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Evaluation of Salivary Alkaline Phosphatase Levels as a Biomarker in Oral Submucous Fibrosis – A Cross-Sectional Observational Study

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Abstract

Salivary alkaline phosphatase (S-ALP) levels have been found to be elevated in cases of oral squamous cell carcinoma and Oral Potentially Malignant Disorder, but their level in Oral Submucous Fibrosis (OSMF) is less explored. The present study aims to investigate salivary alkaline phosphatase levels (S-ALP) as a diagnostic biomarker in oral submucous fibrosis (OSMF). The objective of the study is to measure and compare the S-ALP in individuals with/without the habit of chewing betel nuts and to assess the relationship between S-ALP in individuals with/without betel nut induced oral lesions. The samples were recruited by the stratified random sampling method. A total of 36 patients were divided into two groups, with 18 participants in each group. ***Group A****-control group without habits and without lesions* ***Group B (study group), Group B1****-patients without betel nut induced lesions, and* ***Group B2*** *– Patients with betel nut induced lesions (OSMF). The data obtained were subjected to statistical analysis, and independent t-tests were done. The mean S-ALP was* ***8.2*** *IU/L for normal individuals without tobacco usage,* ***19.5*** *IU/L for patients with the habit of betel nut chewing but without lesions, and* ***49.4*** *IU/L for patients with betel nut chewing induced lesions. The mean difference between the groups was statistically significant (P < 0.001). The results of the present study suggest that S-ALP levels is markedly increased in patients with the habit of using smokeless tobacco suggesting that S-ALP can be used as a reliable non-invasive biomarker to monitor oral submucous fibrosis (OSMF)*

Keywords: *Betel Nut, Oral Submucous Fibrosis (OSMF), Oral Lesions, Salivary Alkaline Phosphatase (S-ALP).*

Introduction

The potentially malignant condition known as Oral Submucous Fibrosis (OSMF) was first identified as "Atropica idiopathica mucosae oris" by Schwartz in 1952 and subsequently by Jens J. Pindborg in 1966. It is defined as “an insidious, chronic disease that affects any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by, or associated with, the formation of vesicles, it is always associated with a juxta-epithelial inflammatory reaction followed by fibroelastic change of the lamina propria and epithelial atrophy that leads to stiffness of the oral mucosa and causes trismus and an inability to eat” [1]. According to recent estimates, the prevalence of OSMF in the Indian subcontinent spans a wide age range, from 11 to 60 years, with males having a prevalence of 0.2 to 2.3% and females having a prevalence of 1.2 to 4.6% [2]. Because oral submucous fibrosis has a high malignant transformation rate (7–13%), early detection and intervention are essential [3]. OSMF can be exacerbated by stimuli including betel quid chewing, consumption of chillies, smoking, individuals who are anaemic, vitamin deficiencies, and some allergic responses triggered by the activation of external antigens

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or genetic factors. According to a Taiwanese study, drinking alcohol raises the chance of OSMF and raises the total risk of OPMD's malignant transformation by 23% [4]. The ingredients of a betel quid in India are betel leaf, areca nut, slaked lime, and dried or fermented tobacco leaf. Though there are many contributing factors to the etiopathogenesis of this disease, as mentioned above, areca nut chewing in any form is the primary cause [2]. The main ingredient in areca nuts is thought to be arecoline, with other alkaloids, polyphenols (tannins), and metallic ions (copper) having supporting roles.

Clinical symptoms of OSMF include ulcers, dry mouth, burning sensations, and mouth opening limitations. As the disease progresses, it may also affect the pharynx and oesophagus, leading to fibrosis of the upper digestive tract. In the early stage of OSMF, oral mucosa whitening is an important clinical feature, while mouth opening limitation is caused by fibrosis of the oral mucosa area in the late stage. The typical symptoms of OSMF patients with limited mouth opening directly affect their quality of life. It should be noticed that many patients with OSMF have anxiety, depression, and stress and show weak social interaction. Though biopsy is still the gold standard for OSMF diagnosis, it is an invasive method, and in some cases, it may aggravate the pain in OSMF patients [5]. Hence, clinical signs and symptoms are used to make the diagnosis of OSMF [6]. Saliva has been used for detecting oral microenvironment changes in OSMF in previous studies. Several studies have indicated that patients with OSMF have higher salivary levels of certain proteins. OSMF biomarkers include, for instance, RNA, protein (LDH), 8-hydroxy-2-deoxyguanosine (8-OHdG), and MDA. No studies have been undertaken utilizing alkaline phosphatase as a biomarker for OSMF.

The present study aims to investigate salivary alkaline phosphatase levels (S-ALP) as a diagnostic biomarker in oral submucous fibrosis (OSMF). The objective of the study is to measure and compare the S-ALP in individuals with or without the habit of chewing betel nuts and to assess the relationship between S-ALP in individuals with or without betel nut-induced oral lesions.

Materials and Methods

The samples were recruited by the stratified random sampling method. A total of 36 patients [male = 35 (97.2%) and female = 1 (2.7%)] were divided into 2 groups, with 18 participants in each group.

Group A (Control Group Without Habits and Without Lesions),

Group B (Study Group)

1. **Group B1: patients without betel nut-induced lesions**
2. **Group B2: patients with betel nut-induced lesions (OSMF)**.

This study was ethically approved by the Institutional Review Board of SRM Dental College (IRB APPROVAL NUMBER: SRMU/M&HS/SRMDC/2023/PG/015). All the participants signed the informed consent forms before beginning the study. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki (2013).

Inclusion Criteria

1. Individuals ranging in age from 18 to 70 years are included in this study.
2. For Group B1 and Group B2, individuals with the habit of betel nut chewing for a minimum period of 6 months were included.
3. Patients who are clinically and histologically diagnosed with OSMF.

Exclusion Criteria

1. Individuals with systemic diseases/conditions such as diabetes, renal failure, liver cirrhosis, and bone disorders

such as rickets, obstructive jaundice, and hyperparathyroidism.

2. Individuals taking medication that could alter salivary characteristics.
3. Individuals with lesions other than OSMF but under OPMDs.

All patients were recruited from the dental OPD of SRM Dental College. A thorough history and clinical examination were performed, and patients with clinical signs and symptoms of OSMF were included. An incisional biopsy was performed for these patients, and a histopathological examination was done to confirm the diagnosis of OSMF.After one week, patients were reviewed. The individuals were explained about the purpose of the study, and informed consent was obtained. Three milliliters of unstimulated saliva were taken from each person using the spitting technique. The subjects were asked not to eat for two hours before having their saliva collected. After being instructed to rinse their mouths with water, participants were instructed to sit up straight for ten minutes, leaning their heads slightly forward to gather saliva on the bottom of their mouths, and then spit into a sample container. The samples were subsequently centrifuged for 15 minutes at 3000 rpm, yielding the saliva supernatant. For the ALP reagent (Alkaline Phosphatase (ALP)-AMP Kit, Biosystems S.A., Barcelona), 20 µl of the supernatant was combined with 1000 µl. The International Federation of Clinical Chemistry and Laboratory Medicine states that the kinetic photometric test was used to detect the amount of ALP based on the concept that p-nitrophenyl phosphate, which was detected at 405 nm, is converted by ALP into phosphate and p-nitrophenol.

Results

Descriptive statistics were done to assess the mean and standard deviation of S-ALP values between the two groups of participants. Inferential statistics were done using an independent-samples t-test to assess the comparison between the groups. To analyse the data, SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp., Released 2019) was used. The significance level was fixed at 5% ($\alpha = 0.05$). A P-value < 0.05 is considered to be statistically significant. The mean values of S-ALP were found to be 8.26 IU/L (standard deviation 6.06) in Group A and 34.52 IU/L in Group B (standard deviation 33.0) (Table 1, Figure 1). The mean difference in S-ALP between Groups A and B was 26.26 IU/L with a standard deviation of 33.42. The mean difference of S-ALP between Groups A and B was significant (P = 0.004) (Table 2). When the mean S-ALP values were compared between the subgroups of B1 and B2, 19.57 ± 5.29 IU/L was obtained in group B1 and 49.49 ± 42.22 IU/L was obtained in group B2 (Table 3, Figure 2). There was no significance in the mean values obtained between the subgroups of B1 and B2 (p = 0.070) (Table 4).

Table 1. Mean Salivary Alkaline Phosphatase levels in Group A and Group B

Variables	Group A	Group B
Mean	8.2670	34.5299
Std. Error of Mean	1.42738	7.77902
Std. Deviation	6.05585	33.00360
Variance	36.673	1089.238
Range	20.53	150.55

Table 2. Comparison of Salivary Alkaline Phosphatase Levels between the Group A and Group B

Groups	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	P-value
				Lower	Upper		
Group A VS Group B	-26.26289	33.41660	7.87637	-42.88057	-9.64520	-3.334	.004*

Table 3. Mean Salivary Alkaline Phosphatase Levels in Group B1 and Group B2

Variables	**Group B1**	**Group B2**
Mean	19.5670	49.4928
Std. Error of Mean	1.76566	14.07450
Std. Deviation	5.29699	42.22351
Variance	28.058	1782.824
Range	17.99	131.20

Table 4. Comparison of Salivary Alkaline Phosphatase Levels between the Group B1 and Group B2

GROUPS	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	P-value
				Lower	Upper		
Group B1 VS Group B2	-29.92578	42.93533	14.31178	-62.92880	3.07724	-2.091	.070

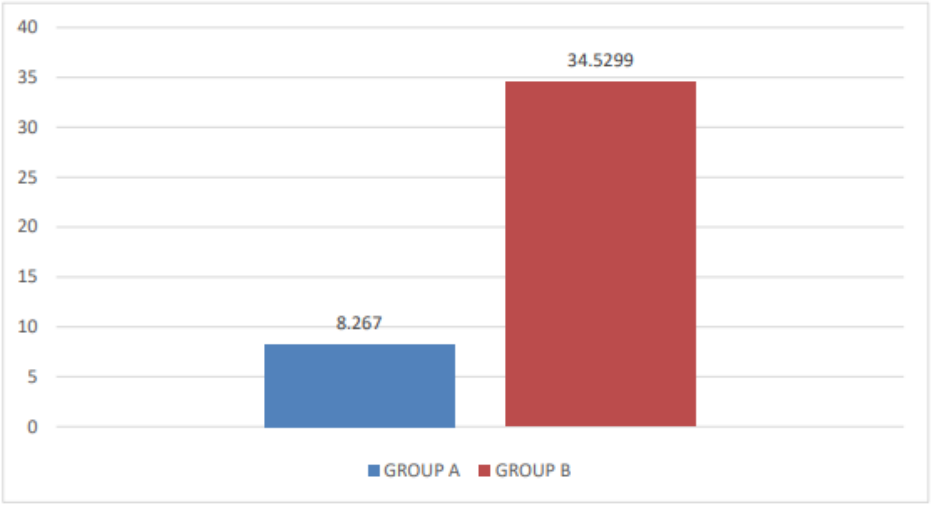

Figure 1. Bar Diagram Showing Mean Salivary Alkaline Phosphatase Levels in Group A and Group B

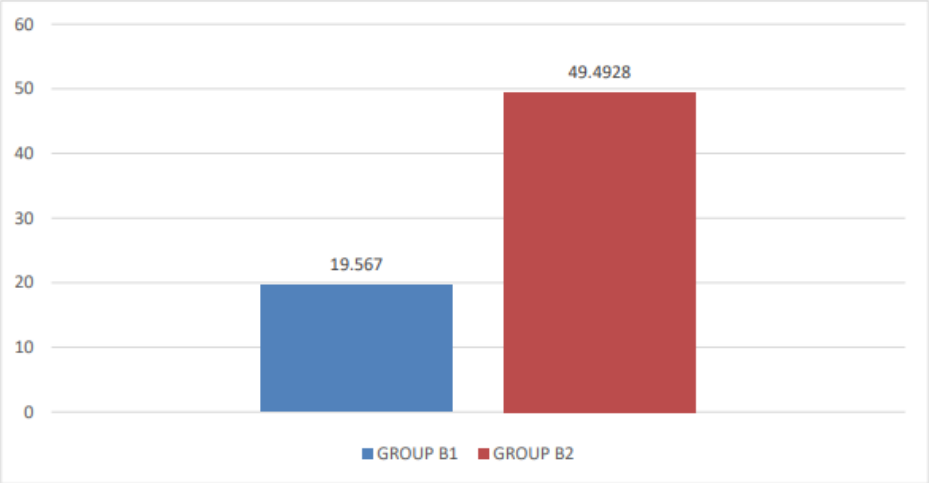

Figure 2. Bar diagram Showing Mean Salivary Alkaline Phosphatase Levels in Group B1 and Group B2

Discussion

Although the etiopathogenesis of OSMF is multifactorial, areca nut-chewing in any formulation is considered the main causative agent. Contributory risk factors suggested include chewing of smokeless tobacco, consumption of chillies, toxic levels of copper in foodstuffs and masticatories, vitamin deficiencies, and malnutrition resulting in low levels of serum proteins, anaemia, and genetic predisposition. The psychoactive and anti-helminthic activities of betel nuts are attributed to the presence of areca alkaloids. According to biochemical research, areca nuts contain four different types of alkaloids: arecoline, arecaidine, guvacine, and guvacoline. These areca nut-specific nitrosamines are metabolized to produce cyanoethyl, which binds to o-methyl guanine in DNA, of which the primary agent is arecoline. These alkaloids counteract weariness and provide euphoria due to their potent parasympathetic effects. Nitrosoguvacoline, nitrosoguvacine, and 3-methyl nitrosominopropionitrile—nitrosamines unique to areca nuts—are formed when arecoline is nitrosated. These nitrosamines contain alkylate deoxyribonucleic acid (DNA). These areca nut-specific nitrosamines are metabolized to produce cyanoethyl, which binds to o-methyl guanine in DNA. Extended contact with this irritant can cause malignant transformations.

Reactive oxygen species (ROS) are molecules with one or more unpaired electrons and at least one oxygen atom that can exist on their own. This category includes free nitrogen radicals and oxygen-free radicals such as superoxide anion radicals, hydroxyl radicals, hydroperoxyl radicals, and singlet oxygen. Under healthy conditions, hepatocytes and macrophages, in particular, produce modest amounts of reactive oxygen species (ROS) during cellular functions, including aerobic respiration and inflammatory processes. Signaling molecules make up the majority of reactive oxygen species. Furthermore, they cause apoptosis and cell differentiation, which accelerate aging naturally. In addition, they control vascular tone, take part in muscle contractions, and assess bactericidal and bacteriostatic activity. Reactive oxygen species (ROS) formed during the auto-oxidation of areca nut (AN) polyphenols in the betel quid chewer's saliva are critical in the beginning and progression of oral cancer [7].

In our study, men are more affected than women when comparing the male-to-female ratio. This is similar to the study conducted by K. Thirumagal et al. on male patients with OSMF (n = 86.45%) and female patients with OSMF (n = 7.36%) [8].And in another study conducted by Nitin Kumar Nigam et al., in the 1000 habitual chewers (678 males and 322

females), the prevalence of OSMF was found to be 6.3% (63 out of 1000) with a male-to-female ratio of 6.88:1 (55:8) (P = 0.0004).

In the study conducted by Nitin Kumar Nigam et al., regarding chewing patterns, among the 63 patients with OSMF, 42 (66.66%) chewed gutkha, 14 (22.22%) chewed pan, and 7 (11.11%) chewed areca nut [9]. Not one patient had inconsistent chewing patterns, which was similar to our study. In another study conducted by Sheshaprasad R. et al., the frequency and duration of a habit do not immediately affect the clinical or histological stages of OSMF disease. More attention should be paid to the patient's genetic makeup and vulnerability to the development and progression of OSMF rather than relying solely on habits and managing the patient accordingly. Seedat et al. (2013) and Debanth et al. (2013) noted that there was no positive correlation between habit duration, frequency, and expression of disease [9].

A class of isoenzymes called alkaline phosphatases (ALPs) is found on the cell membrane's outer layer. In the extracellular area, they catalyse the hydrolysis of organic phosphate esters. This enzyme requires zinc and magnesium as necessary cofactors. ALPs have different physiochemical characteristics and are distributed across a variety of tissues, yet they are all considered to be true isoenzymes because they can all catalyse the same process. Hepatocyte canalicular membranes and the cytoplasm of liver cells both contain ALP. An increasing number of organs, including the kidney, liver, ileal mucosa, placenta, and bone, have decreasing amounts of ALP. The liver and bone account for more than 80% of the ALP in serum, with the colon contributing quite slightly. Despite being found in many different bodily tissues, its concentration is also elevated in saliva. This is because of the response to reactive oxygen species, which are less explored.

In our study, alkaline phosphatase enzymes are taken into consideration, and their levels in saliva are examined. ALP is a member of the hydrolase group of enzymes, which are produced by living cells and used as biocatalysts. ALP works by catalysing the hydrolysis of phosphoric acid monoesters, as well as the transphosphorylation event when phosphate acceptor concentrations are high. [10, 11] Salivary ALP levels typically fall between 5.50 and 12.58 IU/L. Oral epithelial cells, bacteria, and neutrophils are the oral cavity's sources of this enzyme. In the present study, S ALP was found to be about 8.26 IU/L (standard deviation [SD] 6.05585) for Group A, 19.5 IU/L (SD 5.29699) for Group B1, and 49.4 IU/L (SD 42.22351) for Group B2. This was similar to the study conducted by T. R. Menaka et al., which was comprised of 42 individuals and divided into 4 groups. Group I: Individuals without the habit of smoking or chewing tobacco and without any lesion on intraoral examination (n = 12) Group II: Individuals with the habit of chewing tobacco and without any lesion on intraoral examination (n = 10). Group III: Individuals with the habit of smoking and without any lesion on intraoral examination (n = 10). Group IV: Individuals with lesion on intraoral examination with the habit of smoking or chewing tobacco (n = 10) and they concluded the mean values for S ALP were found to be about 18.00 IU/L (standard deviation [SD] 13.376) for Group I (range from 7 to 50 IU/L), 7.50 IU/L (SD 4.353) for Group II (range from 2 to 14 IU/L), 4.60 IU/L (SD 2.011) for Group III (range from 1 to 8 IU/L), and 64.90 IU/L (SD 51.707) for Group IV (range from 10 to 146 IU/L) [12].

In another study conducted by Prakash et al., the mean S-ALP level in smokers without lesions was 4.60 IU/L, which is similar to the results obtained by Kibayashi et al. The mean S-ALP level in tobacco chewers without any lesion was 7.50 IU/L. According to A. Ravi Prakash et al., S-ALP is considered a specific marker in diagnosing oral potentially malignant disorders. Interestingly, alkaline phosphatase levels are high in serum for individuals with

premalignant lesions. In a study conducted by Bhattacharjee et al., elevated serum alkaline phosphatase was seen in leukoplakia and verrucous lesions and was 112.8 ± 53.5 and 101.8 ± 28.4, respectively. [13, 14]

It is possible that the elevated S-ALP levels in OSMF cases are a result of the lesion-related increase in oxidative stress. An increase in reactive oxygen species causes cellular damage, which causes salivary ALP release to increase [15]. The heightened rate of cellular turnover in OSMF, whether as a compensatory mechanism or as a result of genetic mutation, can also cause epithelial cells to produce more ALP. Another possible explanation for the high amounts of S-ALP found could be the enhanced inflammatory response linked to OSMF [16].

Conclusion

The present study concludes that salivary alkaline phosphatase level (S-ALP) is increased in oral submucous fibrosis and can be used as a diagnostic marker in OSMF patients. Further studies can be undertaken to assess and correlate the S-ALP levels in various clinical grades of OSMF and also to assess if S-ALP levels can be a prognostic marker before and after the treatment of OSMF.

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This study received no external funding.

Conflicts of Interest

There are no conflicts of interest.

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