ASSESSMENT OF BRUSH BIOPSY FINDINGS AND SALIVARY LDH LEVELS IN ORAL MUCOSAL LESIONS OF TOBACCO USERS

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DECLARATION BY THE CANDIDATE

TITLE OF DISSERTATION	ASSESSMENT OF BRUSH BIOPSY FINDINGS AND SALIVARY LDH LEVELS IN ORAL MUCOSAL LESIONS OF TOBACCO USERS
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NAME OF THE GUIDE	Dr. Senthil Kumar, M.D.S
HEAD OF THE DEPARTMENT	Dr. (Capt) S. Elangovan , M.D.S

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Dr. (Capt) S. Elangovan, M.D.S.,

PROFESSOR

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DEPARTMENT OF ORAL MEDICINE
K.S.R. INSTITUTE OF DENTAL
SCIENCE & RESEARCH,
TIRUCHENGODE - 637 215.

Signature of the Guide

Dr. Senthil kumar, M.D.S.,

PROFESSOR

K.S.R INSTITUTE OF DENTAL SCIENCE AND RESEARCH TIRUCHENGODE

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Dr. (Capt) S. ELANGOVAN, M.D.S.,

PROFESSOR

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DEPARTMENT OF ORAL MEDICINE
K.S.R. INSTITUTE OF DENTAL
SCIENCE & RESEARCH.
TIRUCHENGODE - 637 215.

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Dr. G.S. KUMAR, M.D.S.,

PRINCIPAL

PRINCIPAL,
K.S.R. INSTITUTE OF DENTAL
SCIENCE & RESEARCH,
K.S.R. KALVI NAGAR,
THOKKAVADI POST,
TIRUCHENGODE - 637 215

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The Tamil Nadu Dr.M.G.R. Medical University

69, Anna Salai, Guindy, Chennai - 600 032.

This certificate is awarded to Dr. S. SAM PONRAJ DAVIDSON

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KSR INSTITUTE OF DENTAL SCIENCE & RESEARCH

KSR Kalvi Nagar, Tiruchengode-637 215. Tamilnadu.
Phone : 04288-274981, Fax : 04288-274761.
email : ksrdentalcollege@yahoo.com

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To

Dr.S.Sam Ponraj Davidson,
Postgraduate Student,
Dept. of Oral Medicine & Radiology,
KSR Institute of Dental Science & Research,

*****

Your dissertational study titled "ASSESSMENT OF BRUSH BIOPSY FINDINGS AND SALIVARY LDH LEVELS IN ORAL MUCOSAL LESIONS OF TOBACCO USERS" presented before the ethical committee on 14th Dec. 2017 has been discussed by the committee members and has been approved.

You are requested to adhere to the ICMR guidelines on Biomedical Research and follow good clinical practice. You are requested to inform the progress of work from time to time and submit a final report on the completion of study.

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(Dr. Philip Robinson)

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“l Dedicate this work to my beloved parents, ***Mr. D.Sunder Singh & Mrs. Regis Sunder Singh*** *and my brother* ***Mr. S.Anand Joshua Daniel*** *for their care, love, support and prayers to overcome all my hardships and relieving me from responsibilities and giving way to make up with my course”*

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Introduction

INTRODUCTION

Cancer is a group of diseases characterized by an abnormal proliferation of any of the different kinds of cells in the body which grow and divide in an uncontrolled manner, invading normal tissues and organs and eventually spreading throughout the body which can metastasize to distant sites. Oral cancer (OC) was first recorded during 600 BC in India. It is the sixth most prevalent type of cancer apart from Lung, Breast, Colorectal, Prostate and Liver cancers and also poses great challenge to the public health in India . 275,000 cases of oral and 130,300 cases of pharyngeal cancers are reported annually around the globe which excludes nasopharyngeal cancers and 168,850 new cases of lip and oral cancers are reported in India. The etiology of oral cancer in India is most commonly due to the frequent use of tobacco, especially the smokeless form, betel (Areca) nut chewing and chronic alcoholism along with poor dental hygiene and malnourishment. The same is seen in areas of Central Asia and also on the rest of the Indian subcontinent due to inadvertent smoking, alcoholism and along with the chronic use of betel quid, with or without smokeless tobacco including poor diet[1-5].

The risk of oral cancer increases by twenty four fold in chronic alcoholics who use tobacco [5]. Oral premalignant disorders are defined as oral cancers in which the tissues are morphologically altered. Most of the oral premalignant disorders (OPMDs) leads to the development of OSCC and one of the most commonly encountered lesion is leukoplakia and its prevalence is estimated to be 2% globally[6,7].

Leukoplakia is defined as a white plaque or patch which cannot be diagnosed pathologically or clinically as any other disease and this definition does not carry any histologic connotation. Oral leukoplakia can be of homogeneous, non-homogeneous and non-homogeneous type has subtypes and they are speckled, nodular, verrucous variants. The homogeneous leukoplakia is a thin white uniform area altering or not with normal mucosa, the speckled type is a white and red lesion, with a majority of white surface, verrucous leukoplakia has an proliferative, corrugated or elevated surface appearance, the nodular type has small polypoid rounded outgrowths predominantly white excrescences. The malignant potential is very high in Non-homogeneous lesions, and on further investigation at the primary site of examination, verrucous carcinoma or squamous cell carcinoma can be evident [8]. Pathohistological examination of leukoplakia can show hyperkeratosis, atrophy, acanthosis and may or may not demonstrate different degrees of epithelial dysplasia. Dysplasia reflects histological changes which are followed by the loss of uniformity of the architecture of the epithelial cells and based on histological examination the presence of dysplasia has been associated with a risk of malignant transformation to oral cancer[8,9,10].

Even though there are more advanced cancer treatments, early detection is the best way to fortify survival of patient, improved quality of life, reduced mortality. So to diagnose the precancerous lesion, the use of less expensive non computer assisted brush cytology or brush biopsy can be performed and can later confirm by using scalpel biopsy[10]. Brush Biopsy (BB) is a technique which involves scraping of surface epithelium and is less

traumatic, can be used as a routine procedure and it was designed to screen innocuous appearing oral epithelial abnormalities for dysplasia or cancer[11]. Brush cytology is a noninvasive method for diagnosing early carcinoma and dysplasia in those patients who are either asymptomatic or in those with minor symptoms who do not warrant immediate biopsy. The mechanism of cytology in the mucosa is based upon the fact that cancerous and dysplastic cells tend to have fewer and less stronger connections to each other and to their adjacent normal cells in the surrounding tissue. Dysplastic and cancerous cells therefore, tend to exfoliate and can easily be collected from the surface of the lesion. A sample from a dysplastic or cancerous lesion, when applied to a microscope slide will often contain abnormalities[12,13,14]. Lactate dehydrogenase (LDH) is a cytoplasmatic enzyme which is present in almost all major organ systems. The extracellular appearance of LDH is mainly used to detect cell damage or cell death. LDH concentration in saliva increases when a lesion affects the integrity of the oral mucosa which can be considered to be a specific diagnostic indicator.

The development of cancer is usually associated with increased activity of glycolysis and because of this process, certain tissues will show a prolific increase in lactate dehydrogenase (LDH) levels. So this study is designed on patients with Oral leukoplakia, oral cancer and tobacco pouch keratosis to assess brush biopsy findings and salivary LDH levels and compare them[15,16,17].

Aims and Objectives

AIM

To assess the efficacy of brush biopsy findings and salivary LDH levels in oral mucosal lesions of tobacco users.

OBJECTIVES

1. The purpose of the study is to evaluate and compare the brush biopsy findings with the corresponding salivary LDH levels.
2. To assess the dysplastic changes in oral mucosal lesions of tobacco users.

Review of Literature

REVIEW OF LITERATURE

Early disease detection is not only vital to reduce disease severity and prevent complications, but also critical to increase success rate of therapy[1]. Saliva has been studied largely as a potential diagnostic tool over the last decade due to its non invasive and ease of accessibility along with its abundance of biomarkers, such as genetic material and proteins. Saliva plays a pivotal role in the pathogenesis of OSCC and its composition has never been studied comprehensively in OSCC patients. Salivary analysis through assessment of its various components originating in the oral and oro-pharyngeal mucosa, as well as components originating in serum allows for the evaluation of local and systemic changes in the body. Salivary analysis can also be used to diagnose specific disease-related alterations such as epithelial tumour markers, which is an indicator of OSCC. Patients various salivary components thus represent both physiological and pathological alterations, thereby contributing to its diagnostic capabilities[16,17].

LDH is an enzyme detectable in the cytoplasm of almost every cell in the human body, which becomes extracellular upon cell death. Therefore, the extracellular presence of salivary LDH is always related to tissue breakdown and cell necrosis. Salivary LDH concentration indicates that the integrity of the oral mucosa is affected by the lesion, where the LDH is expressed in cellular necrosis and a possible correlation may exist between the levels of salivary LDH and the aggressiveness of oral OSCC lesion, as the mitotic rate of more aggressive lesions is higher and thus the salivary LDH is also

expected to increase and thus saliva is an important biomarker can be used as an adjunctive step for diagnosing oral cancers and precancers which improve the prognosis and outcome of the disease process[15,16,17].

The scalpel biopsy is the best and widely accepted and reliable method for definitive diagnosis of oral mucosal lesions. The oral brush biopsy is a technique used for evaluating when cancer or pre-cancer is suspected and dysplastic changes are observed in them. A specially designed brush with hard bristles, is fabricated to access and sample all epithelial layers, with the inclusion of the cells from the basal layer and superficial cells from the lamina propria during oral brush biopsy. A smear is made over the surface of the glass slide which contains material obtained from all epithelial layers in a disaggregated form. The ultimate goal of this procedure is to perform less painful and simpler biopsy unlike scalpel or punch biopsy and shows to be a highly sensitive and specific technique.[13,14].

STUDIES ON BRUSH BIOPSY

Sciubba et al (1999)[18] conducted a large scale study involving 945 patients with oral mucosal lesions The analysis, however, was considered incomplete, as 618 of 945 brush samples, including 517 of the 699 negative brush samples (73.9 %), were not followed with definitive incisional biopsy for diagnostic confirmation and they reported that 7 % of oral brush biopsy specimens were non-diagnostic.

Svirsky et al (2002)[19] conducted a small scale study and compared the results of oral brush biopsy with the standard scalpel. In that the total number of abnormal brush biopsies were 243 and out of which, 93 showed positive dysplasia and 150 showed negative dysplasia. So he concluded that the PPV of an abnormal brush biopsy was only around 38 %.

Christian (2002) [20] reported on the results of oral brush biopsy in 930 dentists and oral hygienists who were screened for oral cancer. Eighty-nine samples with 93 alarming oral lesions were evaluated by brush biopsy. Out of those lesions only 7 showed signs for positive or atypical dysplasia and on them scalpel biopsy was done which finally diagnosed 3 precancerous lesions.

Potter et al (2003)[21] examined all the cases of oral squamous cell carcinoma reported over a 2-year period to the university oral pathology service who had negative brush biopsy results before. They found that out of 115 cases of oral squamous cell

carcinomas, only 4 were seen negative on brush biopsy. The authors noted that, because not all 115 squamous cell carcinomas were preceded by a brush biopsy, they concluded that the false negative rates are more for oral brush biopsy.

Poate and colleagues (2004)[22] reported that of the oral brush biopsies has a sensitivity of 71.4 % in the correct detection of oral epithelial dysplasia and the corresponding specificity to be 32%. The Positive and Negative predictor value was 44.1 % and 60%). These investigators concluded that this non-invasive investigative procedures does detect all potentially malignant diseases in a smaller fashion.

Kujan et al (2006)[23] did a systematic review to see the effectiveness or quality of screening of oral cancer, stated that other screening methods pose neither useful nor harm to the patient. These authors suggested that further more high-quality studies with large sample sizes are needed to evaluate the efficacy and accuracy of other adjuvant methods in the screening of oral cancer.

Driemel and colleagues (2008)[24] assessed the efficacy of oral brush biopsies using hematoxylin and eosin (HE) staining and standardized morphological analysis for the early detection of oral precancers and cancers. Brush biopsies were done in 169 patients with clinically suspicious oral lesions. Out of 62 malignant lesions the cytological analysis identified 49 lesions. Out of 107 samples only seven benign lesions were diagnosed as false-positive. The authors concluded that the efficacy is 80%.

Patton et al (2008)[25] in a systematic review on adjunctive techniques for oral cancer examination and lesion diagnosis evaluated the effectiveness of ViziLite Plus with Toluidine Blue Toluidine blue (TB), Microlux DL, Orascoptic DK, VELscope, ViziLite and OralCDx brush biopsy. These investigators collected and organized the data from 23 articles meeting inclusion and exclusion criteria, including availability of histological outcomes. The most evidence base was seen for TB. A limited number of studies was available for ViziLite Plus, ViziLite, with Toluidine Blue and OralCDx. The authors concluded that there is evidence that Toluidine Blue can be used as a diagnostic adjunct in high-risk populations and suspicious mucosal lesions. OralCDx is comparatively more useful in the assessment of dysplastic changes in clinically alarming premalignant lesions.

Bhoopathi (2009)[26] in a cross-sectional study, assessed the accuracy and efficacy of the oral brush biopsy technique as a diagnostic aid in early identification of dysplastic oral lesions. The author concluded that the computer assisted brush biopsy technique always over-estimated the dysplastic lesions and produced a high and more number of insignificant false-positive results.

Hohlweg-Majert and associates (2009)[27] evaluated the advantages of efficacy of computer-assisted analytical approach of the oral brush biopsy compared with routine standard scalpel biopsy for the early detection of oral premalignant lesions. Both biopsies were performed on 75 patients in that 6 patients had to be eliminated due to

inappropriate results, and out of 69 samples, 43 shown dysplastic epithelium and diagnosing as carcinoma, and 11 as suspicious lesions. According to these results authors concluded that, oral brush biopsy can be used as a standardized, atraumatic method of screening oral lesions.

Trullenque-Eriksson and colleagues (2009)[28] analyzed publications related to examination techniques (ViziLite system and VELscope system) that might maximize the visualization of suspicious premalignant lesions of the oral cavity or that might facilitate the histopathological detection of suspicious lesions using OralCDx. The authors concluded that clinical examination with biopsy and histopathological diagnosis remain the gold standard for the confirmation of oral cancer.

Toyoshima et al (2009)[29] determined the detection of cytokeratin (CK) mRNA in OSCC cells and evaluated the CK relevance for OSCC diagnosis in a brush biopsy test. The authors concluded that brush biopsy properly has more potential for detection of CK mRNA using real-time RT-qPCR. This preliminary study demonstrated the CK 17 possibility for application; however, pivotal studies are needed to confirm CK 17 as a diagnostic marker of OSCC in a brush biopsy test.

Seoane Lestón and Diz Dios (2010)[30] noted that conventional oral examination are still the current gold standard for the screening of oral cancer, while biopsy and other further

examination are absolutely necessary for the detection of clinically suspected lesion. The author concluded that oral brush biopsy slightly over-estimate the results of dysplastic lesions.

In a cross-sectional study, **Bhoopathi and Mascarenhas (2011)**[31] evaluated oral and maxillofacial surgeons' ability in detecting oral dysplastic lesions to that of OralCDx brush biopsy. The authors concluded that the efficiency of the OralCDx brush biopsy was less compared to the oral surgeons' ability in diagnosing oral dysplastic; hence, the author recommended that the patients should be referred to an oral surgeon for further final evaluation.

Mehrotra et al (2011)[32] performed both scalpel biopsy and oral brush biopsies ain 85 patients with minimally suspicious oral lesion. In that 79 samples with brush biopsy samples matched the samples of scalpel biopsies, with 27 samples showed signs of carcinoma or dysplasia and in that 26 samples of which were diagnosed independently with the oral brush biopsy, with a sensitivity of 96.3 %. The authors found that the positive predictive value of 84 % in an abnormal oral brush biopsy

Kujan and associates (2018)[33] examined an innovative oral brush, Orcellex which is based on liquid-based cytology (LBC). The authors concluded that the Orcellex brush, oral liquid-based cytology, may have high accuracy for early detection of potentially malignant oral cancers and disorders.

H Alsarraf and colleagues (2018)[34] in a systematic review analyzed the efficacy and accuracy of oral brush biopsy for the early diagnosis of oral cancer and oral potentially malignant disorders (OPMDs) in published evidence. The authors concluded that the findings from their study has shown that meaningful evidence-based recommendations for the use of a oral brush cytology to be utilized as an adjunctive tool in early detection of oral cancer and OPMDs and subsequent screening are complicated from the reported studies in the literature.

STUDIES ON SALIVARY LACTATE DEHYDROGENASE

Musumeci V et al (1993)[35] conducted a study on Aminotransferases and lactate dehydrogenase in saliva of diabetic patients and they concluded that diabetics have elevated activities in saliva of LDH, probably secondary to the diabetic involvement of salivary glands.

Rafael m. nagler et al (2001)[36] conducted a study to examine the whole saliva for LDH activity before and after exposing it with cigarette smoke. They found that exposure to cigarette smoke had no effect on LDH activity in the plasma. Whole saliva, unlike plasma, contains redox-active metal ions such as iron and copper that may enhance loss of LDH activity. They concluded that whole saliva in the presence of cigarette smoke becomes a potent protein-modifying agent that can destroy some of its endogenous components.

Priya Shirish Joshi et al (2012)[37] on their study in patients with oral leukolakia and oral cancer compared the levels of both Salivary and Serum LDH. They found that Salivary LDH was significantly high comparing with control groups but not as high as Serum LDH and there were few discrepancies and with this they concluded that salivary LDH estimation as a biochemical marker, can be used as an valuable substitute to serum LDH as it has more advantage over it and is an uncomplicated, atraumatic procedure.

Shishir Ram Shetty (2012)[38] in a biochemical study tested the levels of salivary LDH in oral leukoplakia and oral cancer, and found that the salivary LDH levels were significantly different in the samples between oral leukoplakia and oral squamous cell carcinoma and concluded that salivary LDH levels could be a reliable maker for oral cancer.

Priya Shirish Joshi et al (2014)[39] using gel electrophoresis, estimated the levels of lactate dehydrogenase isoenzyme in the saliva of patients with squamous cell carcinoma and oral leukoplakia. They found an overall increased salivary LDH isoenzyme level in the samples of oral leukoplakia and squamous cell carcinoma and concluded that oral precancer and cancer can be monitored by analyzing Salivary LDH levels which provides a valuable, chairside diagnostic tool.

Shrikant Patel et al (2015)[40] in a biochemical study estimated the levels of Salivary LDH in patients with oral leukoplakia and oral cancer, and found that salivary LDH levels increased in Oral Leukoplakia group from healthy control group and further increased in OSCC group. He concluded that an overall altered salivary LDH enzyme levels were seen on salivary analysis for LDH enzyme in oral leukoplakia and oral squamous cell carcinoma cases

Kavyashree Lokesh et al (2016)[41] conducted a study to correlate a relationship between the salivary levels of LDH and its efficiency as a diagnostic biomarker in the detection of OSCC and they concluded that in patients with OSCC the corresponding salivary LDH levels were markedly increased than normal level.

Kumuda Rao et al (2017)[42] conducted a study to estimate the serum and salivary lactate dehydrogenase levels in smokers and non-smokers and they found that, there was a prolific increase in serum and salivary LDH levels for smokers compared to non-smokers and concluded that smoking makes more chances for upregulating the levels of serum and salivary LDH.

Materials and Methods

MATERIALS AND METHODS

SOURCE OF DATA COLLECTION

The size of sample study consist of 80 patients who use tobacco (smoking and smokeless forms) with oral mucosal lesions of both genders who were attending as outpatients in K.S.R dental college, Tamil Nadu, India, between January 2018 to September 2019 were chosen for the study.

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METHODOLOGY

The selection of the subjects would be based on their past deleterious habit and medical history. All the subjects were clinically examined to assess the oral hygiene and to exclude the possibility of any other oral disease or systemic disease with oral manifestation. All the Subjects of the study were informed about the procedure and a written consent was obtained

INCLUSION CRITERIA:

- Patients who had chronic tobacco (smokeless and smoking) use with associated oral mucosal lesions.
- The patients include both males and females in the age group of 20 – 70 years.

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EXCLUSION CRITERIA:

- Patients who had the history of any other systemic diseases were excluded
- Patients who were under any long term medications were excluded.
- Patients who refused to participate were excluded.

SALIVA COLLECTION

Saliva collection was done by spitting method **(Fig-3).** Subjects were comfortably seated in the dental chair and a few minutes of relaxation for the procedure of collecting saliva in a sterile container **(Fig-2)**. During saliva collection, subjects were instructed not to speak or swallow. After salivary collection, the collected salivary samples were centrifuged **(Fig-4)** and stored in a deep freezer. And then the stored saliva samples were analyzed using Spectrophotometer **(Fig-6)** for evaluation of salivary LDH levels.

BRUSH BIOPSY

After saliva collection, Brush biopsy was performed using Cervical cytology brush **(Fig-7)**. Standard universal precautions, including hand washing and the use of gloves were done. The cytology brush is lightly applied to the oral lesion with just enough pressure so that the handle of the brush bows slightly. The cytology brush was rotated 360° on the surface of the lesion. It was then placed on a clean glass slide **(Fig-8)** and again rolled 360° and spreaded over its surface. The slide was immediately sprayed with a cytofixative spray and allowed to dry. The dried slides were stored in a slidebox.

STAINING

The dried, stored slides were stained using RAPID PAP

PAPANICOLAOU STAIN (Fig-9) for cytopathological analysis.

RAPID PAP STAIN AND REAGENT CONTENTS

1	RAPID-PAP NUCLEAR STAIN (Hematoxylin solution)
2A	RAPID-PAP CYTOPLASM STAIN (OG – 6 solution)
2B	RAPID-PAP CYTOPLASM STAIN (Light Green SF – Eosin)
3	BIOFIX-SPRAY (Micro-anatomy fixative)
4	D.P.X (Glass mounting medium)
5	RAPID-PAP DEHYDRANT (Propanol)
6	XYLENE
7	RAPID-PAP WASHBUFFER (Scotte`s Tap water buffer)
8	One empty bottle to prepare working reagent
9	Dropping Plugs

PROCEDURE

HYDRATION – The fixed smear slides were hydrated for 3 – 5 minutes in tap water and then excess water from the slides were blotted out.

NUCLEAR STAINING – The slides were kept on the staining rack and few drops Of RAPID-PAP CYTOPLASM STAIN was added in drops to cover the smear area. After waiting 60 seconds the slides were washed in running tap water.

DEVELOPING – Then 3 to 5 drops of wash buffer was added and then washed with water after 20 seconds. The excess water from the slides were blotted out.

DEHYDRATION – Then dehydration of the slides were done by adding RAPID-PAP DEHYDRANT for 60 seconds.

CYTOPLASM STAINING – Few drops of working CYTOPLASM STAIN which is a mixture of 2A and 2B was added and was spreaded over the smear area. After 60 seconds it was washed in a tap water.

MOUNTING – After drying the slides properly mounting was done using cover glass and a drop of D.P.X

After mounting the slides, all the slides were dried thoroughly.

MICROSCOPIC ANALYSIS

The mounted slides were evaluated using light microscope and the brush biopsy cytological smears were manually examined by two oral pathologists. If there were any discrepancies, a third opinion was obtained and consensus was agreed for a final diagnosis. The smears were analyzed for enlarged nuclei, variation in nuclear/cytoplasmic ratio, number of nuclei, hyperchromatism, and discrepancy in maturation. Smears were categorized as benign, suspicious for malignancy or malignant lesions and atypical lesions.

ARMAMENTARIUM :

SAMPLE COLLECTION :

Sterile Salivary sample container
Centrifuge
Salivary LDH kit
Spectrophotometer
Oral Cytology Brush
Microscopic slides
PAP Stain kit
Stained Slides

FIGURE 1: ARMAMENTARIUM FOR COLLECTING SALIVA

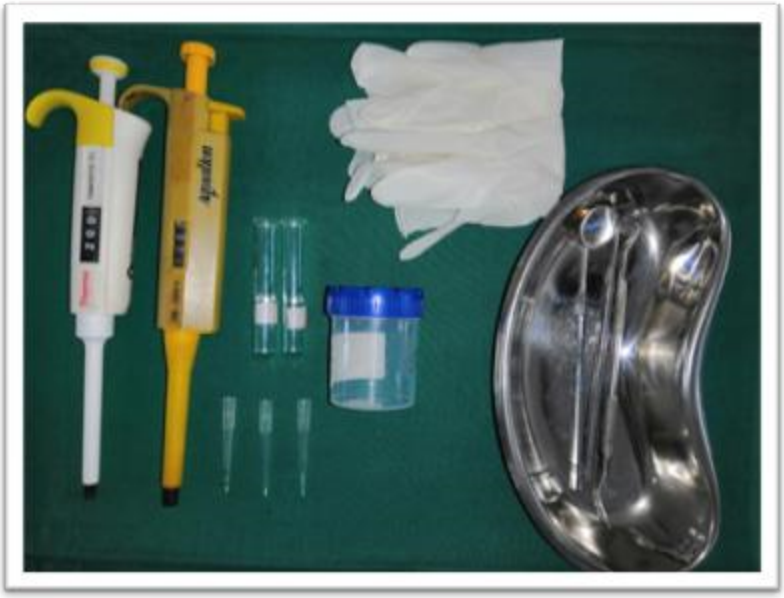


FIGURE 2: STERILE CONTAINERS FOR SALIVA COLLECTION

FIGURE 3 : SALIVA COLLECTION BY SPITTING METHOD

FIGURE 4: CENTRIFUGE

FIGURE 5: SALIVARY LDH KIT

FIGURE 6: SPECTROPHOTOMETER

FIGURE 7: ORAL CYTOLOGY BRUSH

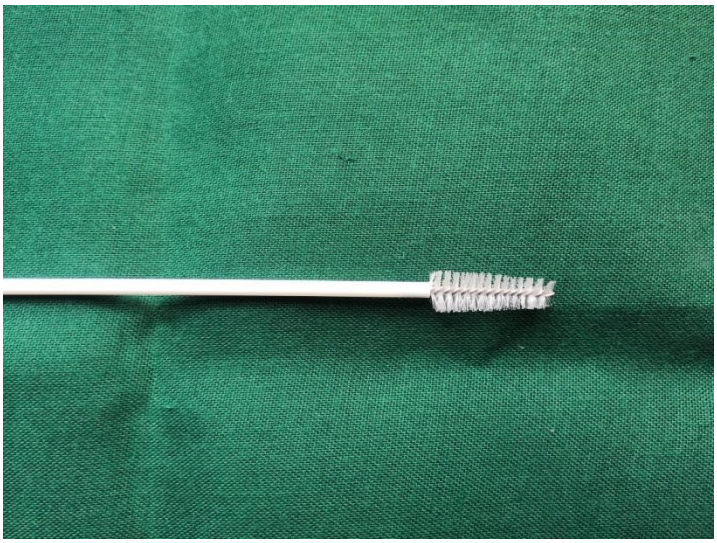


FIGURE 8: MICROSCOPIC SLIDES

FIGURE 9: PAP STAIN KIT

FIGURE 10: STAINED SLIDES

Statistical Analysis

STATISTICAL ANALYSIS

The data obtained from the study was entered in Microsoft Excel and statistical analysis was done. The data was analysed using Statistical Package for Social Sciences (SPSS) software version 16.0 (Windows version 17.0 SPSS Inc.,Chicago,IL,USA).The level of significance (α) was fixed at 5% ($P \leq 0.05$). Statistical analysis was done using the t-test and ANOVA.

t TEST:

Statistical analysis was done using *t*-test. A *t*-test is most commonly applied when the test statistics would follow a normal distribution if the value of a scaling term in the test statistic were known.

ANALYSIS OF VARIANCE (ANOVA):

ANOVA provides a statistical test of whether the population means of several groups are equal, and therefore generalizes the *t*-test to more than two groups. ANOVA is useful in evaluating the statistical significance, by which the mean of two or more group is compared.

Results

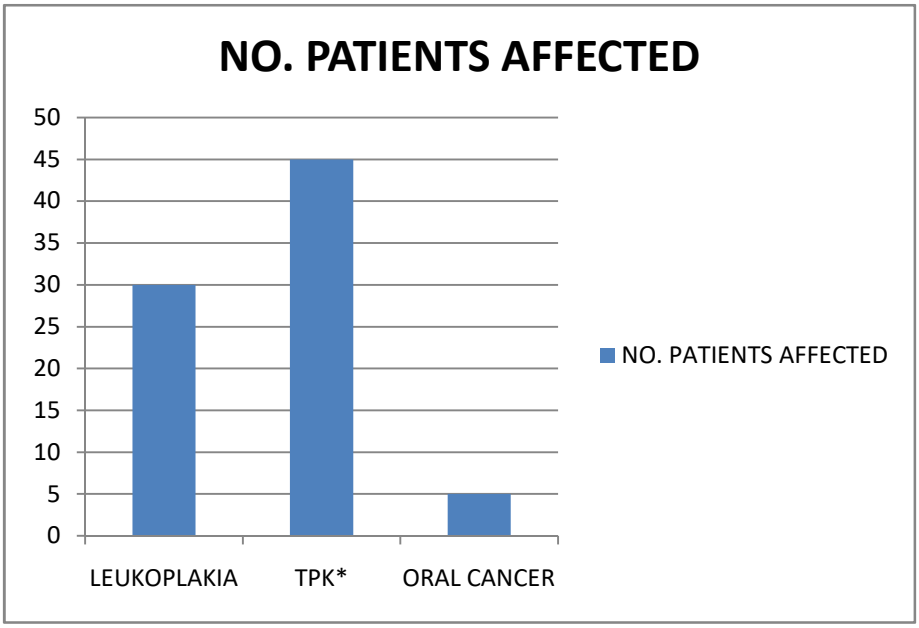
RESULTS

Table 1: Showing total number of patients with Oral Leukoplakia, Tobacco pouch keratosis and Oral cancer.

S.NO	CONDITION	TOTAL NO. PATIENTS
1	LEUKOPLAKIA	30
2	TOBACCO POUCH KERATOSIS	45
3	ORAL CANCER	05

A total of 80 patients who were with tobacco related oral mucosal lesions were selected for the study and out of those, 30 were leukoplakia, 45 were tobacco pouch keratosis and 5 with oral cancer.

Graph 1 : Distribution of subjects in the study group



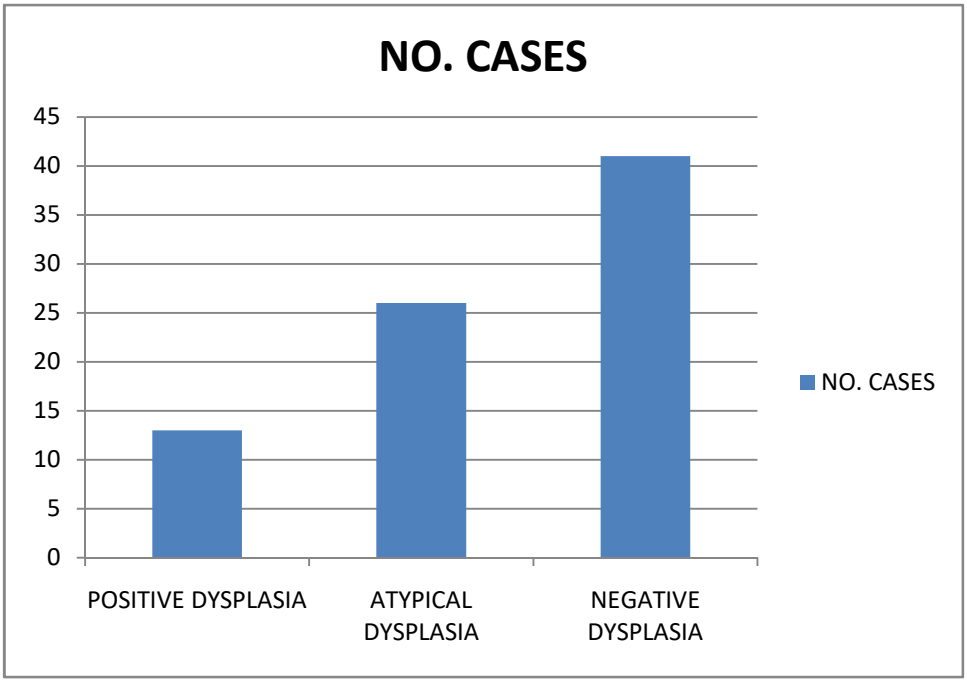
The bar diagram shows that out of 80 samples, tobacco pouch keratosis tops with 45 samples comparing with oral leukoplakia and oral which is 30 and 5 respectively

Table 2 : Showing distribution of dysplastic changes in the study samples

S.NO	DYSPLASTIC CHANGES	NO. CASES
1	POSITIVE DYSPLASIA	13
2	ATYPICAL DYSPLASIA	26
3	NEGATIVE DYSPLASIA	41

Out of those 80 samples, 13 samples showed positive dysplastic changes, 26 samples showed atypical dysplastic changes and 41 samples showed no signs of dysplastic changes and concluded as negative.

Graph 2 : Showing distribution of dysplastic changes in the study samples



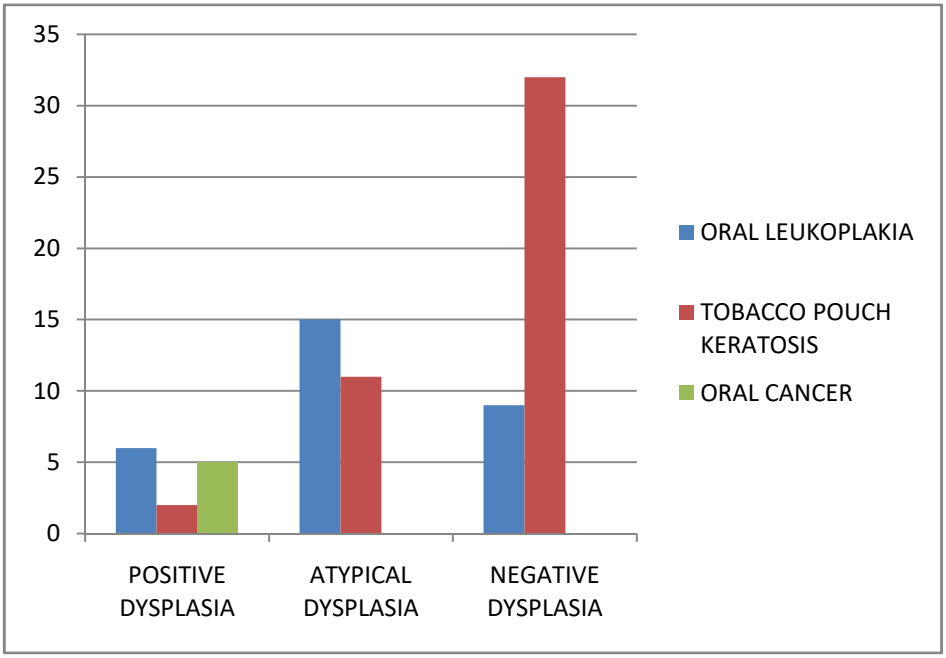
Graph shows the distribution of dysplastic changes among the selected samples showing that the negative dysplasia is more with 41 samples than positive and atypical dysplasia which is 13 and 26 respectively.

Table 3 : Showing levels of dysplastic changes in Oral leukoplakia, Tobacco pouch keratosis and Oral cancer.

S.NO	OUTCOMES	ORAL LEUKOPLAKIA	TOBACCO POUCH KERATOSIS	ORAL CANCER
1	POSITIVE DYSPLASIA	6	2	5
2	ATYPICAL DYSPLASIA	15	11	0
3	NEGATIVE DYSPLASIA	9	32	0

Among 30 samples with oral leukoplakia, 6 showed positive dysplasia, 15 showed atypical dysplasia and 9 showed negative dysplasia. Among 45 samples with tobacco pouch keratosis, 2 showed positive dysplasia, 11 showed atypical dysplasia and 32 samples showed negative results. Among 5 samples of oral cancer, all the 5 showed positive dysplasia

Graph 3 : Showing levels of dysplastic changes in Oral leukoplakia, Tobacco pouch keratosis and Oral cancer



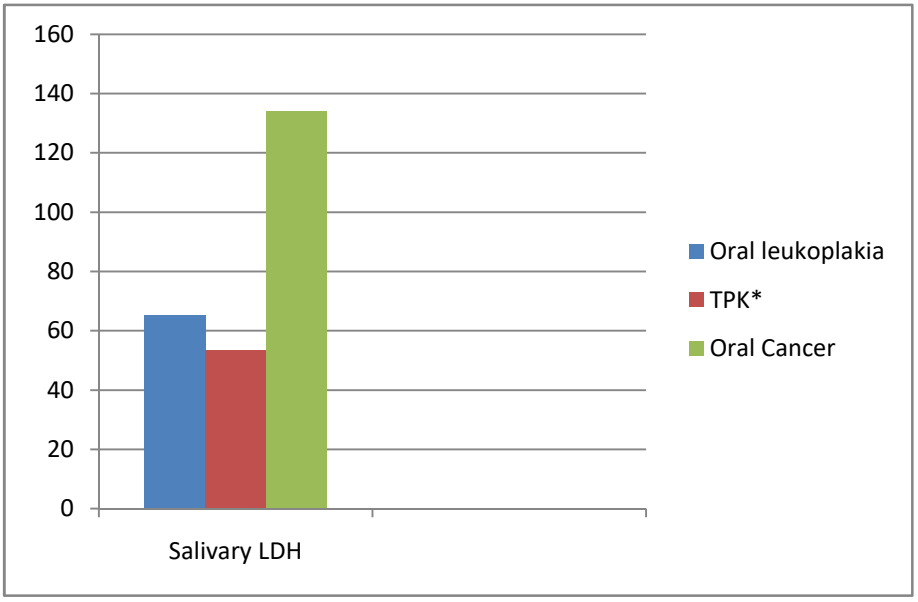
The bar diagram shows the distribution of levels of dysplastic changes In Oral leukoplakia, tobacco pouch keratosis and oral cancer. Positive dysplasia is seen in all the three conditions.

Table 4 : Showing levels of Salivary LDH in Oral leukoplakia, tobacco pouch keratosis and oral cancer

	NUMBER	MEAN ± SD	P VALUE
ORAL LEUKOPLAKIA	30	65.21±17.21	P=0.00
TOBACCO POUCH KERATOSIS	45	53.55±16.79	P=0.00
ORAL CANCER	05	136.01±20.98	P=0.00

Among 80 samples, 30 were oral leukoplakia with a mean salivary LDH level of 65.21, 45 were tobacco pouch keratosis with a mean salivary LDH level of 53.553 and 5 were Oral cancer with a mean salivary LDH level of 134.014 showing significant increase comparing the other two lesions

Graph 4 : Showing levels of Salivary LDH in Oral leukoplakia, tobacco pouch keratosis and oral cancer



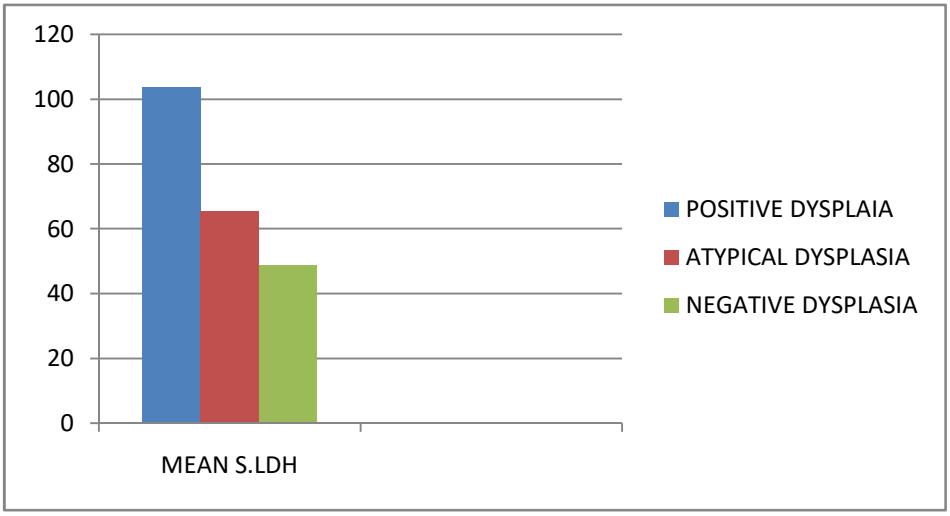
The bar diagram clearly shows that the mean value of salivary LDH for oral cancer is higher when compared to oral leukoplakia and tobacco pouch keratosis and the P – values are statistically significant.

Table 5 : Showing levels of Salivary LDH in Postive, Atypical and Negative dyspasia

	NUMBER	MEAN ± SD	P VALUE
POSITIVE DYSPLASIA	13	103.615±31.69	P=0.00
ATYPCAL DYSPLASIA	26	65.450±14.82	P=0.00
NEGATIVE DYSPLASIA	41	48.721±12.32	P=0.00

On comparing the results of brush biopsy findings and salivary LDH levels the mean salivary LDH value for the samples with positive dysplasia is 103.615, the mean salivary LDH value for the samples with atypical dysplasia is 65.449 and the mean salivary LDH value for the samples with negative dysplasia is 48.721

Graph 5 : Showing levels of Salivary LDH in Postive, Atypical and Negative dyspasia



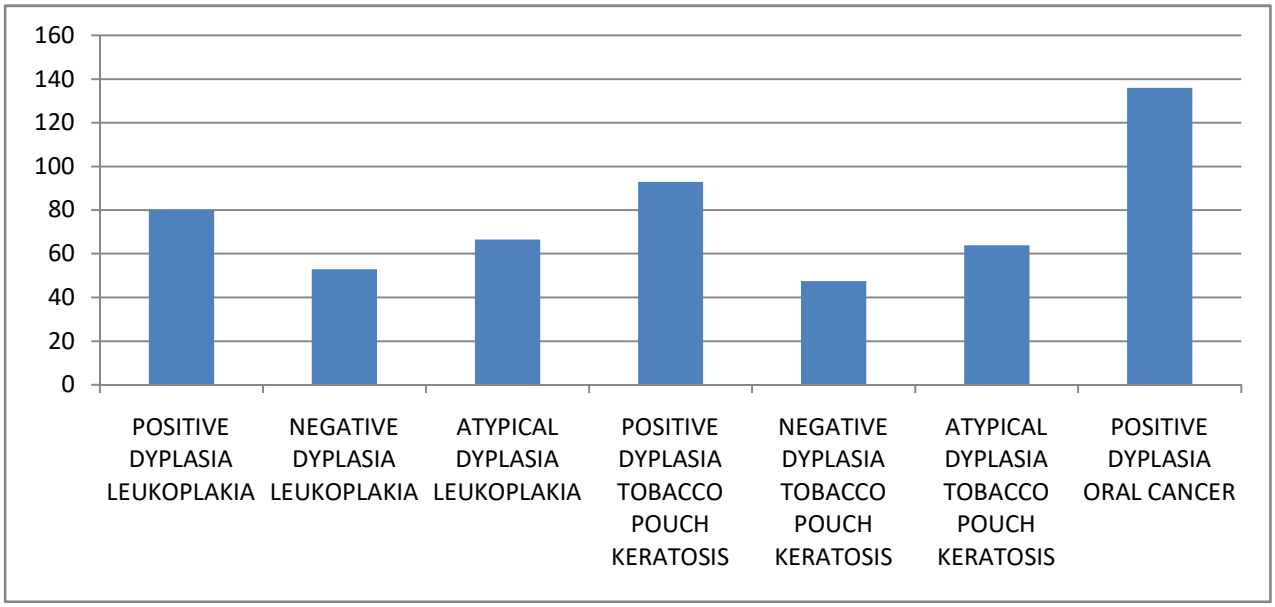
The bar diagram clearly shows that the mean value of salivary LDH with positive dysplasia is higher on comparing atypical and negative dysplasia and the P – value is statistically significant.

Table 6 : Showing the comparison of results of Brush biopsy and salivary LDH levels.

	NUMBER	MEAN±SD	P - VALUE
POSITIVE DYPLASIA LEUKOPLAKIA	6	80.20±17.08	P=0.00
NEGATIVE DYPLASIA LEUKOPLAKIA	9	52.91±17.08	P=0.00
ATYPICAL DYPLASIA LEUKOPLAKIA	15	66.58±15.15	P=0.00
POSITIVE DYPLASIA TOBACCO POUCH KERATOSIS	2	92.85±7.91	P=0.00
NEGATIVE DYPLASIA TOBACCO POUCH KERATOSIS	32	47.54±12.30	P=0.00
ATYPICAL DYPLASIA TOBACCO POUCH KERATOSIS	11	63.89±14.94	P=0.00
POSITIVE DYPLASIA ORAL CANCER	5	136.01±20.98	P=0.00

Among 80 samples the results of brush biopsy were divided into 7 Groups for leukoplakia, tobacco pouch keratosis and oral cancer based on positive, negative and atypical dysplasia. By using t`test mean value for these 7 groups were calculated.

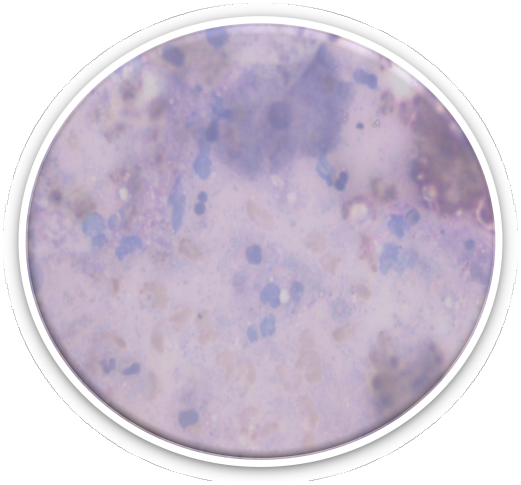
Graph 6 : Showing the comparison of results of Brush biopsy and salivary LDH levels.



The bar diagram clearly shows that the mean value of salivary LDH for oral cancer with positive dysplasia is higher than all the other groups and the P – value is statistically significant.

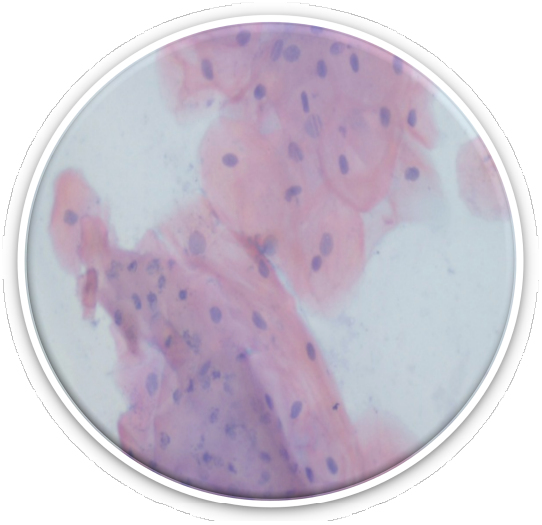
HISTOPATHOLOGY:

Figure 10 : Showing POSITIVE DYSPLASIA



The above histopathological picture shows nuclear hyperchromatism, increased mitotic activity, enlarged nuclei and increased nuclear – cytoplasmic ratio

Figure 11 : Showing ATYPICAL DYSPLASIA



The above histopathological picture shows doubtful or unclear nuclear – cellular cytoplasmic ratio and mitotic activity.

Discussion

DISCUSSION

The term oral cancer encompasses all malignancies that originate in the oral tissues and remains a major public health problem throughout the world as an important case of poor health and illness. The disease is characterized by high degree of morbidity and mortality (about 50%) (44,45) . Oral Squamous cell carcinoma (OSCC) progresses from normal to precancerous lesion (dysplastic cells) and ultimately to squamous cell carcinoma which implies it is a multistage process. The Oral leukoplakia (OL) is a precancerous lesion representing 85%. Even though scalpel biopsy is the gold standard and widely accepted method in diagnosing the premalignant and malignant lesions, it is not possible to use this technique in all suspected oral lesions. In these scenarios, brush cytology acts an alternative technique. Not all patients readily accepts scalpel biopsy because of its invasiveness. Brush cytology is useful in certain scenarios where a medically compromised patients would have possible exposure unwanted surgical risks.(56,57). The oral brush biopsy is an less complicated, easy, less traumatic technique and has the possibility to outpower many of the barriers that had been hindrance in early detection of various cancers and dysplasia (55). Oral cells can be obtained by different methods like scraping the superficial surface of the oral mucosa, by oral cavity rinse or even by collection of saliva samples from the patients. Cells from deeper layers of the epithelium can be yielded by using cytology brush. The mechanism of cytology in the mucosa is based upon the fact that cancerous and dysplastic cells tend to have fewer and less stronger connections to each other and to their adjacent normal cells in the surrounding tissue. Dysplastic and cancerous cells therefore, tend to exfoliate and can easily be collected from the surface of the lesion. In this study, a cervical cytobrush was used to

perform brush biopsy in potentially malignant disorders and malignant lesions of oral cavity and was subjected to histopathological examination for definitve diagnosis(56,57).

A shift from aerobic to anaerobic glycolysis with increased glycolytic activity is seen during the development of cancer. The collateral increase in the lactate dehydrogenase (LDH) enzyme is a reflection of increased glycolytic activity in certain tissues(46,47). Lactate dehydrogenase (LDH) is basically seen in all major organ systems which is an cytoplasmatic enzyme. The extracellular appearance of LDH is used to detect damage to the cells or necrosis.(48).

The LDH in the whole saliva may originate from various sources in the oral cavity, which can be contributed by the major and minor salivary gland secretions, the fluids from the oral epithelium and the periodontium, gastrointestinal reflux materials, and cellular and other debris. The major differences in the composition of serum and saliva is that saliva is distinct its constituents and components may play a major physiological role. The constituents of the salivary LDH is entirely variant from that of plasma but is similar to that found in oral epithelium. This indicates that the major source of salivary LDH is the shedding cells in oral epithelium and it is obvious to assume that pathological alterations of oral epithelium due to dysplasia or cancer may result in alteration of salivary LDH levels. Therefore, possible oral mucosal pathologies can be evaluated by estimating the levels of salivary LDH (49).

In the present study brush biopsy was done to evaluate the dysplastic changes in oral leukoplakia, tobacco pouch keratosis and oral cancer. The "abnormal," "atypical," and "positive" brush biopsies were determined using positive predictive values (PPVs). Out of 80 samples 13 showed positive dysplasia, 26 showed atypical dysplasia and 41 showed negative dysplasia (Table–2, Graph–2). Out of 30 samples of oral leukoplakia, 6 showed positive dysplasia, 15 showed atypical dysplasia and 9 showed negative dysplasia and out of 45 samples of tobacco pouch keratosis, 2 showed positive dysplasia, 11 showed atypical dysplasia and 32 showed negative dysplasia and out of 5 samples of oral cancer, all the 5 showed positive dysplasia (Table-3, Graph-3). The histopathological picture (Figure – 10) showed clear evidence of nuclear hyperchromatism, increased mitotic activity, enlarged nuclei and increased nuclear – cytoplasmic ratio which are the characteristic features of Positive dysplasia. The histopathological picture (Figure – 11) lacked sufficient details for positive dysplasia and it showed doubtful or unclear nuclear - cellular cytoplasmic ratio and mitotic activity suggesting that it is Atypical dysplasia.

On the other hand salivary LDH levels were estimated in patients with Oral Leukoplakia, Oral Cancer and Tobacco pouch keratosis. The results of which showed that in a total of 5 oral cancer patients, all the 5 showed significant increase in salivary LDH levels(Table-4, Graph-4) with a mean value of 136.01. And a 30 samples of Oral leukoplakia also showed significant increase in salivary LDH levels(Table-4, Graph-4) with a mean value of 65.21. All of the above studies showed an accelerated increase in the mean value of salivary LDH levels from oral leukoplakia to oral cancer which is similar to the present

study and regarding the P-values all the groups were having statistically significant results. On comparing the results of brush biopsy findings with the levels of salivary LDH, for the total number of 13 samples with positive dysplasia, the mean value of salivary LDH is 103.615, for the total number of 26 samples of atypical dysplasia, the mean value of salivary LDH is 65.449 and for the total number of 41 samples of negative dysplasia, the mean value of salivary LDH is 48.721 which clearly shows that there is significant increase in the salivary LDH levels of samples of positive dysplasia comparing the other two groups (Table-5, Graph-5) and the P-values are statistically significant.

The above results of brush biopsy correlates with the studies done by Christian et al. (2002) and Bhoopathi et al. (2009) and regarding the results of salivary LDH levels, this study correlates with the studies done by Masahiro et al. (1971), Hariharan et al. (1977), Muralidhar et al. (1998), Shetty et al. (2012) and Joshi et al. (2012)

(Table-6, Graph-6) shows the comparison of combined results of brush biopsy and salivary LDH levels. The results of brush biopsy were divided into 7 groups for oral leukoplakia, tobacco pouch keratosis and oral cancer based on positive, negative and atypical dysplasias. The total number of samples with positive dysplasia in leukoplakia is 6 with mean salivary LDH level of 80.20. The total number of samples with negative dysplasia in leukoplakia is 9 with mean salivary LDH level of 52.91. The total number of samples with atypical dysplasia in leukoplakia is 15 with mean salivary LDH level of 66.58. The total number of samples with positive dysplasia in tobacco pouch keratosis is

2 with mean salivary LDH level of 92.85. The total number of samples with negative dysplasia in tobacco pouch keratosis is 32 with mean salivary LDH level of 47.54. The total number of samples with atypical dysplasia in tobacco pouch keratosis is 11 with mean salivary LDH level of 47.54. The total number of samples with positive dysplasia in oral cancer is 5 with mean salivary LDH level of 136.01 and regarding the P-values all the groups were having statistically significant results.

On comparing the results of findings of brush biopsy and salivary LDH levels, it clearly shows that brush biopsy gives significant results of aiding in finding dysplasias and significant rise in salivary LDH levels in positive dysplasias and results of both were statistically significant.

Summary and Conclusion

SUMMARY

We started our study with an aim to evaluate the efficacy of brush biopsy findings and salivary LDH levels in oral mucosal lesions of tobacco users and to compare the results of both and assess the dysplastic changes in oral mucosal lesions of tobacco users. The patients were selected for the study from the Oral Medicine and Radiology department and 80 patients were included in the study according to the inclusion and exclusion criteria only after obtaining their informed consent. The patients with oral leukoplakia, tobacco pouch keratosis and oral cancer were included in the study. After detailed history and examinations all the patients were counseled to quit the deleterious habits. Saliva samples were collected by spitting method and samples were collected in a sterile container and stored in a deep freezer and analyzed by spectrophotometer at the same time brush biopsy was performed for each patient and slides were made which were then fixed, stained with PAP stain and were analyzed for dysplastic changes. The results were analyzed using t'test and statistical analysis were done which showed that the mean value of salivary LDH were significantly increases in oral cancer than other two groups and 39 samples showed positive and atypical dysplasia combined out of 80 and the results were statistically significant.

CONCLUSION

The role of brush biopsy in detection of oral cancer is possible even at early stage of the lesion. When comparing with other noninvasive techniques, brush biopsy showed increased sensitivity and specificity in detecting premalignant lesions. This can be used as an ideal screening tool even in a normal clinical setup without requiring sophisticated instruments. Early detection of malignant diseases of the oral cavity can be done by estimating Salivary LDH levels which can be used as a diagnostic biomarker and its detection can serve as a potent diagnostic aid. Based on the results of this study we conclude that brush biopsy showed positive results in detecting premalignant lesions and salivary LDH levels were elevated significantly in oral mucosal lesions of tobacco users.

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Annexures

ANNEXURE-I

INFORMED CONSENT FORM

KSR INSTITUTE OF DENTAL SCIENCE & RESEARCH

ASSESSMENT OF BRUSH BIOPSY FINDINGS AND SALIVARY LDH LEVELS IN ORAL MUCOSAL LESIONS OF TOBACCO USERS

I ……. hereby declare that I clearly understood the procedures of the study. Also, I declare that I give permission for the above mentioned individual/organization/hospital to do the procedure to the individual/organization listed above.

Signature ……. Date……..

I have explained the above and answered all questions asked by the participant.

Signature…….. Date……..

ANNEXURE-II

ஒப்புகை வாக்குமூலம்

..... ஆகிய நான் மேற்கூறிய ஆராய்ச்சி படிப்பின் வழிமுறைகளைத் தெளிவாகப் புரிந்து கொண்டேன். மேலும் நான் இந்த ஆராய்ச்சிப் படிப்புக்கான வழிமுறைகளை மேற்கொள்வதற்கும். அதன் பரிசோதனை முடிவுகளை தெரிந்து கொள்ளவும் முழுமையாக அனுமதிக்கிறேன்.

.....

நோயாளியின் கையொப்பம் தேதி.....

நான் மேற்கூறிய ஆராய்ச்சிப் படிப்பிற்கான விதிமுறைகள் மற்றும் அது குறித்த நோயாளியின் சந்தேகங்களையும் தெளிவாக விளக்கியுள்ளேன்.

.....

மருத்துவரின் கையொப்பம் தேதி.....

ANNEXURE III

A. RESULTS OF BRUSH BIOPSY IN ORAL LEUKOPLAKIA, TOBACCO POUCH KERATOSIS AND ORAL CANCER

S. NO	POSITIVE	ATYPICAL	NEGATIVE
1			1
2			1
3		1	
4		1	
5		1	
6			1
7		1	
8			1
9		1	
10		1	
11		1	
12		1	
13		1	
14		1	
15			1
16		1	
17	1		
18			1
19			1
20			1
21			1
22		1	
23		1	
24		1	
25		1	
26			1
27		1	
28		1	
29			1
30		1	

A. RESULTS OF BRUSH BIOPSY IN ORAL LEUKOPLAKIA, TOBACCO POUCH KERATOSIS AND ORAL CANCER (*Continue…)*

S.NO	POSITIVE	ATYPICAL	NEGATIVE
31		1	
32			1
33		1	
34		1	
35			1
36			1
37			1
38			1
39			1
40		1	
41		1	
42		1	
43		1	
44			1
45			1
46		1	
47		1	
48		1	
49		1	
50		1	
51			1
52		1	
53		1	
54			1
55		1	
56		1	
57		1	
58	1		
59	1		
60		1	

A. RESULTS OF BRUSH BIOPSY IN ORAL LEUKOPLAKIA, TOBACCO POUCH KERATOSIS AND ORAL CANCER (*Continue…)*

S.NO	POSITIVE	ATYPICAL	NEGATIVE
61		1	
62			1
63			1
64	1		
65	1		
66	1		
67		1	
68			1
69	1		
70		1	
71		1	
72		1	
73			1
74	1		
75	1		
76			1
77	1		
78	1		
79	1		
80	1		

`1` - PRESENT

ANNEXURE IV

B. SALIVARY LDH VALUES

SAMPLE	SALIVARY LDH LEVELS
1	48.457
2	52.618
3	40.147
4	39.428
5	52.618
6	59.55
7	41.488
8	65.89
9	42.135
10	48.356
11	42.897
12	45.359
13	46.482
14	52.421
15	55.732
16	39.889
17	98.75
18	56.665
19	52.864
20	66.743
21	64.76
22	45.882
23	50.753
24	51.564
25	60.754
26	75.998
27	42.249
28	65.745
29	56.665
30	56.665

B. SALIVARY LDH VALUES (Continue..)

S.NO	SALIVARY LDH LEVELS
31	56.665
32	82.654
33	72.855
34	42.456
35	70.896
36	72.652
37	72.855
38	85.45
39	79.106
40	84.302
41	51.256
42	40.841
43	56.702
44	60.256
45	70.21
46	40.256
47	42.256
48	65.254
49	40.475
50	45.358
51	48.57
52	42.654
53	55.425
54	86.452
55	35.452
56	47.251
57	45.658
58	98.452
59	87.256
60	24.285

B. SALIVARY LDH VALUES (Continue..)

S.NO	SALIVARY LDH LEVELS
61	35.256
62	55.452
63	85.256
64	65.256
65	95.425
66	86.421
67	41.203
68	35.412
69	55.256
70	85.256
71	41.256
72	56.214
73	95.279
74	153.805
75	113.33
76	45.257
77	121.425
78	129.52
79	161.99
80	80.12
MEAN	**63.078**