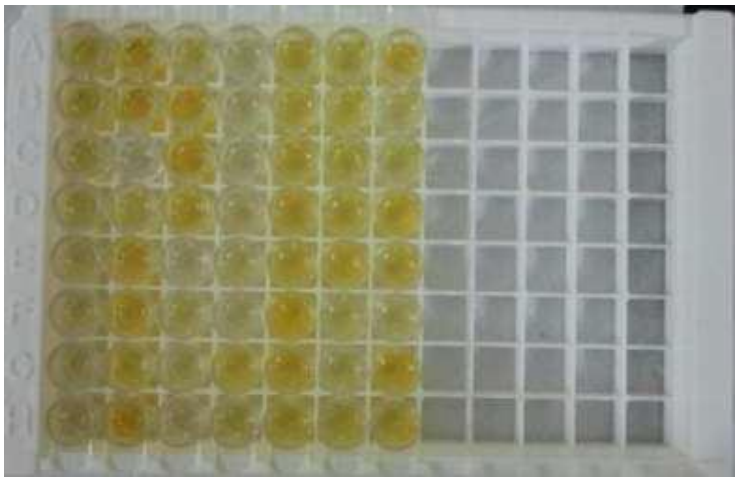


FIG.16. <u>RESULTS PRINTED OUT BY THE IMMUNO ASSAY ANALYZER</u>

Results

RESULTS

This study was carried out in 80 patients with same demographic and socio economic status who reported to the out patient section, Department of Periodontics, Ragas dental college and hospital Chennai. The age distribution of the patients ranged from 30 to 45 years with a mean range of 32.6 years. Depending on calculus scores, patients were divided into mild/ no calculus, moderate and severe groups. The saliva samples were collected and analyzed for the concentration of pyrophosphate using pyrophosphate assay kit by the Elisa method.

The mean concentration of pyrophosphate in three groups mild/ no calculus, moderate and severe calculus formers was found to be 15.81, 6.69 and 2.17 µM respectively There was a statistically significant difference between the mild/ no calculus group and moderate and severe groups. ($p< 0.05$). The mean concentration of pyrophosphate was found to be lower in the severe calculus formers and higher in case of mild/no calculus formers. (Refer fig 2).This result correlates with the finding that pyrophosphate concentration is inversely proportional to the severity of calculus formation.

The mean bleeding scores using Gingival bleeding index in three groups mild/ no calculus, moderate and severe was found to be 12.36, 21.89 and 42.55 respectively. The difference in the bleeding scores between three groups was found to be statistically significant. The bleeding score was found to be higher in severe calculus formers.

Tables & Graphs

TABLE 1 : MILD/ NO CALCULUS GROUP

S.NO	PATIENT NAME	AGE/SEX	PYROPHOSPHATE CONC.IN µM
1.	Saranya	30/F	23
2.	Gomathi	19/F	23.53
3.	Sowjanya	30/F	23.1
4.	Siva	33/M	5.34
5.	Bhavani	29/F	5.2
6.	Kumar	39/M	4.53
7.	Sana	26/F	9.99
8.	Sureshkumar	25/M	4.2
9.	Mohan	37/M	22.51
10.	Sugendran	30/M	9.45
11.	Pratap	30/M	17.31
12.	Kalpana	20/F	16.75
13.	Kumar	42/M	9.48
14.	Vijayapriya	28/F	16.14
15.	Davis	31/M	17.77
16.	Senthil kumar	40/M	16.41
17.	Mohd. Guddu	30/M	28.71
18.	Priya	23/F	12.11
19.	Amila	18/F	7.74
20.	Raja	46/M	19.8
21.	Manju	26/F	3.32
22.	Kottaiammal	42/F	2.31
23.	Balaji	33/M	24.57
24.	Prasanth	30/M	16.75
25.	Geetha	30/F	24.03
26.	Anuruthran	23/M	40.93
27.	Yuvaraj	19/M	37.56
28.	Usharani	37/F	0.36

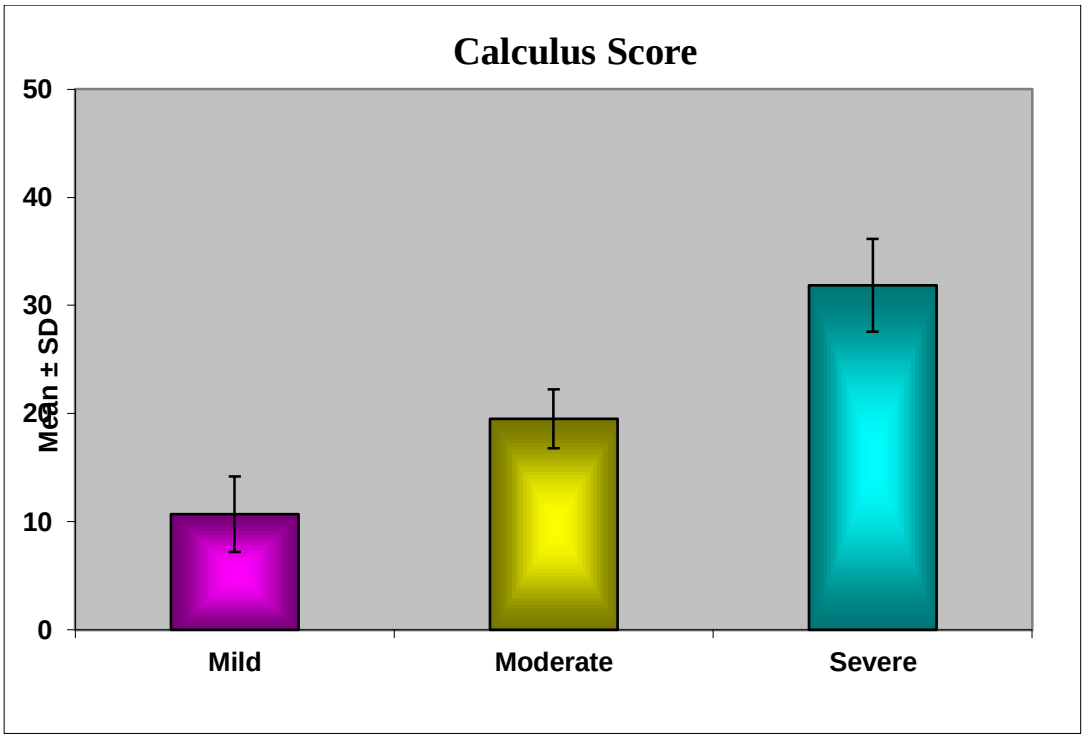
TABLE 2 : MODERATE CALCULUS GROUP

S.NO	PATIENT NAME	AGE/SEX	PYROPHOSPHATE CONC.IN µM
1.	Deivanayaki	42/F	4.14
2.	Kuppusamy	46/M	3.58
3.	Mahendrakumar	50/M	6.39
4.	Jeni	31/F	5.4
5.	Thooyavan	22/M	7.25
6.	Rani Jaganathan	37/F	5.46
7.	Malan basha	32/M	5.43
8.	Muthu	30/M	3.42
9.	Sivakumar	30/M	3.46
10.	Almas begum	30/F	2.61
11.	Mohanraj	27/M	3.3
12.	Sivaprakasam	21/M	3.3
13.	Vijayanand	27/M	4.36
14.	Goutham	25/M	3.41
15.	Rajasekaran	35/M	3.45
16.	Dinesh	26/M	4.14
17.	Gowtham	20/M	3.2
18.	Thenmozhi	27/F	3.75
19.	Ponvignesh	19/M	2.68
20.	Mahesh	27/M	9.99
21.	Usha	36/F	12.52
22.	Sathishkumar	35/M	12.54
23.	Periasamy	21/M	4.53
24.	Meena	33/F	12.86
25.	Kajamohdeen	34/M	9.81
26.	Elsi John	47/F	1.43
27.	Perumal	48/M	0.6
28.	Sabikha	26/F	12.47
29.	Sathish	23/M	0.36
30.	Phaikin	29/F	22.01
31.	Mukul biswas	24/M	29.54

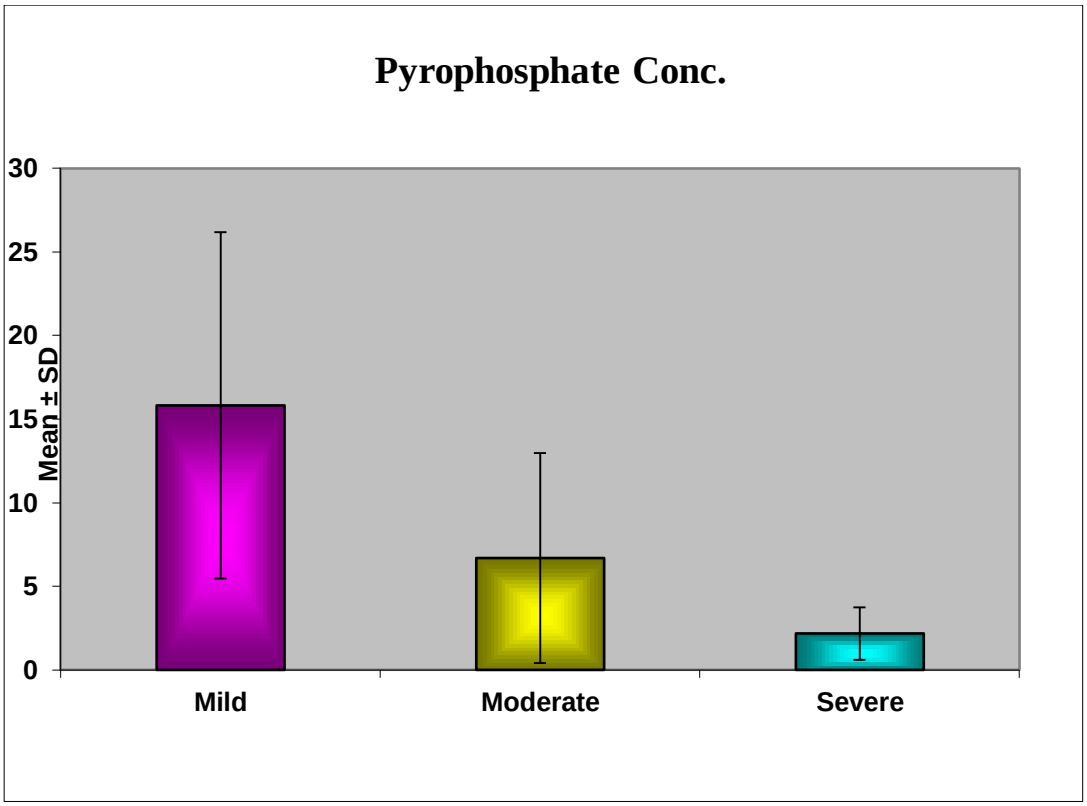
TABLE 3: SEVERE CALCULUS GROUP

S.No	PATIENT NAME	AGE/SEX	PYROPHOSPHATE CONC.IN µM
1.	Usha devi	45/F	5.43
2.	Banumathi	45/F	3.68
3.	Ganeshkumar	44/M	2.1
4.	Shubham	19/M	5.36
5.	Maniammal	26/F	0.28
6.	Maheswari	34/F	2.81
7.	Parvathi	65/F	2.36
8.	Gunalan	68/M	1.19
9.	Salomi	27/F	2.73
10.	Rani	45/F	1.72
11.	Parameswari	46/F	1.9
12.	Ansari	30/M	2.38
13.	Suresh	36/M	2.91
14.	Farveen	27/F	3.86
15.	Devi	32/F	2.6
16.	Raja	35/M	0.27
17.	Thomas mathew	35/M	0.29
18.	Murugadas	26/M	0.58
19.	Karthick	19/M	2.63
20.	Umashankar	25/M	0.09
21.	Rajeswari	28/F	0.6

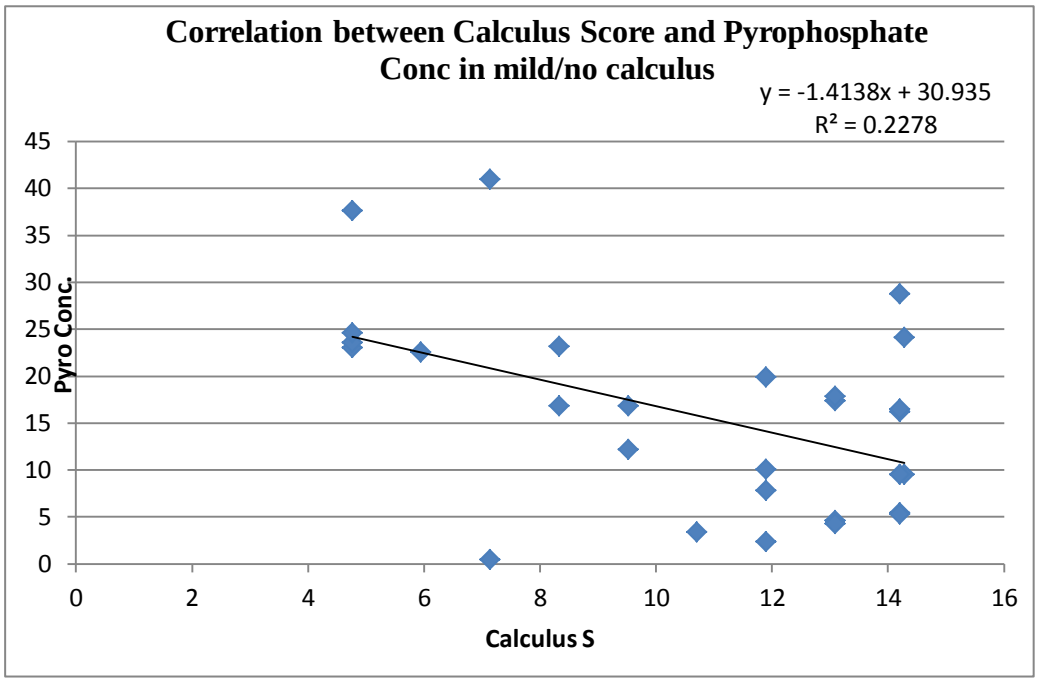
GRAPH 1: CALCULUS SCORE



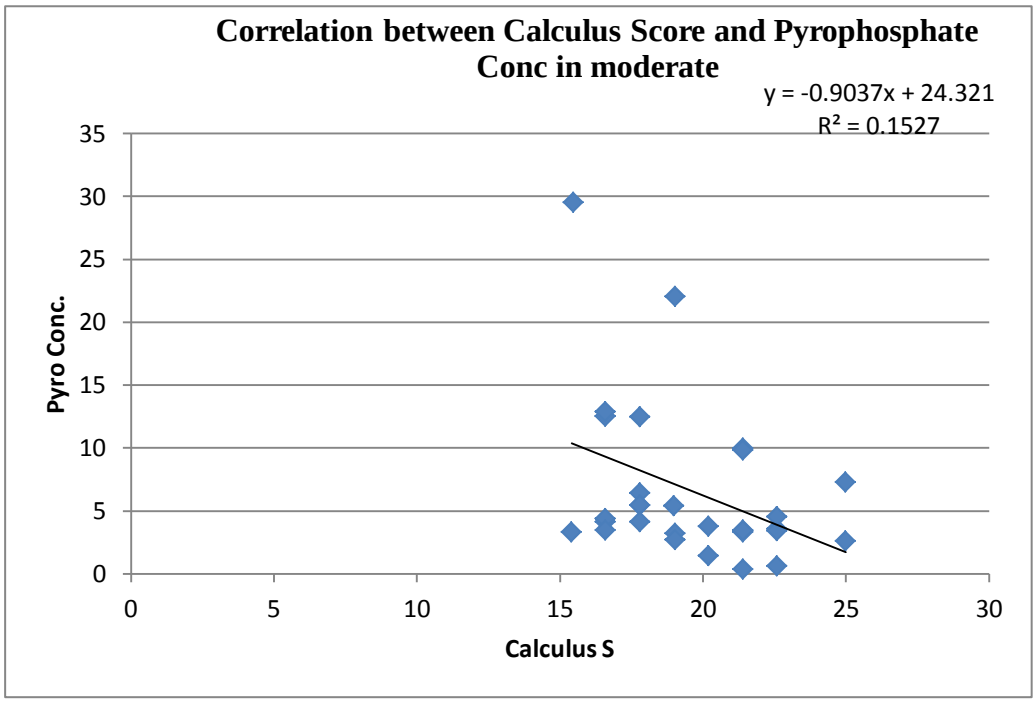
GRAPH 2: PYROPHOSPHATE CONCENTRATION



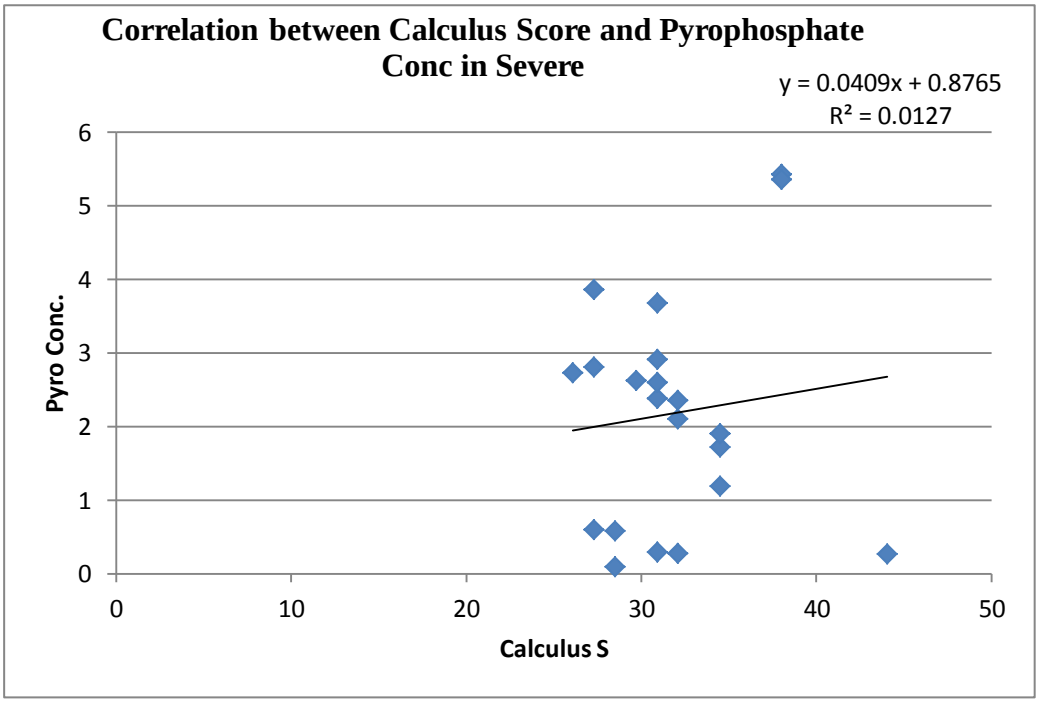
GRAPH 3: CORRELATION BETWEEN CALCULUS SCORE AND PYROPHOSPHATE CONCENTRATION IN MILD/ NO CALCULUS GROUP



GRAPH 4: CORRELATION BETWEEN CALCULUS SCORE AND PYROPHOSPHATE CONCENTRATION IN MODERATE GROUP



GRAPH 5: CORRELATION BETWEEN CALCULUS SCORE AND PYROPHOSPHATE CONCENTRATION IN SEVERE GROUP



Discussion

DISCUSSION

The initial damage to the gingival margin in periodontal disease is due to the immunologic and/or enzymatic effects of microorganisms in plaque. (**Schroeder**, 1969)[68] However, the process is enhanced by the supragingival calculus, which provides retention and thus promotes new plaque accumulations. It is difficult to separate the effects of plaque and calculus on the gingiva because calculus is always covered with a non-mineralized layer of plaque. Various longitudinal and crosssectional epidemiological studies have demonstrated clear association between the presence of calculus and periodontitis. **(Grossi et al, 1994)**[31]

Calculus formation and inhibition is the result of interaction of certain components present in saliva, namely pyrophosphate, pyrophosphatase and orthophosphate. Enzyme alkaline phosphatase present in saliva and in plaque releases inorganic orthophosphate from organic phosphate, increasing concentration of orthophosphate locally, which reacts with calcium ions leading to precipitation of insoluble calcium apatite crystals. **(Jenkins et al, 1978)**[41]. Pyrophosphate present in saliva competes with orthophosphate for minerals, thus having a strong inhibitory effect on plaque mineralization.

The composition of saliva can vary rapidly according to the flow rate, the type of stimulation and the time of day. At higher flow rates, phosphate concentration decreases in saliva. **(Shannon et al, 1962)**[71] Parotid saliva is

relatively low in calcium and high in phosphate. In this study, flow rate of saliva is maintained constant by collection of unstimulated whole saliva using Navazesh technique. Salivary flow rate shows a circadian rhythm of high amplitude, with an acrophase in the afternoon **(Dawes et al, 1974),** it is preferable that the time of day for saliva collection be standardized. Significant rhythms were detected in inorganic phosphate content in both stimulated and unstimulated saliva. Hence, saliva collection was done at the same time (10 am – 12 pm) in all the subjects included in this study to minimize the effects of circadian rhythm. Centrifugation of saliva was done to remove the salivary proteins, both cellular and bacterial proteins were removed during the centrifugation process. **(Campbell et al, 2012)** Therefore, standardization of saliva collection and processing techniques were done for minimizing the effects on variations in salivary pyrophosphate concentration.

The influence of oral hygiene practices on plaque and calculus formation is well established. The samples included in this study were derived from population pool that reported to the out patient Department of Periodontics, Ragas Dental College and Hospital. All these patient had similar socioeconomic and demographics, as a result of which they had similar lifestyle, dietary patterns and oral hygiene practices. History revealed that all of them brush once a day using horizontal strokes with tooth paste and did not use interdental aids or chemical plaque control methods. Only patients who had no previous history of scaling in past 1 year where included in the study.

All of them visited dental clinics sporadically and only in case of pain/ discomfort.

In this study the demographic data regarding heterogenecity was obtained to establish that

1. Difference in severity of calculus formation was not due to difference in oral hygiene practices alone.
2. There was no systemic or salivary gland related factors that could influence pyrophosphate levels.

   It must however be noted that perfect standardization of brushing time and pattern is almost impossible to achieve. It cannot therefore be positively stated that oral hygiene had no influence at all on the presence of calculus, we are only suggesting that it may not be the only variable.

Among the patients examined for the study everybody included in the study had atleast some calculus deposits in the oral cavity as per the OHI index used for this purpose. Index teeth as per OHI was not used in study because discrepancies in partial mouth scoring and full mouth scoring leading to over/under representation of periodontal disease have been previously reported. Mild calculus group was therefore combined with the no calculus and was treated as control for comparison between moderate and severe calculus groups.

As per well established literature, all the patients included in the study exhibited plaque deposits on the calculus. The bleeding scores closely correlated with severity of calculus, once again underscoring the fact that calculus may act as a primary plaque retentive factor and thereby promote gingival inflammation. This indicates that severity of calculus formation plays an important role in periodontal disease progression.

In this study, an attempt was made to identify the influence of pyrophosphate in saliva on calculus formation and also to ascertain the significant relationship between them in mild/ no calculus, moderate and severe calculus formers. Pyrophosphate assay was used because it is a simple and convenient method for determination of pyrophosphate. Elisa method was used to determine the concentration of pyrophosphate in the salivary samples. The results indicate that in mild/no calculus group, mean level of pyrophosphate is 15.81µM, in moderate group it is 6.69µM and in severe group it is 2.17µM. This difference is statistically significant. It is observed that the mean of pyrophosphate declined considerably in severe calculus group. Thus, the results are indicative that the pyrophosphate present in saliva have a significant role to play in inhibition of calculus formation.

This observation is in agreement with the observations of **Sawinski and cole**[66] that the severity of calculus formation is inversely proportional to the pyrophosphate content. As the concentration of pyrophosphate increases, the calculus score show a steady decrease. This is highly suggestive of the

inhibitory role of pyrophosphate in formation of calculus. Similar observations in relation to pyrophosphate have been reported by **Vogel and Amdur**[83]**, Bisaz et al**[17]**, and Edgar and Jenkins**[20] in parotid and submaxillary saliva, except for the fact that there is variation in the concentration of pyrophosphate in whole saliva than in the other two sources. A therapeutic implication of this study is that pyrophosphate may serve as a target for prevention, treatment of periodontal disease.

Pyrophosphate was first tested as an anticalculus agent by **Kinoshita and Muhlemann**[42]. A decrease in calculus formation of was observed in the test group, but this difference was not statistically significant. Bisphosphonates represent another group of synthetic pyrophosphate analogs that inhibit crystal growth and prevent calculus formation, and have been used as potential anticalculus agents. Inhibitory effect of TRK-530, was studied and was found to inhibit the formation of dental calculus **(Sikder et al, 2004)**

A number of anticalculus dentrifices containing pyrophosphates and bisphosphonates have been developed and proved to be efficacious. **Herforth**[36]**, Sturzenberger**[76] and **Suomi et al**[78]**,** studied the effects of EHDP dentrifice and found that sodium etidronate dentrifice reduced preformed calculus levels. **Gaffer and Moreno**[23] tested the effect of PBTA on crystal growth and demonstrated a significant decrease in the incidence of calculus formation. Recently, the anticalculus effect of a triclosan mouthwash

containing phytate was tested and it was found to be an effective anticalculus agent. **(Grases et al, 2009)**[27]

Previous reports have indicated that pyrophosphate may be a target for diseases other than that in the oral cavity. In 2000, **Ho et al**[37], reported work on the gene for progressive joint ankylosis in mice, known as ANK. Recent data showed that human analog of this ANKH gene may play a role in familial calcium pyrophosphate dehydrate deposition (CPPD) disease **(Pendelton et al, 2002)**[60]. Probenicid was found to decrease the amount of pyrophosphate released by cultured chondrocytes and inhibit transport by the ANK protein. **(Rosenthal et al, 1994)**[65]

Within the limits of study, we have established that salivary pyrophosphate levels may influence calculus formation even in patients with similar life style and oral hygiene practices. We have also reaffirmed the literature evidence that has correlated calculus deposits with gingival inflammation. The mean bleeding scores using gingival bleeding index in three groups, mild/ no calculus, moderate and severe was found to be 12.36, 21.89 and 42.55 respectively. The difference in bleeding scores between three groups was found to be statistically significant. ($p < 0.05$) The bleeding score was found to be higher in severe calculus formers. Gingivitis has been long reported to be an important factor that determines periodontal disease progression. Several authors have indicated that inflammation in the gingiva may be a sign of disease activity as well as prognostic indicator for therapeutic

intervention. In this study therefore, salivary pyrophosphate may be linked indirectly to disease progression. The etiopathogenic role may be through plaque deposits on the calculus that favour gingival inflammation.

However, there are several other factors present in saliva that may play important role in calculus formation such as protein polysaccharide complexes, proteolipids, proteoglycans, phosphatases and esterases released by microorganisms, urea and ammonia which causes increase in salivary and plaque pH, seeding agents from plaque bacteria and differences in carbondioxide tension in saliva. A more detailed assessment of all these factors taken together will give a clearer indication of the effect of pyrophosphate in calculus formation and whether it is advisable for use as a therapeutic target.

Further longitudinal studies with assessment of all the mineralization regulators are required to obtain a clearer picture.

Summary and Conclusion

SUMMARY AND CONCLUSION

Calculus was once regarded to be the primary etiologic factor in periodontal disease. The findings that mineralized plaque was covered by an unmineralized bacterial layer in supagingival and subgingival calculus changed this perception. This study consisted of 80 patients with same demographic and socio economic status. Based on calculus scores, patients were divided into mild/ no calculus, moderate and severe groups. Salivary pyrophosphate levels in mild/no calculus, moderate and severe groups were 15.81, 6.69 and 2.17 µM respectively. There was a statistically significant difference between mild/ no calculus group and moderate group and severe groups ($p < 0.05$).

These results indicate that salivary pyrophosphate may inhibit calculus formation and indirectly affect periodontal disease progression. Pyrophosphate containing dentrifices could be potential anticalculus agents. Further studies should be carried out to assess the activity of salivary pyrophosphate in calculus formation and periodontal disease progression.

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Annexures

ANNEXURE I

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA
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TO WHOM SO EVER IT MAY CONCERN

Date: 20.12.2017

Place: Chennai

From

The Institutional Review Board

Ragas Dental College & Hospital

Uthandi,

Chennai- 600119.

The dissertation topic titled "**INFLUENCE OF SALIVARY PYROPHOSPHATE LEVELS ON CALCULUS FORMATION AND PERIODONTAL DISEASE PROGRESSION**." submitted by **Dr. N. SAKTHI GANESH** has been approved by the Institutional Ethics Board of Ragas Dental College and Hospital.

DR. N.S.AZHAGARASAN, MDS.,

Member Secretary,

Institutional Ethics Board,

Ragas Dental College & Hospital,

Uthandi,

Chennai- 600119

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI-600 119.

ANNEXURE II

URKUND

Urkund Analysis Result

Analysed Document: PYRO THESIS .docx (D35070989)
Submitted: 1/27/2018 7:39:00 AM
Submitted By: drsakthiganeshn@gmail.com
Significance: 7 %

Sources included in the report:

COMPARISON OF SALIVARY LEVELS OF AMYLASE AND MUCIN IN CHRONIC GENERALISED PERIODONTITIS PATIENTS BEFORE AND AFTER PHASE I~1.pdf (D34332681)
https://pocketdentistry.com/7-the-role-of-dental-calculus-and-other-local-predisposing-factors/

Instances where selected sources appear:

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