ESTIMATION OF LEVELS OF ORTHOPHOSPHATE, PYROPHOSPHATE AND PYROPHOSPHATASE IN SALIVA – A BIOCHEMICAL STUDY

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MASTER OF DENTAL SURGERY

BRANCH II

DEPARTMENT OF PERIODONTICS

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CERTIFICATE

This is to certify that this dissertation titled "**ESTIMATION OF LEVELS OF ORTHOPHOSPHATE, PYROPHOSPHATE AND PYROPHOSPHATASE IN SALIVA – A BIOCHEMICAL STUDY**" is a bonafide record of work done by **Dr. M. AZWEER HUSSAIN** under our guidance and to our satisfaction, during his postgraduate study period of 2011 – 2014.

This dissertation is submitted to **THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY** in partial fulfillment for the award of the degree of **MASTER OF DENTAL SURGERY – PERIODONTICS, BRANCH II.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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DECLARATION

TITLE OF DISSERTATION	**ESTIMATION OF LEVELS OF ORTHOPHOSPHATE, PYROPHOSPHATE, AND PYROPHOSPHATASE IN SALIVA – A BIOCHEMICAL STUDY.**
PLACE OF STUDY	**SRI RAMAKRISHNA DENTAL COLLEGE & HOSPITAL, COIMBATORE – 641006**
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I hereby declare that no part of the dissertation will be utilized for gaining financial assistance/any promotion without obtaining prior permission of the Principal, Sri Ramakrishna Dental College and Hospital, Coimbatore. In addition, I declare that no part of this work will be published either or in electronic media without the permission of the guide who has been actively involved in the dissertation. The author has the rights to publish the work solely with the prior permission of the Principal, Sri Ramakrishna Dental College and Hospital, Coimbatore.

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ABSTRACT

AIM: The aim of this study was to estimate the levels of orthophosphate, pyrophosphate, and enzyme pyrophosphatase from unstimulated saliva in humans using biochemical analysis, and to evaluate their activity with regard to the formation and inhibition of the dental calculus with the reference to the amount of orthophosphate, pyrophosphate, and enzyme pyrophosphatase present in the human saliva.

MATERIALS & METHODS: This clinico-biochemical prospective cross-sectional study was conducted in Department of Periodontics, Sri Ramakrishna Dental College & Hospital, Coimbatore. The study included 60 systemically healthy subjects, age ranging from 15 – 30 years, with presence of chronic generalized marginal gingivitis. The subjects were divided into 4 groups. Group I consisted of 15 subjects who had calculus index score of 0.40 to ≤1.00. Group II consisted of 15 subjects who had calculus index score of >1.00 to ≤1.30. Group III consisted of 15 subjects who had calculus index score of >1.30 to ≤1.80. Plaque group was considered as Group IV included 15 subjects. Statistical analysis was calculated by using analysis of variable (ANOVA) and inter group comparison was done using Post Hoc test of Tukey HSD method.

RESULTS: As the calculus index score increases, there was a significant gradual increase in the levels of orthophosphate and enzyme pyrophosphatase. Eventually, there was a significant decrease in the levels of pyrophosphate in each of the calculus group. Comparatively, in the plaque group, the levels of pyrophosphate is increased significantly. And the orthophosphate and enzyme pyrophosphatase were in very low levels when compared to the values from the calculus groups.

CONCLUSION: The present study concludes that orthophosphate, pyrophosphate, and the enzyme pyrophosphatase play a significant role in formation and inhibition of the dental calculus. It is also evident that pyrophosphate being the strong inhibitor in formation of dental calculus acts as an anticalculus agent present in the human saliva.

KEYWORDS: Dental calculus; orthophosphate; inorganic pyrophosphate; pyrophosphatase; dental plaque; saliva.

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ANOVA – ANALYSIS OF VARIANCE

Hcl – HYDROCHLORIC ACID

HSD – HONESTLY SIGNIFICANT DIFFERENCE

mM – MILLIMOLAR

N – NORMALITY

PDI – PERIODONTAL DISEASE INDEX

RPM – REVOLUTIONS PER MINUTE

TCA – TRICHLOROACETIC ACID

INTRODUCTION

A Greek physician named Hippocrates in 460 – 377 BC who was the founder of the modern medicine, noticed the deleterious effects on the teeth and the gums of the pituita, which are the hard deposits on the tooth surface. The arabian physician and surgeon Albucasis in 936 – 1013 AD had noticed the relationship between the calculus and the gingival diseases and the need for the removal of the deposits which was considered to be the causative factor for the gingival disease. **(Robert J. Genco).**[1]

The gingival disease has been broadly classified into two large categories, which depends on the presence or the absence of dental plaque. Further, the two categories of plaque-induced gingival diseases are those, that are affected by local factors and those which are modified by specific systemic factors along with the local factors that occurs in the host, which includes the endocrine system, hematologic diseases, drugs, or malnutrition. Plaque-induced gingivitis is an inflammation of the gingiva resulting from bacteria located at the gingival margin. **(Angelo Mariotti 1999).**[2]

The supragingival plaque has been generally recognised as the primary etiological factor for gingivitis, along with other predisposing factors like the dental calculus, faulty restorations, complications associated with orthodontic therapy, self-inflicted injuries, use of tobacco, and others. **(Theilade J & Schroeder H 1966).**[3]

The dental calculus is a hard deposit that is formed by mineralization of dental plaque, which is generally covered by a layer of unmineralized plaque on its superficial surface. **(Stanley P. Hazen 1995).**[4]

The dental calculus is classified as supragingival calculus and subgingival calculus, according to the relation to the marginal gingiva. The supragingival and subgingival calculus contains 37% and 58% mineral content by its volume, respectively. **(Ye Jin & Hak-Kong Yip 2002).**[5] The dental calculus is the mineralized dental plaque, which consist of crystals of various calcium phosphates. **(Schroeder H 1969).**[6]

The formation and inhibition of the dental calculus occurs as a result of the interaction between certain important inorganic components which are present in saliva, like orthophosphate, pyrophosphate, and enzyme pyrophosphatase. **(Pradeep AR et al. 2011).**[7]

Orthophosphate is directly related to the formation of the dental calculus by competing with the pyrophosphate. It has the capability to alter the effect of the pyrophosphate on inhibition of calcification and creates an environment, which is highly suitable for the deposition of calculus.

The enzyme pyrophosphatase inhibits the action of pyrophosphate and helps in calculus formation indirectly, by converting pyrophosphate to orthophosphate. **(Vogel JJ & Amdur BH 1967).**[8]

The enzyme alkaline phosphatase, which is present in the saliva and in the dental plaque, releases the inorganic orthophosphate from organic phosphate. It increases the concentration of orthophosphate locally, which can react with calcium ions and leads to the precipitation of insoluble calcium apatite crystals on the tooth surface.

Pyrophosphate, which is present in saliva inhibits crystallization of minerals and competes with orthophosphate, and it has an inhibitory effect on the mineralization of the dental plaque. **(Pradeep AR et al. 2011).**[7]

Hence the activity of orthophosphate, pyrophosphate, and enzyme pyrophosphatase is being evaluated in the unstimulated whole saliva and compared between the calculus-forming groups and plaque-forming groups.

AIM & OBJECTIVES

AIM:

To evaluate the presence of orthophosphate, pyrophosphate and enzyme pyrophosphatase in unstimulated whole saliva in humans, with reference to the formation and inhibition of dental calculus.

OBJECTIVES:

• To estimate the levels of orthophosphate, pyrophosphate and enzyme pyrophosphatase in the whole saliva of humans.

• To compare the levels of orthophosphate, pyrophosphate and enzyme pyrophosphatase between calculus-forming groups and plaque-forming groups.

• To study the activity of orthophosphate, pyrophosphate and enzyme pyrophosphatase in formation of dental calculus and its inhibition.

REVIEW OF LITERATURE

DEFINITION AND CLASSIFICATION OF DENTAL CALCULUS:

In **1683, Van Leeuwenhoek** described the micro-organisms in tartar, which is present on the tooth surface as animalcules. **Schroeder HE (1969)[6]** defined dental calculus as mineralized dental plaque that is permeated with crystals of various calcium phosphates.

The dental calculus is a hard deposit that forms by mineralization of dental plaque, it is generally covered by a layer of unmineralized plaque, which forms on the surfaces of natural teeth and dental prostheses.

Dental calculus is classified as the supragingival and the subgingival calculus, according to its relation to the gingival margin. Supragingival calculus is located coronal to the gingival margin and therefore they are visible in the oral cavity. It is usually white or whitish yellow in colour, hard with clay like consistency and easily detached from the tooth surface. Since the salivary secretions are the main source of mineral salt, supragingival calculus is most frequently formed in the lingual surfaces of the mandibular anterior teeth, which are present opposite to the openings of the wharton's duct of the submandibular salivary gland, and on the buccal surfaces of the maxillary molars, which are present opposite to the openings of the stenson's duct of the parotid salivary gland.

Subgingival calculus is located below the crest of the marginal gingiva and therefore it can be evaluated by careful tactile perception with a delicate dental instrument such as an explorer No.17. It is possible clinically for subgingival calculus to become supragingival when the gingiva recedes and also the possibility of supragingival calculus to become subgingival in periodontal disease involvement such as gingival enlargement.

Subgingival calculus are predominantly found on the root surface with a periodontal pocket. Since the saliva is not involved in the subgingival calculus formation, the distribution of subgingival calculus is unrelated to supragingival deposits. Morphologically it occurs in ring-like or ledge-like forms on the root surfaces of the tooth.

COMPOSITION :

Glock GE & Murray MM (1938)[9] described that, the dental calculus, which is primarily composed of minerals as well as inorganic and organic components. The inorganic component constitutes about 70 to 90%, which includes

- 75.9% Calcium phosphate,
- 3.1% Calcium carbonate,
- 4% magnesium phosphate, and
- Trace amounts of other minerals.

The principal inorganic components are,

- Calcium 39%,
- Phosphorus 19%,
- Carbon-di-oxide 1.9%,
- Magnesium 0.8% and
- Trace amounts of other minerals such as
    - Sodium,
    - Zinc,
    - Strontium,
    - Bromine,
    - Copper,
    - Manganese,
    - Tungsten,
    - Gold,
    - Aluminium,
    - Silicon,
    - Iron, and
    - Fluorine.

The organic component consists of,

- Protein-polysaccahride complexes,
- Desquamated epithelial cells,
- leukocytes, and
- other microorganisms.

The organic components constitutes,

- Carbohydrates constitutes between 1.9 to 9.1% that includes,
  - galactose,
  - glucose,
  - rhamnose,
  - mannose,
  - glucuronic acid,
  - galactosamine.

- Proteins constitutes 5.9 to 8.2% in forms of most amino acids,

- Lipids constitutes 0.2% in forms of,
  - Neutral fats,
  - Free fatty acids,
  - Cholestrol,
  - Cholestrol esters, and
  - Phospholipids.

Mighailov MG (1958)[10] analyzed with 20 samples of salivary calculus and confirmed Glock & Murray findings and gave the following percentages of minerals present:

Phosphate: 12-16%,

Calcium: 32-36% and

Magnesium: 3-3.5%.

Schroeder HE (1969)[6] explained four different crystalline forms of and their percentage are:

Hydroxyapatite, approximately 58%

Magnesium whitlockite, approximately 21%

Octacalcium phosphate, approximately 12%

Brushite, approximately 9%

Sundberg JR & Friskopp J (1985)[11] explained that, the supragingival calculus predominantly consists of octacalcium phosphate and hydroxyapatite, where the hydroxyapatite is dominantly seen in the inner layers of the old calculus and the outer layer is covered by octacalcium phosphate. Whitlockite is found in small proportion. Brushite is seen in recently formed calculus which is not older than 2 weeks. Brushite is commonly seen in lingual surfaces of mandibular anterior region and magnesium whitlockite predominantly occurs in lingual surfaces of mandibular posterior region.

Friskopp J & Isacsson C (1984)[12] stated that, the dental calculus is primarily composed of mineral as well as inorganic and organic components. Supragingival and subgingival calculus contain 37% and 58% mineral content by volume, respectively.

Goldfine H et al (1972)[13] described that, the matrix of supragingival calculus constitutes 15.7% of the calculus dry weight and contains 54.9% protein and 10.2% lipid. Of the total lipids, 61.8% are neutral lipids, including a high content of free fatty acids and a smaller amount of triglycerides. Glycolipids account for 28% of the total lipids and are composed of 17.2% simple glycosphingolipids, mainly lactosyl- and glucosylceramine, and of 82.8% neutral and sulphated glyceroglucolipids. Phospholipids, representing 10.2% of the total lipid, contain 34.2% phosphatidyl-ethanolamine, 25.5% diphosphatidylglycerol, 2.3% phosphatidylinositol, and 1.7% phosphatidylserine. Phosphatidylinositol and phosphatidylserine are two important classes of acidic phospholipids but are only minor phospholipid components of bacterial cell membrane.

THEORIES OF CALCULUS FORMATION:

BOOSTER MECHANISM:

Calcification will occur in a particular locus when the local pH and calcium, phosphorus concentration is high enough to allow for precipitation of calcium phosphate salts.

LOSS OF CARBON DIOXIDE:

Magitot E (1878)[14] explained that, the dental calculus constitutes predominantly mineral matter which is formed by deposition of carbonates and phosphates from the saliva which is of alkaline medium. The minerals include organic matter, fatty globules, epithelial cells, leukocytes, filiform algae and infusoriae.

Burchard HH (1895)[15] stated that, the calcification occurs in a particular locus when the local pH and calcium and phosphorus concentrations are high enough to allow for precipitation of the calcium phosphate salt. such factors as loss of carbon di oxide and production of ammonia could be the causative factor for elevation in pH : acid or alkaline phosphatise activity could result in a higher phosphate concentration, which results in liberation of bound or complexed calcium from the salivary proteins, which further leads to increase in calcium levels in the saliva.

Hodge HC & Leung SW (1950)[16] supported Burchard's theory and demonstrated in experiments that upon loss of carbon dioxide from a supersaturated saliva, precipitate would form. Contrarily, no precipitate formed with a high carbon dioxide tension.

Rapp GW (1946)[17] explained that, the carbonic anhydrase present in saliva causes increase in uptake and liberation of carbon dioxide from the saliva. This results in precipitation of calcium salts caused by the alkalisation of saliva and loss of carbon dioxide.

ROLE OF BACTERIA:

Goodrich HI & Mosley (1916)[18] explained that, there was presence of long, thick, unbranching filamentous microstructures called Leptothrix which plays an important role in formation and deposition of calculus.

Bulleid A (1925)[19] explained that, there was presence of organisms such as *Leptotrichia* and *Leptotrichia buccalis* in calculus. He identified that *Leptotrichia buccalis* plays an important role in calculus formation. He investigated that *Leptotrichia buccalis* was the only organism, of several cultured, that formed a precipitate from a calcifying medium and bacteria must be present for precipitation to take place.

Naeslund CA (1925)[20] stated that, *Actinomyces* and *Leptotrichia* were important organisms in calculus formation. The *Leptotrichia* form the superficial layer over the deeper colonies formed which are formed by the *Actinomyces.* He explained that the growth of these organisms produced certain biochemical changes that lead to a precipitation of calcium salts from the saliva or the serum or exudate. These precipitates could readily be trapped by the bacteria already attached to surfaces of the teeth. He stated that there was no calculus formation in the absence of bacteria.

Bibby BG (1935)[21] explained that, *Leptotrichia* was an important organism in calculus formation. The cause for calculus formation was due to a combination of physic-chemical process in which there is an initial precipitation of calcium salts from the saliva and bacterial growths, which plays an important role in precipitation, fixation of the calculus to the teeth.

Yardeni J (1948)[22] stated that, there were very few viable organisms in the deep, well mineralized portions of the calculus. The bulk of the calculus mostly contained gram positive filaments of the actinomyces type.

COLLOIDAL PRECIPITATION:

Prinz H (1921)[23] postulated that, the colloidal substances in saliva became viscous and formed a matrix for the precipitation of calculus. These colloidal substances condensed around an 'inanimate nucleus', and inorganic calcium and magnesium salts precipitated out at right angles to this surface, making a radiating form. Colloidal particles then filled in around these salts to produce a laminated appearance. For calculus formation the alkalinity of saliva was essential and this was due to ammonia produced from protein decomposition and not due to loss of carbon di oxide from the saliva.

ENZYMATIC THEORIES:

Adamson KT (1929)[24] stated that, the enzyme phosphatase present in the gingival tissue was important in the hydrolysis of organic phosphates present in the saliva, to produce inorganic phosphates that could be precipitated as calcium salts in calculus.

Bowen WH (1959)[25] explained that, low concentrations of calcium and magnesium increased the enzyme activity. He demonstrated that there was an increase in the inorganic phosphate concentration from 0.8-2.0 mg% in incubated cultures of *Actinomyces* with parotid saliva. He explained that the *Actinomyces* could produce phosphatase, which might be important in liberating inorganic phosphates from the organic phosphates of the saliva and thus become precipitated as calcium salts in calculus.

Martland M & Robison R (1926)[26] stated that, the enzyme phosphatase could hydrolyze phosphoric esters of the blood to produce the local increase of phosphate ions until a precipitate of calcium phosphate would result.

EPITAXIC CONCEPT:

Boskey AL (1981)[27] described that, concentration of calcium and phosphate ions is not high enough in tissue fluids and saliva to precipitate spontaneously. But, it is sufficient to support the growth of a hydroxyapatite crystal once an initial seed or nucleus is formed. Hence the tissue fluids in saliva are called metastable solutions. The formation of the initial crystal or nucleus is called nucleation and it occurs when a proper organic matrix is available on which the nucleus can crystallize in the exact structural configuration.

The matrix provides the architectural template or geometric configuration for the initial hydroxyapatite crystal. The crystal growth then proceeds in the presence of a metastable solution. A number of nucleating molecules including some types of collagen and proteoglycans are the metastable solution. Calcium-phospholipid-phosphate complexes are important nucleators in normal and ectopic calcifications, including salivary gland stones and bacteria from salivary calculus.

INHIBITION THEORY:

Russell RG & Fleisch H (1970)[28] explained that, calcification occurs only at specific sites because of existence of inhibiting mechanism at non calcifying sites. The inhibitor is apparently moved or altered where calcification occurs. The inhibiting substance is found to be pyrophosphate and enzyme alkaline phosphatase, which can hydrolyze the pyrophosphate to phosphate. The pyrophosphate inhibits calcification by preventing the initial nucleus from growing by poisoning the growth centers of the crystal.

TRANSFORMATION THEORY:

Eanes ED et al. (1970)[29] postulated that, amorphous noncrystalline deposits and brushite can be transferred to octocalcium phosphate and then to hydroxyapatite. The controlling mechanism in the transformation process is the pyrophosphate. In salivary calculus brushite may develop spontaneously as a result of local elevation of pH, calcium and phosphate and then in the maturing process it is further modified to crystals of higher calcium to phosphate ratios. Early amorphous deposits is transferred to more crystalline material. The nucleating substance arising from the bacterial or the salivary proteins and lipids will also initiate calcification and lead to hydroxyapatite in early deposits.

MISCELLANEOUS THEORIES:

Schroeder HE (1969)[6] stated that, there are higher levels of calcium and phosphorus in heavy calculus formers than in light calculus formers within a few days after prophylaxis. A tendency towards heavy calculus formation could be due to,

1) Elevation in pH
2) Elevation in concentration of homogenous nucleators
3) Elevation in heterogenous nucleators
4) Low level of inhibitors

Berke JD (1935)[30] stated that, the deposition of the dental calculus on the tooth surface might be due to some systemic factor or a pathological condition of the person. **Badanes B & Parodneck C (1927)**[31] suggested that, the emotional status of an individual may play a role increased amount of the deposition of the dental calculus on the tooth surface. **Black GV (1915)**[32] explained that, higher the quantity of food intake, which is directly proportional to the amount of deposition of the dental calculus on the tooth surface.

ATTACHMENT OF CALCULUS:

Leung SW (1951)[33] described, that the initially present calculus enhance the precipitation of calcium and phosphates from the saliva, predominantly at the areas of tooth surface which is situated opposite to orifices of the salivary gland ducts, which receives the greatest concentration of precipitation of the inorganic salts.

Zander HA (1953)[34] described four types of calculus attachment on to the tooth surface, which are:

- The organic matrix of the calculus which is attached to the secondary cuticle over the tooth surface,
- When there is absence of cuticle, the calculus matrix gets attached to the irregularities over the cemental surface,
- Organisms penetrated into the cementum and were continuous with organisms in the calculus matrix, and
- Calculus can be interlocked mechanically in the undercut areas of the cementum resorption.

King JD (1954)[35] proposed that, the dental calculus originated on sheltered surfaces of tooth surface, which are close to ductal orifices, in areas where nasmyth's membrane had been retained. He assumed that nasmyth's membrane along with epithelial keratinization, food particles and salivary mucin would provide a good substrate for initial calculus deposition and mineralization.

Shroff FR (1955)[36] described that, the type of the attachment of the dental calculus probably depends on the longevity of time that the calculus has been adherent to the tooth surface. Initially the organic matrix of the deposit attached in some manner to the tooth surface, either by bacterial or chemical means. Later there may be changes in the cementum underlying the calculus that might change the type of attachment.

Everett FG (1956)[37] suggested that, the position of the tongue against the lingual surfaces of the mandibular anterior teeth contributes to formation of calculus by preventing certain food particles of a low pH from reaching lingual surfaces.

Parfitt G (1959)[38] supported Everett's concept of location of greatest amount of supragingival calculus is present on the lingual surfaces of mandibular anterior teeth and decreases toward the third molars. In the maxilla, supragingival calculus frequently forms on the buccal surfaces of the first molars.

Dawes C et al. (1989)[39] reported that, the velocity of deposition of calculus according to the salivary flow where for unstimulated salivary flow, the velocity of the film over tooth surfaces is estimated to vary between 0.8 and 8 mm/min, depending on different oral regions and status of saliva; for stimulated salivary flow, the velocity is from 1.3 to about 350 mm/min. The lowest film velocity of 0.8 to 1.3 mm/min occurs on the facial surfaces of the upper incisors, while the highest salivary film velocities are observed on the lingual surfaces of teeth.

Corbett TL & Dawes C (1998)[40] postulated that, the levels of subgingival calculus are significantly higher on the lingual than on the buccal surfaces. For the lingual surfaces of the teeth, the lower first molars have the most subgingival calculus. For the buccal surfaces of the teeth, the mandibular anterior teeth and maxillary molar teeth have the greatest amount of subgingival calculus.

PREVALENCE:

Anerud KE et al. (1983)[41] described in a study comparing the prevalence of dental calculus in adult males, aged 19-30 years, was undertaken in the United States, Norway and Sri Lanka. Where, all the subjects are from the higher socioeconomic status in these countries. Despite differences in geography, race and oral hygiene, calculus accumulated most frequently on lingual surfaces of the mandibular incisor and buccal surfaces of the maxillary molar teeth in all three young adult populations.

The lowest calculus scores were found in Norwegians, who opt for frequent dental visits than the individuals in Sri Lanka and the United States. The frequency of supragingival calculus alone remained constant with age, whereas the percentage of surfaces with subgingival calculus, with or without the presence of supragingival calculus increases with age.

Anerud A et al. (1991)[42] proposed in a longitudinal study comprising a group of Sri Lankan tea workers with no access to dental care and practicing no oral hygiene is compared with the group of Norwegians who performed tooth brushing twice-daily and received regular dental care.

However, only 6% of teeth were with absence of calculus in Sri Lankans compared to 74% of teeth in Norwegians. Fewer than 1 percent of Sri Lankan population had only supragingival calculus compared with 56 percentage of Norwegians. However, the Norwegian population had a 17 percentage of teeth with only supragingival calculus compared to only 6 percentage in Sri Lankan population.

All Sri Lankans had subgingival calculus on almost all teeth, whereas only 36 percentage of Norwegians had subgingival deposits involving an average of 9 percentage of teeth. In the youngest Sri Lankan group, aged between 14-17 years, supragingival calculus only was found on 8.5% of the tooth surfaces, and 80-90% of the mandibular anterior teeth had supra- or subgingival calculus or both. There was absence of calculus in about 20% of population of age between 16 – 20 years in Norwegian population. Approximately one-third of the population within age of 16-

to 17 years had supragingival calculus, which was 6 times more prevalent on lingual surfaces of mandibular incisors than on the buccal surfaces maxillary molars and was rarely seen on other teeth. Throughout 30 years of adult life (16-50 years) supragingival calculus did not increase significantly in Norwegian individuals.

FORMATION OF CALCULUS:

The formation of supragingival calculus is complex and involves both biological and physical-chemical processes. Since supragingival calculus is essentially mineralized plaque, the initial stages of its formation involve the selective adsorption of salivary proteins on the tooth surface and the subsequent attachment and colonization of the pellicle by bacteria. This biofilm of pellicle, bacteria and interbacterial matrix provides an environment within which mineralization may occur with the resultant of calculus deposition.

Schroeder HE (1969)[6] described that, the process of mineralization, which commences in the interbacterial matrix, which is composed of mineral-nucleating proteolipids. When there is increase in plaque accumulation, the deeper layers of organisms enters into a stationary or death phase and mineralization occurs in both within and between bacteria.

According to **Gilbert S (1969)[43]** vitamin A and calcium intake in diet has shown higher percentage of calculus formation, whereas ascorbic acid is higher in non calculus formers.

Ralph R et al (1969)[44] reported that, after the administration of antibiotic erythromycin for seven days, there was decrease in amount of plaque formation by 35%. And spirochetes which were preent in the subjects before antibiotic administration significantly disappeared for a period of 5-18 weeks after administration of antibiotic erythromycin.

Sheila J Jones (1972)[45] reported that, the initiation and spread of calculus is predominant on enamel pits and outcroppings of the incremental lines at the leading edges of the perikymata. The initial deposition of calculus was seen at the crevices between the mineralised ends of the sharpey's fibres.

According to **Sideaway DA (1978)**[46] the frequency of phase of calculus formation probably reflects the fact, that the oral salivary environment is supersaturated with calcium and phosphate ions, and inhibitors of calculus formation diffuses gradually through the biofilm.

Bercy P & Vreven J (1979)4[7] reported that, there is a close relationship of alkaline phosphatase with biomineralisation in bones and has reported a positive correlation between amount of formation of dental calculus and presence of salivary phosphatases. It has also been reported about the positive correlation between alkaline pyrophosphatase activity occurring in dental plaque with the calculus formation, despite the fact that the pH of dental plaque does not favor alkaline pyrophosphatase activity (pH optimum, 8.5).

According to **Hay DI et al. (1982)**[48] the parotid and the submandibular saliva are supersaturated with respect to various calcium phosphates. But it shows minimum tendency to spontaneous precipitation of minerals, during the short time that saliva would be in contact with plaque on the lingual surfaces of the mandibular incisors and the buccal surfaces of the maxillary molars. However, in these locations the abundant secretion of urea from the saliva and the high salivary film velocity tend to increase the incidence of formation of base over the dental plaque and the precipitation of calcium phosphate.

Pellat BP & Grand M (1986)[49] demonstrated that, similar to phosphatase, acid and alkaline pyrophosphatases can also promote crystal growth by hydrolyzing pyrophosphate.

Watanabe T et al. (1982)[50] described that, the calculus level was positively correlated with protease activity in human saliva. **Morita M & Watanabe T (1986)**[51] reported that, the supragingival plaque from calculus formers has been found to show significantly higher protease activity than that from non-calculus formers since protease in saliva and plaque can degrade calcification inhibitors such as statherin and proline rich protein.

White DJ (1991)[52] suggested that, the minerals appears to be deposited in layers which states that the process of mineralization is occurs alternatively

with periods of mineralization being interspersed with periods during which further deposits of salivary protein and bacteria accumulate on the tooth surface.

Stanley P.Hazen (1995)[4] described that the formation of supragingival calculus is by,

(i) An attachment phase in which a cuticle-like structure is precipitated from the saliva onto the tooth surface which becomes the medium for which the microorganisms gets attached to the tooth surface or an irregular tooth surface may support the direct attachment of microorganisms.

(ii) A colonization phase in which microorganisms develop colonies within an intermicrobial matrix. These colonies may represent many types of microorganisms and variable in size of the deposit as well as it varies from specimen to specimen even in the same individual. The early colonies appear to be primarily coccal forms, which gets converted into filamentous forms more rapidly.

(iii) A mineralization phase in which calcification gets initiated anywhere over the initial deposition over the tooth surface. These organized "niduses" mature and increase in size, which gradually coalesce to form the crusty material called as the calculus. The simple precipitation of calcium salts into the deposit can be observed, but it is a minor factor in the development of the matured calcified mass.

INHIBITION OF FORMATION OF CALCULUS:

The formation of supragingival calculus may be prevented by,

(i) reducing the amount of plaque present, which are to be mineralized using antimicrobial agents and enzymes,

(ii) modifying the attachment of plaque by anti-adhesive agents and

(iii) inhibiting the process of mineralization by crystal growth inhibitors.

Draus FJ et al. (1968)[53] stated that, pyrophosphate is an effective inhibitor of hydroxyapatite formation in vitro. **White DJ et al. (1989)**[54] demonstrated that, the increase in the concentration of pyrophosphate has been shown to increase its retention and to enhance the clinical efficacy.

Bubani G (1980)[55] demonstrated that, the toothpaste containing 1.15% azacycloheptane-2,2-diphosponic acid was compared with a placebo; after 2 months of use of test products, mean calculus scores declined by 23% compared to a placebo toothpaste which is statistically significant.

According to **Harrap GJ et al. (1984)**[56] zinc has a property to inhibit hydroxyapatite crystals in vitro and inhibits plaque formation. Thus inhibiting the formation of dental calculus on the tooth surface.

Nilsson B & Holm G (1986)[57] demonstrated with 1.0% azacycloheptane-2, 2-diphosponic acid formulation tested over 4 months resulted in a 30% reduction in mean calculus scores relative to a placebo. Thus it has been demonstrated that diphosphonates also have anticalculus efficacy.

Gaffar A et al. (1987)[58] described that, the incorporation of a copolymer of polyvinylmethyl ether and maleic acid also enhances the efficacy of pyrophosphate by inhibiting alkaline phosphatase and pyrophosphatase activity which has enabled a lower concentration of pyrophosphate (1.3%) and maintain an effective level of inhibition of calculus deposition.

Gilbert RJ (1987)[59] stated that, zinc is cationic and is consequently retained within the oral cavity. **Gilbert RJ & Ingram GS (1988)**[60] demonstrated that, following the use of a 0.5% zinc citrate dentifrice, the oral retention varied from 24-38%. Reasonable plaque levels of zinc were found 4 hours after oral hygiene procedure and concentrations were elevated in both plaque fluid and plaque residue. The levels of zinc in calculus from individuals who had used dentifrices containing 0.5% or 1.0% zinc citrate for 3 months were well in excess of those shown to inhibit crystal growth in vitro.

Gaffar A et al. (1989)[61] stated that, mineralization inhibitors include chemicals such as pyrophosphates, diphosphonates and zinc salts that adsorb to the surface of crystals, causing reduction in the rate of crystal growth and phase transformations of calcium phosphate salts.

Moreno EC et al. (1989)[62] stated that, pyrophosphate is a small molecule and has been reported to inhibit crystal growth by binding to the surface of crystal. Pyrophosphate binds to two sites on the hydroxyapatite surface, and one of the two sites needs to be bound by phosphate ion to permit crystal growth to occur. If this site is bound by pyrophosphate, phosphate ion cannot absorb onto crystal, and thus crystal growth is inhibited. To inhibit crystal growth effectively, the concentration of pyrophosphate has to reach a critical level. Below this level, the addition of sodium flouride can induce a short period of slow precipitation, which is followed by rapid crystal growth.

White DJ et al. (1989)[54] reported that, along with the inhibitory effect on crystal growth pyrophosphate has the capacity to delay the initiation of converson of dicalcium phosphate dihydrate to hydroxyapetite by more than three folds and reducing acquired pellicle formation.

Sammons MC et al. (1989)[63] demonstrated that, the uptake and retention of pyrophosphate from a dentifrice containing 5% pyrophosphate was greater than from a formulation containing 3.3% pyrophosphate.

Schiff T et al. (1990)[64] explained that, silica and alumina abrasive based formulations produced similar, statistically significant, reductions in mean calculus scores compared with a silica based control. Lobene et al, 1990 reported that the percentage reductions were 23% and 26% for the silica and alumina formulations.

Lobene RR et al. (1991)[65] reported that, there is a reduction of 26% of calculus formation compared to the placebo after 3 months and a 36% reduction in calculus formation after 6 months. **Volpe AR et al. (1992)**[66] reported Significant reductions in calculus for the triclosan - copolymer formulation of 36% after 3 months. Thus antimicrobial agents like tricloson (0.3%), polyvinylmethyl ether and maleic acid (2.0%) has anticalculus efficacy.

Schaeken MJM & van der Hoeven JS. (1993)[67] suggested that, the reduction in calculus scores of approximately 45% relative to a control dentifrice after 3 months study. Calcium lactate has been shown to possess anticalculus activity although its mechanism of action is unclear.

ETIOLOGIC SIGNIFICANCE:

The dental calculus is superficially covered with a layer of unmineralized dental plaque. Hence it is difficult to distinguish between the effects of calculus and dental plaque on the gingiva. There is a positive correlation between the presence of calculus and the prevalence of gingivitis. But the correlation is least significant compared to the presence of the dental plaque with the prevalence of gingivitis.

The periodontal conditions in young subjects are closely related to plaque accumulation then to the calculus. But as the age progresses this condition is reversed. As the age increases, there is increase in incidence of presence of dental calculus and occurrence of gingivitis and periodontal diseases. Presence of subgingival calculus has a positive correlation with the presence of periodontal pockets in adults.

The principal irritant is the unmineralized dental plaque which is present on the superficial surface of the dental calculus. But the significant contributing factors are the underlying calcified portion of the dental calculus. The calcified portion of the dental calculus does not irritate the gingiva directly, but leads to a provision of a fixed nidus for the continued accumulation of dental plaque and retaining it close to the marginal gingiva. The periodontal pocket leads to formation of subgingival calculus rather than the subgingival calculus being the cause for formation of the periodontal pockets.

The gingival inflammation is initiated with continued plaque formation which further leads to formation of the periodontal pockets. And, this periodontal pocket in turn provides a sheltered area for the accumulation of dental plaque and the bacterial microorganisms. The flow of gingival crevicular fluid is increased with increase in the gingival inflammation which provides the minerals that convert the continually accumulating dental plaque into subgingival calculus.

Albandar J et al. (1998)[68] observed among 156 young adolescents with history of aggressive periodontitis, that the loss of periodontal attachment have a positive correlation with the presence of subgingival calculus than in the sites that did not initially exhibit presence of subgingival calculus.

The etiologic factor in development of periodontal disease is bacterial plaque. But the removal of subgingival plaque and calculus plays an important role in periodontal therapy. Presence of dental calculus is important in maintaining the plaque in close contact with the marginal gingival tissue and creates areas where plaque removal is impossible, leading to increase in incidence of periodontal disease. **(Newman).**[69]

MATERIALS & METHODS

This study was conducted in Department of Periodontics, Sri Ramakrishna Dental College & Hospital, Coimbatore. It was carried out with the written informed consent from the subjects and was approved by institutional Ethical committee.

The study included 60 systemically healthy subjects, with presence of chronic generalized marginal gingivitis, who were divided into 4 groups according to presence of calculus and plaque, with age ranging from 15 – 30 years.

STUDY DESIGN AND PATIENT SELECTION:

The subjects included in the study have to satisfy the following criteria:

CRITERIA OF INCLUSION:

1.Patient of age 15-30 years, with full complement of teeth.

2.Patient should not have undergone oral prophylaxis for past 1 year.

3.Patients with diagnosis of chronic generalized marginal gingivitis.

CRITERIA OF EXCLUSION:

1.Patient with any systemic disorders and oral diseases.

2.Tobacco users whether in a smoking and/or smokeless form.

3.Patients who are consuming alcohol.

4.Patients who had undergone oral prophylaxis within past 1 year.

Diagnosis of chronic generalized marginal gingivitis was made using observation of clinical signs and parameters, and did not have periodontitis at the time of sample collection and also did not present a history of previous periodontal disease.

ARMAMENTARIUM:

Dental Mouth Mirror, explorer, surgical mask, gloves, sterile cotton pellets, disclosing solution (AlphaPlac) **(FIG.4)**, patient apron, Test tube, glass funnel, graduated pipettes, photoelectric calorimeter **(FIG.2)**, centrifuge **(FIG.3)**.

REAGENTS USED:

Trichloroacetic acid 10%, Molybdate solution, Phosphorus reagent, Veroneal acetate buffer solution, Sodium pyrophosphate substrate, Magnesium chloride, Hydrochloric acid (1N), Distilled water. **(FIG.1)**

CLINICAL EXAMINATION:

During the examination of the patient the following clinical parameters were assessed:

Plaque score : The plaque score was assessed, according to the plaque component from the Periodontal Disease Index given by **Ramfjord (1959)**.[70]

Calculus score : The assessment of presence of calculus done by using the calculus component from the Periodontal Disease Index given by **Ramfjord (1959)**.[70]

The calculus component and the plaque component assesses the presence of and the extent of the calculus and the plaque present on the facial (Buccal/Labial) and lingual/palatal surfaces of 6 index teeth. The index teeth examined are,

Tooth #16 (Maxillary right first molar),

Tooth #21 (Maxillary left central incisor),

Tooth #24 (Maxillary left first bicuspid),

Tooth #36 (Mandibular left first molar),

Tooth #41 (Mandibular right central incisor), and

Tooth #44 (Mandibular right first bicuspid).

GROUPING OF THE SUBJECTS:

These 60 study subjects were classified into 4 groups, with 15 subjects in each group. Group I consisted of 15 subjects with calculus index score between 0.40 to ≤ 1.00. Group II consisted of 15 subjects with calculus index score between >1.00 to ≤ 1.30. Group III consisted of 15 subjects with calculus index score between >1.30 to ≤ 1.80. The plaque group was considered as Group IV consisted of 15 subjects.

SAMPLE COLLECTION:

The patient is advised to refrain from intake of any food or beverage (water exempted) one hour before the session. The subject is advised to rinse his or her mouth several times with water and then adviced to relax for five minutes. Saliva collected in the first few minutes was discarded. 5ml of unstimulated saliva collected from each patient in a 10ml test tube and sent to Department of Biochemistry, Sri Ramakrishna Dental College and Hospital for centrifugation and further laboratory biochemical analysis.

LABORATORY BIOCHEMICAL ANALYSIS:

The laboratory analysis was done in Department of Biochemistry, Sri Ramakrishna Dental College and Hospital, Coimbatore. Initially deproteination of saliva was done. The saliva samples collected from each subjects were transferred to a sterile centrifugal test tube. A total of 3 ml of the saliva was taken separately, which was treated with 5 ml of 10% trichloroacetic acid. Then, it was centrifuged at 3,000 rpm for 15 minutes at room temperature to remove the salivary proteins. The clear, supernatant saliva was analyzed for orthophosphate, pyrophosphate, and enzyme pyrophosphatase.

Estimation of orthophosphate was done according to the procedure by **Flynn et al (1954)**[71] by using phosphorus reagent. The reagents used were molybdate solution and phosphorus reagents of the **Fiske & Subbarow (1925)**[72] for the determination of orthophosphate.

For the estimation of the orthophosphate, two aliquots of 0.1ml of supernatant saliva were pipetted out into a sterile test tube and 1 ml of the molybdate solution and 0.5 ml of the phosphorus reagent were added, turning the colour of the solution into blue. The volume was then increased to 10 ml by adding 8.4 ml of distilled water. A blank solution was prepared using 1 ml of molybdate solution, 0.5 ml of phosphorus reagent, and 8.5 ml of distilled water. The readings were recorded using photoelectric calorimeter and compared with the standard solution.

Estimation of pyrophosphate was done as per the procedure of **Flynn et al (1954)**[71] where, two aliquots of 0.1 ml saliva were taken in a test tube and to that solution 0.1 ml of 1 N HCl was added and heated for 10 minutes at 100°C using water bath for the hydrolysis of the remaining pyrophosphate to orthophosphate. After heating, the aliquots were cooled at room temperature and were analyzed to give a measure of the total phosphate in the saliva. The difference of the values of the phosphate between the hydrolyzed saliva (A) and the unhydrolyzed saliva (B) was taken as pyrophosphate. A blank solution was prepared and the readings were recorded separately.

For estimation of the enzyme pyrophosphatase, test sample was prepared by **Heppel & Hilmore (1951).**[73] A total of 1.16 ml and 1.21 ml of veroneal acetate buffer was pipetted out to the sterile test tubes, and 0.05 ml of magnesium chloride was added. To the test tube of veroneal acetate buffer 1.16 ml, 0.05 ml of sodium pyrophosphate substrate was added and later 0.04 ml of supernatant saliva was added to both the test tubes. The assay mixture was mixed well, and kept stable for

15 minutes. Then the reaction was arrested by adding 0.2 ml of 10% trichloroacetic acid. Further, the aliquots were analyzed for orthophosphate as per the procedure of **Flynn et al (1954).**[71] The difference in values between the aliquot with the substrate and without the substrate gave the presence of enzyme pyrophosphatase in saliva. Before the readings were being taken, a blank solution was also prepared which does not contain either the substrate or the supernatant saliva, for standardizing the calorimetric value to 0.

SRI RAMAKRISHNA DENTAL COLLEGE AND HOSPITAL, COIMBATORE.

DEPARTMENT OF PERIODONTICS

Estimation of levels of Orthophosphate, Pyrophosphate, and Pyrophosphatase in Saliva – A Biochemical study

PROFORMA

FORM I - SCREENING PROFORMA

NAME:

AGE:

SEX:

OCCUPATION:

POSTAL ADDRESS:

TELEPHONE NUMBER:

PATIENT SELECTION CRITERIA:

CRITERIA OF INCLUSION:

- Patients of age 15-30years, with full complement of teeth.
- Patients should not have undergone oral prophylaxis for past 1 year.
- Patient with full complement of teeth.

CRITERIA OF EXCLUSION:

- Patient with any systemic disorders and oral diseases.
- Tobacco users whether in a smoking and/or smokeless form.
- Patients who are consuming alcohol.
- Patients who had undergone oral prophylaxis within past 1 year.

FORM II- HISTORY PROFORMA

PERSONAL HISTORY:

1. Diet: Veg: ☐ Non – veg: ☐

2 .Brushing habit:

3. Any other (specify):

1. PLAQUE COMPONENT OF PDI **(**Ramfjord 1959**):**

DATE: _____

16 21 24

44 41 36

PLAQUE SCORE PER TEETH

PLAQUE SCORE OF AN INDIVIDUAL- ————————————————————

NUMBER OF TEETH EXAMINED

P0 = No plaque present.

P1 = Plaque present on some but not on all of the interproximal and gingival surfaces of the tooth.

P2 = Plaque present on all interproximal and gingival surfaces, but covering less than one-half of entire clinical crown.

P3 = Plaque extending over all interproximal and gingival surfaces covering more than one-half of the entire clinical crown.

2. CALCULUS COMPONENT OF PDI **(**Ramfjord 1959**):**

DATE: _____

16 21 24

44 41 36

CALCULUS SCORE PER TEETH

CALCULUS SCORE PER PERSON- ——————————————————

NUMBER OF TEETH EXAMINED

0 = Absence of calculus.

1 = Supragingival calculus extending only slightly below the free gingival margin (not more than 1mm).

2 = Moderate amount of supragingival & sub gingival calculus or sub gingival calculus alone.

3 = An abundance of supragingival and sub gingival calculus.

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CERTIFICATE BY INVESTIGATOR

I certify that I have disclosed all details about the study in the terms easily understood by the patient

Dated _____ Signature_____

Name_____

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DEPARTMENT OF PERIODONTICS

CONSENT FORM

I have been informed to my satisfaction, by the attending investigator, the purpose of the clinical trial, follow up including the laboratory investigation to be performed to monitor and safe guard my body functions

I exercising my free power of choice hereby give my consent to be included as a subject in this clinical trial.

Date: Signature of the patient

PHOTOGRAPHS

FIG.1: REAGENTS USED

FIG.2: PHOTOELECTRIC CALORIMETER

FIG.3: CENTRIFUGE

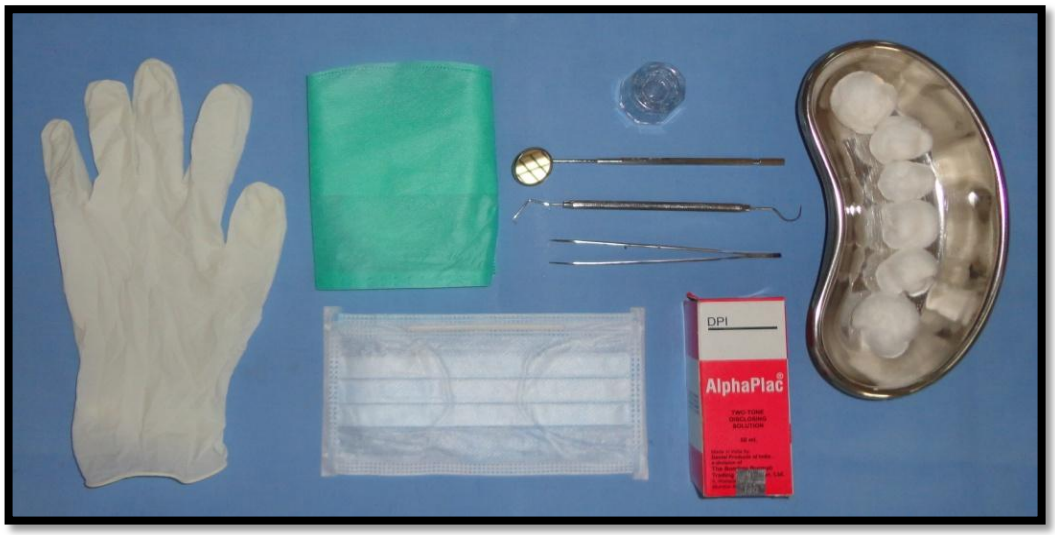

FIG.4: ARMAMENTARIUM FOR CLINICAL EXAMINATION

RESULTS

STUDY POPULATION CHARACTERISTICS:

This study was a clinico-biochemical prospective cross-sectional study, which included 60 systemically healthy subjects having full complement of teeth, with age range between 15 to 30 years, who had not undergone oral prophylaxis for the past 1 year, before the commencement of the study. Table 1 shows the descriptive statistics of study population. This study included 31 (51.7%) males and 29 (48.3%) females. Those 60 subjects were divided into 4 groups, with 15 subjects in each group. Group I consisted of 15 subjects with calculus index score between 0.40 to ≤ 1.00, which included 8 (53.3%) males and 7 (46.7%) females. Group II consisted of 15 subjects with calculus index score between > 1.00 to ≤ 1.30, which included 7 (46.7%) males and 8 (53.3%) females. Group III consisted of 15 subjects with calculus index score between > 1.30 to ≤ 1.80, which included 8 (53.3%) males and 7 (46.7%) females. The plaque group was considered as Group IV consisted of 15 subjects, which included 8 (53.3%) males and 7 (46.7%) females.

Statistical analysis was calculated by using analysis of variable (ANOVA) and inter group comparison was done using Post Hoc test of Scheffe´ method. Table 2 shows the mean age of the study population was 22.6167 years. The mean plaque score of total study population was 1.2772. The mean calculus score of total study population was 1.0234.

Table 3 shows the descriptive statistics of orthophosphate, pyrophosphate, and Pyrophosphatase and the FIG.5 shows its graphical representation. The mean values of orthophosphate, pyrophosphate, and pyrophosphatase for the calculus index group I which includes the subjects with calculus index score values between 0.40 to ≤ 1.00 were 1.152 mM, 0.176 mM, 11.359 units/ml respectively. The mean values of orthophosphate, pyrophosphate, and pyrophosphatase for the calculus index group II which includes the subjects with calculus index score values between > 1.00 to ≤ 1.30 were 1.372 mM, 0.109 mM, 15.237 units/ml respectively. The mean values of orthophosphate, pyrophosphate, and pyrophosphatase for the calculus index group III which includes the subjects with calculus index score values between > 1.30 to ≤ 1.80 were 1.666 mM, 0.068 mM, 21.451 units/ml respectively. The mean values of orthophosphate, pyrophosphate, and pyrophosphatase for the group IV were 0.897 mM, 0.257 mM, 9.034 units/ml respectively.

Table 4 shows the inter group comparison of orthophosphate using Tukey HSD method. With regard to the orthophosphate, the mean difference in values between calculus index group I and calculus index group II was -0.22, which was statistically significant (p= 0.027). The mean difference in values between calculus index group I and calculus index group III was -0.514, which has a high statistical significance (p= <0.001). The mean difference in values between calculus index group I and group IV was -0.2553, which was statistically significant (p= 0.008).

The mean difference in values between calculus index group II and calculus index group III was -0.294, which was statistically significant (p= 0.002). The mean difference in values between calculus index group II and group IV was -0.4753, which has a high statistical significance (p= <0.001). The mean difference in values between calculus index group III and group IV was -0.7693, which has a high statistical significance (p= <0.001).

Table 5 shows the inter group comparison of pyrophosphate using Tukey HSD method. With regard to the pyrophosphate, the mean difference in values between calculus index group I and calculus index group II was 0.0673, which was not statistically significant (p= 0.103). The mean difference in values between calculus index group I and calculus index group III was 0.108, which was statistically significant (p= 0.002). The mean difference in values between calculus index group I and group IV was 0.0806, which was statistically significant (p= 0.035). The mean difference in values between calculus index group II and calculus index group III was 0.0406, which was not statistically significant (p= 0.500). The mean difference in values between calculus index group II and group IV was 0.148, which has a high statistical significance (p= <0.001). The mean difference in values between calculus index group III and group IV was 0.1886, which has a high statistical significance (p= <0.001).

Table 6 shows the inter group comparison of pyrophosphatase using Tukey HSD method. With regard to the enzyme pyrophosphatase, the mean difference in values between calculus index group I and calculus index group II was -3.8785, which was not statistically significant (p= 0.063). The mean difference in values between calculus index group I and calculus index group III was -10.0926, which has a high statistical significance (p= <0.001). The mean difference in values between calculus index group I and group IV was -2.3243, which was not statistically significant (p= 0.427). The mean difference in values between calculus index group II and calculus index group III was -6.21406, which has a high statistical significance (p= 0.001). The mean difference in values between calculus index group II and group IV was -6.20286, which has a high statistical significance (p= 0.001). The mean difference in values between calculus index group III and group IV was -12.41693, which was statistically highly significant (p= <0.001).

The graphical representations of the mean values of the orthophosphate, pyrophosphate, and pyrophosphatase for all the calculus groups and plaque group were given in FIG.6, FIG.7 and FIG.8.

TABLE 1: GENDER VARIATIONS OF STUDY POPULATION

			GROUP				Overall Gender classification
			Group I (0.40-≤1.00)	Group II (>1.00 to ≤1.30)	Group III (>1.30 to ≤1.80)	Group IV (Plaque group)	
GENDER	Male	N	8	7	8	8	31
		%	53.3%	46.7%	53.3%	53.3%	51.7%
	Female	N	7	8	7	7	29
		%	46.7%	53.3%	46.7%	46.7%	48.3%

TABLE 2: MEAN AGE, PLAQUE AND CALCULUS SCORE OF THE STUDY POPULATION

	Mean	**Std. Deviation**
AGE (YEARS)	22.6167	4.46110
PLAQUE SCORE	1.2772	0.38393
CALCULUS SCORE	1.0234	0.39973

TABLE 3: DESCRIPTIVE STATISTICS OF ORTHOPHOSPHATE, PYROPHOSPHATE, AND PYROPHOSPHATASE

	N	Orthophosphate (mM)	Pyrophosphate (mM)	Pyrophosphatase (units/ml)
Group I (0.40 to ≤1.00)	15	1.152±0.147	0.176±0.096	11.359±2.843
Group II (>1.00 to ≤1.30)	15	1.372±0.116	0.109±0.034	15.237±3.723
Group III (>1.30 to ≤1.80)	15	1.666±0.163	0.068±0.043	21.451±4.819
Group IV (Plaque group)	15	0.897±0.335	0.257±0.113	9.034±4.915

TABLE 4: Tukey HSD Test (ORTHOPHOSPHATE)

Groups	Mean Difference	P value
I and II	-0.22	**0.027***
I and III	-0.514	**<0.001***
I and IV	-0.2553	**0.008***
II and III	-0.294	**0.002***
II and IV	-0.4753	**<0.001***
III and IV	-0.7693	**<0.001***

**** p <0.05***

TABLE 5: Tukey HSD Test (PYROPHOSPHATE)

Groups	Mean Difference	P value
I and II	0.0673	0.103
I and III	0.108	**0.002***
I and IV	0.0806	**0.035***
II and III	0.0406	0.500
II and IV	0.148	**<0.001***
III and IV	0.1886	**<0.001***

**** p <0.05***

TABLE 6: Tukey HSD Test (PYROPHOSPHATASE)

Groups	Mean Difference	P value
I and II	-3.87853	0.063
I and III	-10.0926	**<0.001***
I and IV	-2.3243	0.427
II and III	-6.21406	**0.001***
II and IV	-6.20286	**0.001***
III and IV	-12.41693	**<0.001***

**** p <0.05***

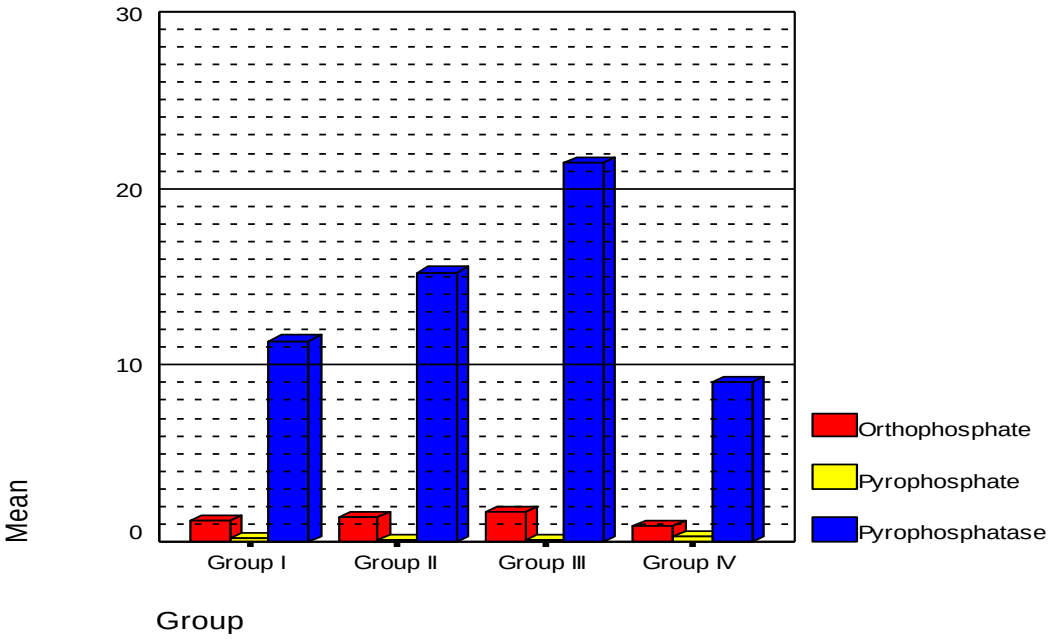

FIG.5: DESCRIPTIVE STATISTICS OF ORTHOPHOSPHATE, PYROPHOSPHATE AND PYROPHOSPHATASE

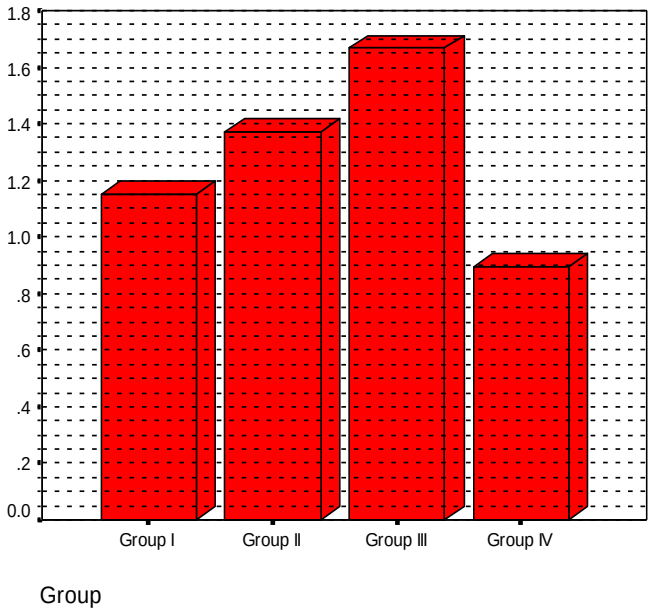

FIG.6: MEAN VALUES OF ORTHOPHOSPHATE

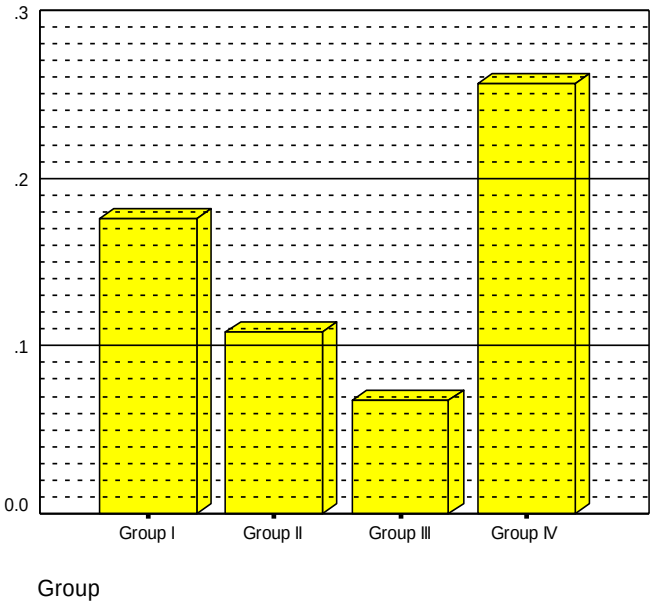

FIG.7: MEAN VALUES OF PYROPHOSPHATE

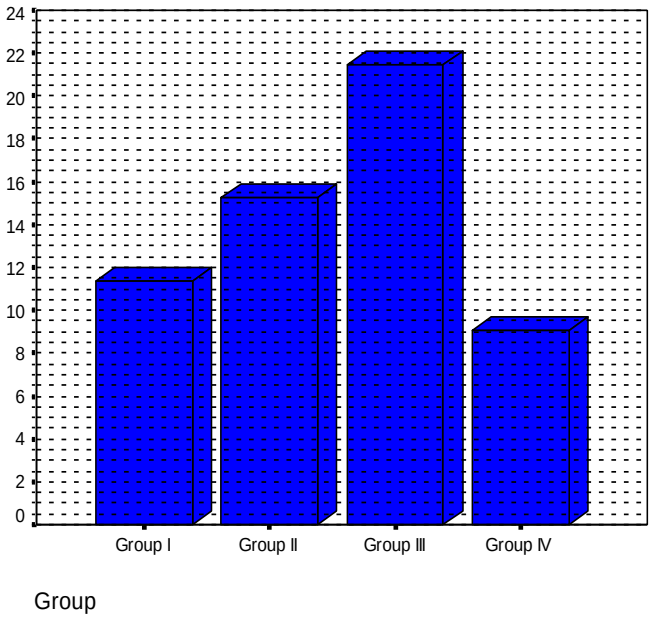

FIG.8: MEAN VALUES OF PYROPHOSPHATASE

DISCUSSION

The aim of this study was to estimate the levels of orthophosphate, pyrophosphate, and enzyme pyrophosphatase from unstimulated saliva in humans using biochemical analysis, and to evaluate their activity with regard to the formation and inhibition of the dental calculus with the reference to the amount of orthophosphate, pyrophosphate, and enzyme pyrophosphatase present in the human saliva.

The inorganic components such as orthophosphate, pyrophosphate, and enzyme pyrophosphatase which are present in the human saliva helps in formation and inhibition of the dental calculus through the interaction between these components. **(Pradeep AR et al. 2011).**[7]

The enzyme alkaline phosphatase which is present in saliva and dental plaque releases inorganic orthophosphate from organic phosphate, and by increasing concentration of orthophosphate locally, reacts with the calcium ions in the saliva leading to the precipitation of the insoluble calcium apatite crystals on the surface of the teeth. Pyrophosphate, which are the byproducts of many biosynthetic reactions is present in saliva and helps in inhibition of the crystallization, and competes with orthophosphate for minerals. The enzyme pyrophosphatase plays an important role in the formation of dental calculus by hydrolysing pyrophosphate to orthophosphate, thereby removing its inhibitory efficiency and simultaneously converting into booster by increasing the concentration of the orthophosphate.

The present clinico-biochemical prospective cross-sectional study consisted of 60 systemically healthy subjects, having full complement of teeth, with the age group ranging between 15 to 30 years, with a mean age of 22.61 years. The subjects had no systemic diseases or oral diseases, and had not undergone oral prophylaxis for the past 1 year from the time of the commencement of the study.

In the present study, the subjects were divided into 4 groups. Group I consisted of 15 subjects who had calculus index score of 0.40 to ≤1.00. Group II consisted of 15 subjects who had calculus index score of >1.00 to ≤1.30. Group III consisted of 15 subjects who had calculus index score of >1.30 to ≤1.80. Plaque group was considered as Group IV, which included 15 subjects.

5 ml of unstimulated whole saliva was collected from all the 4 groups of subjects by the method given by **Mahvash Navazesh and Satish K.S. Kumar (2008)**[74] and deproteination of the saliva was done using 10% TCA and the sample was centrifuged at 3000 rpm for 15 mins at room temperature. The supernatant saliva was obtained for the biochemical analysis.

The estimation of orthophosphate, pyrophosphate was done using the procedure by **Flynn et al (1954)**[71] by using phosphorus reagent. For the evaluation of orthophosphate, the reagents used in this study were molybdate solution and the phosphorus reagents, which was prepared according to **Fiske & Subbarow (1925)**[72] The test sample for the assay of the enzyme pyrophosphatase was prepared using

procedure given by **Heppel & Hilmore (1951).**[73] The enzyme pyrophophatase was converted to orthophosphate and was analyzed as per the procedure by **Flynn et al (1954).**[71] This procedure was followed as it was a simple and convenient method. It was a colorimetric method that involves simple reagents easily available in the laboratory and it was also cost effective, when a large study population was involved. The calorimetric values were recorded from each of the samples. Statistical analysis was calculated by using analysis of variable (ANOVA) and inter group comparison was done using Post Hoc test of Tukey HSD method.

In this present study, as the calculus index score increases ranging from 0.40 to 1.80, the mean values of orthophosphate were 1.152 mM, 1.372 mM, 1.666 mM for the calculus groups. The mean pyrophosphate levels were 0.176 mM, 0.109 mM, 0.068 mM for the calculus groups. The mean pyrophosphatase levels showed the values 11.359, 15.237, 21.451 (units/ml) for the calculus groups. The group IV (plaque group) showed the mean values of 0.897 mM, 0.257 mM, 9.034 units/ml for orthophosphate, pyrophosphate, and pyrophosphatase respectively.

Hence, from the results of the present study, it was observed that, when the calculus index score increases, there was a significant gradual increase in the levels of orthophosphate and also there is an increase in the levels of enzyme pyrophosphatase. Eventually, there was a significant decrease in the levels of pyrophosphate in each of the calculus groups. Comparatively, in the group IV (plaque group), the levels of pyrophosphate were increased (0.257 mM). And the

orthophosphate and enzyme pyrophosphatase in the plaque group were in low levels (0.897 mM, 9.034 units/ml) in comparison with the values from the calculus groups.

In the present study, the levels of orthophosphate and enzyme pyrophosphatase increased significantly and the level of pyrophosphate decreased when calculus index score increases from 0.40 to 1.80, which was in correlation with the results from the study by **Pradeep AR et al. (2011).**[7] who observed that levels of orthophosphate and enzyme pyrophosphatase increased, when the calculus index score increases, whereas the level of pyrophosphate was decreased.

These observations were also in agreement with the findings from the study by **Sawinski VJ & Cole DF (1965).**[75] where the severity of the calculus formation is directly proportional to the content of orthophosphate and the enzyme pyrophophatase present in saliva and pyrophosphate had an inhibitory role in the formation of calculus.

The values of orthophosphate in present study were significantly lower than those observed by **Vogel JJ & Amdur BH (1967).**[8] It was suggestive of high levels of intake of carbohydrate rich food substances, which has more capacity to lower the plasma phosphate levels, causing decrease in the orthophosphate concentration levels of human saliva. **(Jenkins GN 1978).**[76] This could be the causative factor for lower values of orthophosphate concentration in the present study population, as consumption of carbohydrates rich food substances are common

among the Indian population, particularly in the South Indian population. **(Pradeep AR et al. 2011).**[7]

The results from the present study, were suggestive of pyrophosphate being the strong inhibitor in formation of the dental calculus. Since, the acid and alkaline phosphatase were found in intracellular and intercellular components of dental plaque, the phosphatases were associated with small vesicles of microbial origin in extracellular matrix. The acid phosphatase was found to be concentrated on cell walls of gram positive and gram negative bacteria. This localization of phosphatases affects the natural inhibition of plaque calcification by pyrophosphate and phosphorylated proteins **(White DJ 1997)**[77] And, the higher levels of the orthophosphate and enzyme pyrophosphatase increases the incidence of the formation of the dental calculus.

SUMMARY & CONCLUSION

This clinico-biochemical prospective cross-sectional study estimated the levels of orthophosphate, pyrophosphate and enzyme pyrophosphatase in unstimulated human saliva, and evaluated its activity with regard to the formation and inhibition of the dental calculus. The study included 60 systemically healthy subjects, who had full complement of teeth. They were divided into 4 groups according to the calculus index score which consisted of 3 groups and a plaque group. The age group of the study population was ranging between 15 to 30 years. The study was conducted in Department of Periodontology, Sri Ramakrishna Dental College and Hospital, Coimbatore, Tamilnadu, India.

The mean age of the study population was 22.6167 ± 4.46110 years. The mean plaque index score of total study population was 1.2772 ± 0.38393. The mean calculus index score of total study population was 1.0234 ± 0.39973.

In this present study, it was observed that, as the calculus index score increases, there was a significant gradual increase in the levels of orthophosphate and enzyme pyrophosphatase. Simultaneously, there was a decrease in the levels of pyrophosphate from group I to group III. Relatively, in the plaque group, when compared to the values from the calculus groups, the levels of pyrophosphate is increased significantly. And, the values of orthophosphate and enzyme pyrophosphatase were decreased.

Hence, it was conclusive that orthophosphate, and the enzyme pyrophosphatase plays a significant role in formation the dental calculus. And, it is evident that pyrophosphate being a strong inhibitor in formation of the dental calculus, acts as an anticalculus agent, present in the human saliva.

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