Research Article

An *in vitro* analysis to ascertain whether smokeless tobacco has any detrimental effect on the diagnostic efficacy of salivary lactate dehydrogenase

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ABSTRACT

Background: Salivary diagnostics promise an easily accessible, non-invasive approach for analyzing and monitoring various oral diseases including cancer. **Objectives:** This study was undertaken to establish the baseline salivary lactate dehydrogenase (LDH) levels in healthy controls and to discern the effect of smokeless tobacco (SLT) on the diagnostic efficacy of salivary LDH. **Materials and Methods:** Thirty-seven systemically and periodontally healthy individuals in the age group of 19-27 years without any deleterious habit and oral lesion participated in this study. After explaining the procedure to the participants, unstimulated whole saliva was collected by spit method under sterile conditions. Baseline LDH activity was measured spectrophotometrically. After evaluating baseline activity, 1 ml of saliva was mixed with 1 g of SLT and incubated at 37°C. LDH activity was measured at different time intervals. **Results:** The median value of baseline LDH levels at rest was 338 IU/L. The gender-based variation in LDH activity was found to be non-significant. Exposure of whole saliva to SLT resulted in an initial increase followed by reduction in the LDH activity. **Conclusion:** This study provides additional rationale for the use of salivary LDH as a biomarker to investigate the aggressiveness and biological nature of oral pathological conditions. Interpretation of changes in salivary LDH activity should be done with caution in individuals habituated to tobacco use.

KEY WORDS: Biomarker; Lactate Dehydrogenase; Saliva; Smokeless Tobacco; Tumor

INTRODUCTION

The ultimate goal of health-care professionals has always been detection of the disease at an early stage to reduce the gravity of its impact and associated future complications on an individual's life. An imperative tool for diagnosing a disease is investigating the activity of various biomarkers, a measurable indicator of the physiologic or pathologic state of a living organism in biofluids. The two biofluids, serum and saliva, harbor a melange of organic and inorganic constituents which have been recognized as potential biomarkers. However, in contrast to serum, salivary diagnostics promise an easily accessible, non-invasive approach for analyzing and monitoring various diseases including oral cancer.[1-3] Oral cancer, the sixth most common malignancy, constitutes neoplasm of various cellular origins that can arise in the oral cavity, of which more than 90% are oral squamous cell carcinoma (OSCC). The etiology of OSCC is multifactorial. Among numerous factors characterized to be associated with OSCC, consumption of tobacco and alcohol is acknowledged as the major risk factor.[4] Tobacco is either smoked or chewed, and its mutagenic effects are dose, frequency as well as duration dependent.[5] An array of smokeless tobacco (SLT) products is consumed by individuals of all ages worldwide.[6]

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The first biofluid to encounter the diverse mutagenic chemicals present in tobacco is saliva. Saliva is a complex biological fluid composed of serous and mucous secretions from major and minor salivary glands. Oral mucosal cells

are constantly drenched in saliva which consists of a myriad of organic, inorganic, non-protein, protein, hormone, lipid molecules, and various biomarkers with diagnostic efficiency and therapeutic efficacy.[7] The effect of tobacco on salivary biomarkers is relatively underexplored. One of the markers of cellular damage present in saliva is lactate dehydrogenase (LDH), an oxidoreductase enzyme which catalyzes the reversible interconversion of pyruvate and lactate. An increase in the levels of this protein biomarker has been reported in the saliva of patients with oral potentially malignant disorders (OPMD) and cancer.[8-10] Therefore, it is essential to know the effects of various exogenous chemical mutagens such as tobacco on its activity. Existing literature about the effect of cigarette smoke on salivary LDH reports a marked reduction in its activity.[11-15] Since tobacco is one of the established causes of OSCC and the effect of tobacco smoke on LDH has been investigated previously, we formulated an *in vitro* analysis to evaluate the effect of SLT on LDH. In addition, limited information is available in literature on the various practical and laboratorial aspects of salivary LDH that can affect its essence as a biomarker.

To the best of our knowledge, we have not come across any study that has evaluated the effect of SLT on salivary LDH. The results of this study will provide an insight into the effect of SLT on LDH activity and these changes should be considered while using LDH as a biomarker in patients habituated to the consumption of tobacco.

MATERIALS AND METHODS

This research was approved by the Institutional Ethics Committee. Before sampling, the procedure was described to the participants.

Patients

Thirty-seven healthy individuals (males 16; females 21) in the age group of 19-27 years without any deleterious habit and oral lesion participated in this study.

Saliva Sampling

Saliva was collected by spit method as previously described.[16] Sampling was done in the morning between 10 am and noon to minimize the variability in salivary composition and flow rate between different patients. No oral stimulus was permitted for 60 min before collection. Forcible spitting was avoided. After the collection of unstimulated saliva, the sample was centrifuged at 1500 rpm for 5 min, and LDH activity was measured in the supernatant.

Measuring LDH Activity in Samples

Analysis of LDH activity was done using standard kinetic spectrophotometric method in accordance with the recommendations of the International Federation of Clinical Chemistry with the help of 3000 EVOLUTION semi-automatic biochemistry analyzer for clinical chemistry tests (Figure 1d), using the commercial LDH (P-L) Kit (The Tulip Group, India).

SLT

The SLT used in this study was a popular, easily accessible commercial Indian product "Hathi Chhap tobacco" (Figure 1a).

Exposure of Saliva to SLT

To evaluate the effect of SLT on LDH activity in a time-dependent manner, we analyzed the changes in LDH activity at 1 h and 3 hrs after mixing with SLT.

Briefly, 1 g of SLT was mixed thoroughly with 1 ml of saliva supernatant using LYZER™ (Figure 1b and c) and was placed in the incubator set at a temperature of 37°C. 1 ml of saliva supernatant without SLT was incubated simultaneously at the same temperature for the same time to rule out, if any, the effect of temperature on LDH activity. After 1 h and 3 h of incubation, LDH activity was measured in both the saliva samples, with and without SLT, using the same protocol as described above.

Statistical Analysis

The data were analyzed using Microsoft Excel 2010 and Statistical package for Social Sciences (SPSS version 20;

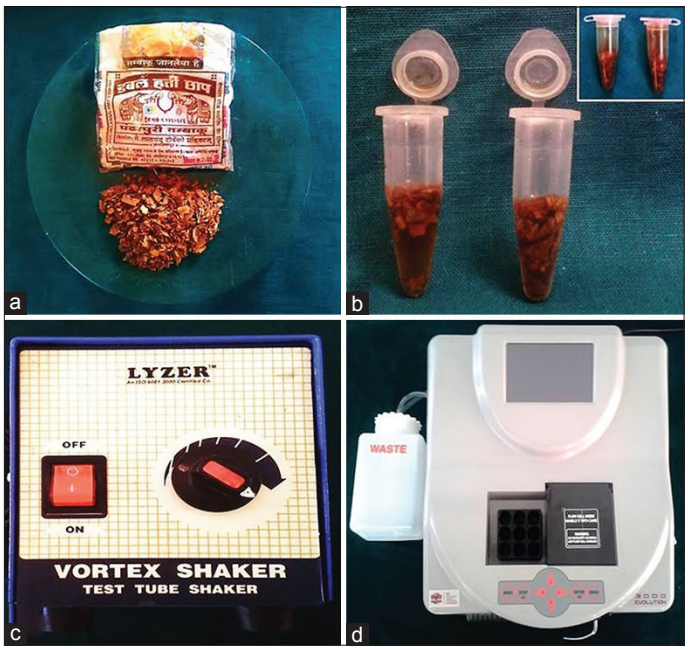

Figure 1: (a) Locally available and widely used smokeless form of tobacco; (b) smokeless tobacco (1 g) mixed with saliva supernatant (1 ml) after centrifugation; (c) Lyzer™ (Vortex mixer); (d) 3000 EVOLUTION semi-automatic laboratory analyzer

IBM Software group, Chicago, U.S.A). Descriptive statistics, namely, mean, standard deviation, median, interquartile range, minimum, maximum as well as graphical displays were obtained for different levels of measurement. Normality tests were carried out on LDH activity at 0 h (baseline levels), 1 h (with and without SLT), and 3 h (with and without SLT), before opting a particular parametric or non-parametric alternative. Statistical tests used were Friedman test for repeated measures and Mann–Whitney U-test with significance level of 0.05.

RESULTS

Statistical analysis revealed that the data for LDH activity at each time point were not normally distributed (Table 1). Hence, non-parametric tests were used for analysis. No significant gender-based difference was found in the LDH activity (P = 0.818). The median LDH activity reported in males and females was 315 IU/L and 342 IU/L, respectively. The baseline median salivary LDH activity in whole saliva of 37 healthy participants under resting condition was found to be 338 IU/L which in the presence of SLT showed a median percent increase of 4.7% and decrease of 15.08% after 1 h and 3 h, respectively (Table 2). However, without exposing saliva to SLT, the median percent decrease observed at 1 h and 3 h was 2.66% and 7.69%, respectively.

For comparing LDH activity at different time intervals, we segregated all the comparisons into two groups. Group 1 included comparisons at different time intervals without exposing saliva to SLT and group 2 included comparisons after exposure to SLT.

The following comparisons were made in the two groups for different time intervals:

1. Baseline activity and activity at 1 h
2. Baseline activity and activity at 3 h
3. Activity at 1 h and at 3 h.

In Group 1 and Group 2, the results of Friedman test show that the median LDH activity showed a statistically significant difference ($P < 0.05$) for different time intervals. To examine where the differences actually lie, Wilcoxon signed-rank test (Table 3) on the different combinations of each group was done separately. Bonferroni adjustment on the results obtained from the Wilcoxon tests in each group was used to avoid Type 1 error. The new significance level for each group after Bonferroni adjustment was 0.017. However, the difference in the median values of LDH activity with and

Table 1: Evaluating distribution of the data obtained for salivary LDH activity with and without SLT at different time intervals using normality tests (Kolmogorov–Smirnov and Shapiro–Wilk)

Tests of normality						
Salivary LDH activity (IU/L)	Kolmogorov–Smirnov			Shapiro–Wilk		
	Statistic	df	Significant	Statistic	df	Significant
Baseline	0.167	37	0.011	0.902	37	0.003
After 1 h	0.150	37	0.035	0.892	37	0.002
After 1 h+SLT	0.153	37	0.028	0.897	37	0.002
After 3 h	0.156	37	0.023	0.896	37	0.002
After 3 h+SLT	0.152	37	0.031	0.908	37	0.005

LDH: Lactate dehydrogenase, SLT: Smokeless tobacco

Table 2: Descriptive statistics for salivary LDH activity at baseline, 1 h (with and without smokeless tobacco) and 3 h (with and without smokeless tobacco)

Descriptive statistics	Baseline activity (IU/L)	1 h after incubation		3 h after incubation	
		Activity with SLT	Activity without SLT	Activity with SLT	Activity without SLT
Mean±SD	411.95±217.712	427.89±216.074	394.30±217.005	332.97±178.255	378.76±206.566
Median (IQR*)	338 (256-548)	354 (248-553)	329 (235-512)	287 (182-448)	312 (226-513)

*Interquartile range, LDH: Lactate dehydrogenase, SLT: Smokeless tobacco, SD: Standard deviation

Table 3: The median salivary LDH activity at different time intervals and result of Wilcoxon signed-rank test on different combinations of each group

Group	Activity (IU/L) median (IQR)			Intragroup comparison *P* value (Z)		
	Baseline (B)	After 1 h	After 3 h	B-1 h	B-3 h	1-3 h
Group 1 (without SLT)	338 (256-548)	329 (235-512)	312 (226-513)	0.013* (−2.475)	0.0001* −3.795)	0.009* (−2.608)
Group 2 (with SLT)		354 (248-553)	287 (182-448)	0.029 (−2.188)	<0.0005* (−4.971)	<0.0005* (−5.303)

*Significant difference. LDH: Lactate dehydrogenase, SLT: Smokeless tobacco, IQR: Interquartile range

without SLT for 1 h (Figure 2a) as well as for 3 h (Figure 2b) was not statistically significant ($P > 0.05$).

DISCUSSION

LDH is a ubiquitous medically significant enzyme which exists in five isoforms composed of H and M protein subunits encoded by LDHA and LDHB gene, respectively.[17] Interestingly, the isozyme profile of serum and salivary LDH differs considerably. While LDH-1 and LDH-2 are the main isozymes present in serum, LDH-4 and LDH-5 predominate in saliva. Over a decade ago, Nagler et al.[11] demonstrated the similarity between the isozyme profile of salivary LDH and oral epithelium, indicating that the main source of salivary LDH is oral epithelial cells. Cell lysis and an increase in cell membrane permeability are the two possible ways that lead to LDH release from cells.[18,19] LDH, being a marker of cellular destruction, estimating its activity in OSCC, would reflect the extent of tissue damage as well as tumor aggressiveness by serving as an indirect measurement for the metabolic reprograming of cancer cells, as reviewed recently.[20] Thus, estimating LDH activity in OSCC patients would offer diagnostic and prognostic advantages. However, to successfully accomplish the accurate estimation of LDH, it is necessary to have the knowledge of factors which negate its diagnostic efficaciousness. One such factor which is invariably present in OSCC cases is tobacco.

Tobacco consists of innumerable chemical adjuncts and preservatives with carcinogenic potential responsible for multitude of oral and systemic maladies.[6] Among various carcinogens known to be present in SLT, the main culprits are the tobacco-specific nitrosamines, namely N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). A recent case–control study has proven the association of OPMD with the use of SLT and that its use could be more detrimental than the smoked form.[21] Toxicity of SLT is attributed not only to its nicotine content but also to various other biologically active substances such as aldehydes and reactive oxygen species (ROS).[6,22] The nicotine content of the tobacco used in the present study has been reported to be 39.5 mg/g while NNN and NNK concentrations as 2.75 and 0.85 μg/g, respectively.[23] In the present analysis, an increase of 3.72% in LDH activity observed 1 h after incubation with SLT could be due to the cytotoxic effect of SLT on cells present in saliva leading to ROS-mediated cellular damage and LDH release.

It has been found that exposure of biofluids to aldehydes at a concentration present in cigarette smoke leads to an increase in protein carbonyl concentration as a result of the reaction between -SH groups of proteins and aldehydes.[11,12,24] Nagler et al.[11,12] have reported that exposure of saliva to cigarette smoke lead to a significant reduction in the activity of LDH, amylase, and acid phosphatase.[13-15] They ascribed this loss to aldehyde reacting with -SH group necessary for enzyme activity.[25] Since SLT also contains aldehyde, it can be concluded that saliva in the presence of SLT becomes a potent protein-modifying agent and causes destruction of its endogenous components. We observed a reduction of 19.17% and 22.18% in LDH activity with SLT from 0-3 h to 1-3 h, respectively (Table 2). Therefore, it can be speculated that the effect of SLT on saliva occurred in two phases. Initially, SLT-mediated cytodamage leading to LDH release was the prominent phase. However, with time, the concentration of aldehydes released from SLT resulted in an increase in carbonyl concentration, thereby rendering its protein constituents inactive.

Broadening our perspective on the factors that can alter salivary LDH activity, we incubated saliva samples at 37°C (conducive temperature for bacterial growth) and concluded that not only tobacco but storage conditions can also affect the activity of salivary constituents.[26] A marked reduction observed in LDH activity at 37°C could be secondary to time-dependent increase in bacterial protease activity leading to degradation of salivary constituents. This time-dependent

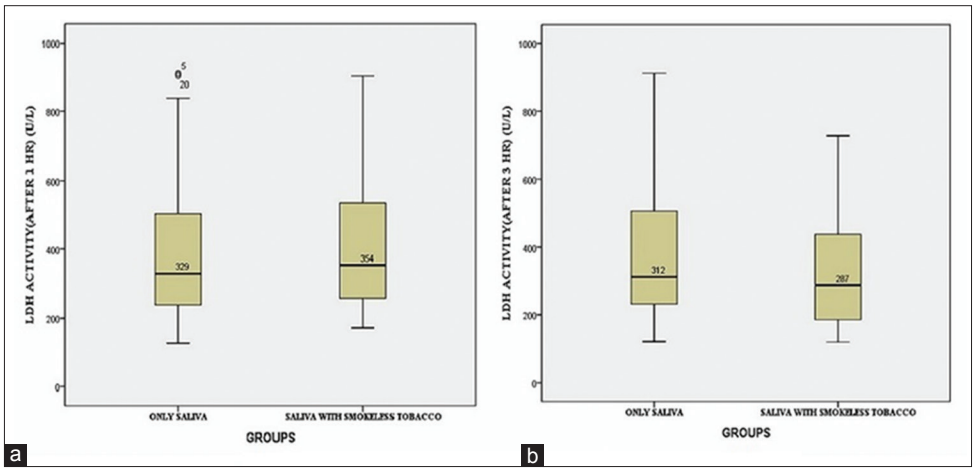

Figure 2: (a) Evaluation of salivary lactate dehydrogenase activity with and without smokeless tobacco for 1 h and (b) 3 h, respectively, using Mann–Whitney U-test

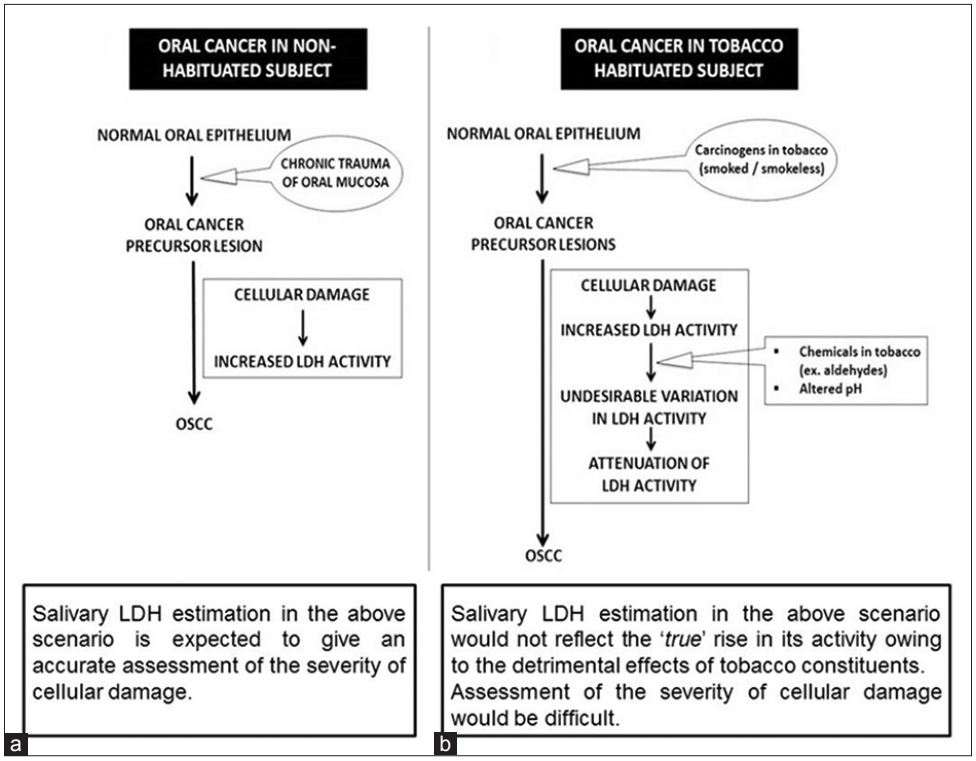

Figure 3: (a and b) The tobacco usage tends to reduce the diagnostic efficacy of salivary lactate dehydrogenase

deterioration in LDH activity highlights the importance of sample storage at an optimum temperature and the use of protease inhibitors to minimize the loss in biological activity of biomarkers.[27]

Traditionally available SLT products are buffered to an alkaline pH as a high pH raises the readily absorbable unprotonated form of nicotine.[28] The variation in pH from the optimal required value can have detrimental effects on LDH activity.[29] Therefore, an increase in pH of saliva due to tobacco usage will lead to time-dependent reduction in LDH activity, as observed after 3 hrs in this study. Although we found a significant decrease in LDH activity, further study on a larger sample size is needed for establishing a definitive relationship. The results of this study are anticipated to lay down the groundwork for refining the basis of assessing the diagnostic proficiency of salivary biomarkers in tobacco-habituated cases.

To summarize, tobacco constituents are not only responsible for inducing genetic mutations in the cells driving them toward malignant phenotype but also affect salivary components. Considering two situations, one in which a non-habituated individual develops OSCC secondary to chronic mucosal trauma[30] and another in which tobacco consumption leads to OSCC (Figure 3), evaluating and interpreting LDH activity in the latter case might conceal important diagnostic information (Figure 3b) as it will not divulge the actual rise in LDH above normal. However, no such changes are expected to occur in the former case (Figure 3a). The findings of this study indicate that the inhibitory effects of tobacco products on LDH should be given due consideration while its use as a biomarker in the investigative protocol of cancer patients.

The main strength of the present analysis was its consideration of the effect of SLT on a clinically significant salivary constituent. The results of this study lay emphasis on the fact that any aberrant change in the activity of salivary biomarkers, such as LDH, secondary to tobacco can limit their diagnostic and/or prognostic efficacy. However, this study has some potential limitations too. First, the sample size was small. Second, the effect of only one SLT product (marketed in India) was assessed and finally, the effect of SLT on different isoforms of LDH was not evaluated.

CONCLUSION

To conclude, the emerging diagnostic methods based on non-invasive techniques for precise prediction of the outcome of disease require the cognizance of the practical facet of laboratory-based conditions and handling of biofluids. Irrespective of the findings of the current study, LDH screening has substantial advantages. Therefore, it is essential to gain insights into the toxicology of tobacco affecting the clinical utility of salivary biomarkers. Since tobacco is the main culprit behind OSCC, its inhibitory effect on LDH will mask an important increase in its activity, thereby reducing its efficacy as a biomarker. Research on a large sample size is warranted for a better understanding of the toxicology of various chemical mutagens and their effects on salivary biomarkers.

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