Original Article

# To evaluate the role of lactate dehydrogenase as biomarker in the progression of potentially malignant disorders in smokers and gutkha chewers

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## Abstract

**Aim:** To evaluate the role of lactate dehydrogenase (LDH) as a biomarker in the progression of potentially malignant disorders in smokers and gutkha chewers.

**Material and Methods:** The total of 70 individuals having the habit of chewing gutkha and smoking were selected for the study and divided into four groups. Group 1: 20 individuals having the habit of smoking, Group 2: 20 individuals having the habit of chewing gutkha, Group 3: 20 individuals having the habit of smoking and gutkha chewing, and Group 4: 10 healthy individuals.

**Results:** In the present study showed that unstimulated whole saliva from group 1, group 2, and group 3 individuals, there was a significant decrease in isoenzymes LDH-1 and LDH-2 and there was no effect on LDH-3 isoenzyme. In the present study, there is a significant increase in salivary isoenzyme LDH-4. The result indicated that there is an effect of carcinogen (tobacco)–enhanced activity on salivary LDH. The present study showed 70 volunteers reported a statistical significant correlation among the groups. The individuals with habit of smoking and gutkha chewing showed $P$ value $<.001$ with a significant increase in the isoenzyme LDH-4. A significant increase in the buccal micronuclei of exfoliated cells in the oral mucosal cells of smokers and gutkha chewers. This shows a positive relationship between LDH isoenzymes and the individuals with the smoking and chewing of tobacco.

**Conclusion:** There was a significant correlation between the levels of salivary LDH isoenzymes and cytomorphometric analysis of oral epithelium in smokers and gutkha chewers.

**Keywords:** Gutkha chewers, LDH, malignant disorders, smokers

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## INTRODUCTION

Oral cancer (OC) is one of the major global threats to public health. The development of OC is a tobacco-related multistep and multifocal process involving field cancerization and carcinogenesis.[1] Tobacco is regarded as one of the most potential carcinogen and available in various forms as smoke and smokeless.[2] The carcinogenic potential of tobacco is mainly attributed to its main

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component called nicotine.[3] Smoke constitutes one of the largest environmental sources of human exposure to saturated (e.g., acetaldehyde) and a, b-unsaturated aldehydes (e.g., acrolein, crotonaldehyde) and reactive nitrogen species (e.g., nitric oxide and peroxynitrite).[4]

In recent years it has become clear that oxidative damage caused by reactive nitrogen, oxygen, and aldehyde species is an important element in inflammation development causing tissue injury.[5] Additionally, carbonylation caused by oxidative stress leads to protein dysfunction and an increase in disease onset, including cancerous transformation processes.[6] In the oral cavity, there will be many changes and few biochemical changes in the saliva.

The progressive changes in the oral epithelium to precancerous conditions are by many factors, one of it is increased levels of salivary lactate dehydrogenase (LDH).[7] The enzyme LDH is an ubiquitous enzyme that was discovered in early periods of enzymology.[8] This enzyme catalyzes the reaction of lactate production via pyruvate reduction during anaerobic glycolysis.[9] The salivary LDH isoenzymes profile is similar to that of LDH isoenzymes present in the oral epithelium, which indicates that the major source of LDH is the oral epithelium probably, which is derived from the surface exfoliated cells. Consequently, LDH concentration in saliva as an expression of cellular necrosis can be considered to be a specific indicator for lesions affecting the integrity of the oral mucosa.[9] LDH displays different isoenzyme profiles in different body fluids including serum, saliva, cerebrospinal fluid, etc. It is observed LDH-1 and LDH-2, which predominate in plasma, could be detected to only a limited extent in saliva secreted under resting conditions, whereas LDH-1 in saliva could barely be detected under stimulated conditions.[10] Isoenzymes LDH-4 and LDH-5 were found to predominate in saliva, with higher specific activity than in plasma. LDH estimation can evolve as a noninvasive technique for routine screening in assessing oral diseases.[3]

Few studies have been done in cancerous and precancerous conditions which showed a profound increase in this enzyme profile (salivary LDH).[3] The studies have comprehensively analyzed saliva in OC and they found total salivary LDH (88%, $P = .002$) to be elevated in comparison to normal healthy individuals.[2] Although many attempts have been made to recognize the role of salivary LDH in using, it has an early diagnostic tool in many premalignant conditions and lesions. Our study is focused on much earlier diagnosis based on habits of the individual. The study is designed to estimate the levels of salivary LDH in individuals with smoking and chewing habits which are known to induce the changes in the oral mucosal epithelial cells well before the evidence of premalignant conditions.

## MATERIALS AND METHODS

This study carried out in the department of Oral Maxillofacial Pathology, MNR Dental College and Hospital and Biognosys, Hyderabad. Approval of the institutional ethical committee board was taken before pursuing the research project. The total of 70 individuals having the habit of chewing gutkha and smoking were selected for the study and divided into four groups. Group 1: 20 individuals having the habit of smoking, Group 2: 20 individuals having the habit of chewing gutkha, Group 3: 20 individuals having the habit of smoking and gutkha chewing, and Group 4: 10 healthy individuals. Patients treated for cancers (surgery, chemotherapy, radiotherapy). Systemic diseases known to increase serum LDH levels such as myocardial infarction, liver diseases, renal disease, and muscle dystrophy and other oral conditions known to increase salivary LDH levels like periodontitis and patients having received dental treatment 48 hours prior to the study were excluded from the study.

### Inclusion criteria

1. Individuals giving the positive history of tobacco intake in the form of smoke since 10 years.
2. Individuals giving a history of tobacco intake in the form of gutkha.

### Exclusion criteria

1. Patients treated for cancers (surgery, chemotherapy, radiotherapy). Systemic diseases known to increase serum LDH levels such as myocardial infarction, liver diseases, renal disease, and muscle dystrophy.
2. Other oral conditions known to increase salivary LDH levels like periodontitis and patients having received dental treatment 48 hours prior to the study.
3. Patients with premalignant conditions and lesions (oral submucous fibrosis [OSMF], leukoplakia lichen planus, and erythroplakia).

### Sample collection and storage

An informed written consent was obtained from the patients prior to saliva collection. Three milliliters of unstimulated whole saliva was aseptically collected in a wide mouthed container by spit method [Figure 1].

### Sample preparation

Following the collection, saliva was centrifuged at 1,000 rpm for 10 min and the resulting supernatant [Figure 2b] was used for biochemical estimation of LDH isoenzymes by using agarose gel electrophoresis method (SEBIA-HYDRAGEL ISO-LDH K-20 kit) [Figures 1 and 2a].

Samples to avoid: Frozen samples were avoided.

Oral buccal mucosal cells were smeared on to the slide [Figures 7 and 8] with a sterile wooden spatula and cytomorphometric analysis of oral epithelial cells was done to analyze the carcinogenic effect of tobacco on the oral epithelial cells [Figure 6].

## Procedure

### I. Migration step [Table 1]

1. HYDRAGEL K20 applicator carrier was placed on a flat surface and part of the applicator carrier was raised with the numbered notches.
2. Pooling of 120 μL distilled or deionized water was poured on the lower third of the frame printed on the HYDRAGEL K20 applicator carrier.
3. Unpacked the HYDRAGEL agarose gel plate.
4. Rolled quickly and uniformly one thin filter paper onto the gel surface to absorb the excess of liquid and removed the paper immediately.
5. The gel plate (the gel side-up) was placed with its edge against the stop at the bottom of the printed frame.
6. The gel was bent and lowered down onto the water pool. Care was taken to avoid the entrapment of air bubbles, water was spread underneath the entire gel plate, and the gel was lined up with the printed frame.
7. The applicator carrier was lowered on to the numbered notches down to the intermediate position with the switch in high position.
8. One applicator was placed on a flat surface with the well numbers in the right side-up position.
9. 10 μL of supernatant saliva was loaded into the applicator wells within 2 minutes [Figure 3a and b].
10. The applicator teeth's protection frame was snapped off.
11. The sample applicator was into position number 6 on the applicator carrier [Figure 4].
12. The applicator with the switch was lowered on to the gel surface [Figure 4b].
13. After 5 minutes, the switch was turned to rise up the applicator and the applicator was removed and discarded.
14. Put the gel into an appropriate electrophoresis chamber, as per the polarity indicated on the gel, the lower side of the gel on the cathodic side. When using SEBIA K20 chamber, place the HYDRAGEL on the bridge with the gel side facing down; the gel should dip about 1 cm into the buffer on each side.
15. Plug the chamber to the power supply.
16. After migration, the chamber was unplugged and gel plate was removed.

**Table 1: Migration conditions for agarose gel electrophoresis**

| Migration Conditions | Sebia K20 |
|---|---|
| Volume of buffer per compartment | 150 mL |
| Total buffer volume | 300 mL |
| Migration time | 25 minutes |
| Constant voltage | 80 V |
| Initial current (per gel) | 10±2 mA |

### II. PREPARATION FOR INCUBATION WITH ISO-LDH SUBSTRATE

The incubator dryer was preheated for 5 minutes at

**Figure 1:** Collection of saliva sample in labelled sterile container along with reagents of Sebia Hydragel Iso Ldh20k

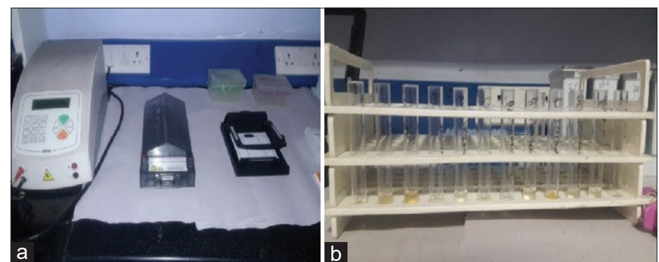

**Figure 2:** Steps for the detection of salivary LDH using salivary LDH KIT 20k kit, (a) Equipment of agrose gel electrophoresis, (b) Supernatant of saliva in the test tubes

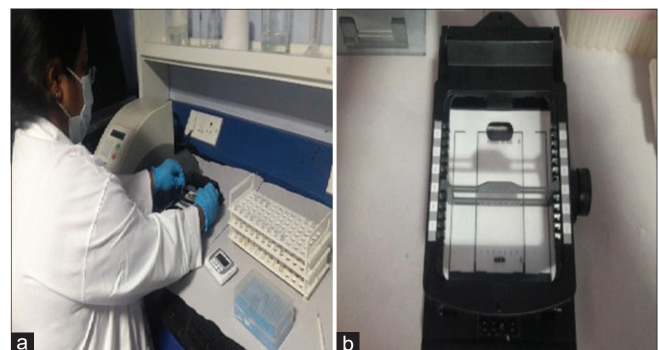

**Figure 3:** Collection of saliva into the micropipette & amp; loading of supernatant saliva into the applicator wells, (a) Collection of saliva into the micropipette, (b) Loading of supernatant saliva into the applicator wells

the temperature indicated below, before placing the template carrier K20 and the gel to incubate.

1. The template carrier was placed on a flat surface and the cover was removed.
2. Deionized water of 120 μL was pooled on the lower third of the frame printed on the template carrier.
3. The gel plate (the gel side-up) was placed with its edge against the stop at the bottom of the printed frame.
4. The gel was bent and lowered down onto the water pool carefully without entrapment of air bubbles, water was spread underneath the entire gel plate, and the gel is lined up with the printed frame.
5. The reagent application template ENZ 4 mL was set on the template carrier as follows:
   - The application template on the anchoring clips was positioned.
   - Holding the handle of the template and lowering the template onto the gel.
6. 2.25 mL of ISO-LDH substrate solution was applied, as follows:
   - Holding the pipette vertically.
   - Lightly pressed the tip of the pipette into the hole of the template.
   - Carefully and progressively the reagent was injected without introducing air bubbles under the template.
7. The template carrier cover was closed.
8. The template carrier in the incubator dryer was placed at 51°C for 25 minutes.
9. After incubation, the template carrier was removed from the incubator dryer and placed it on a flat surface.
10. The cover was opened and the template carrier was left at room temperature for 5 minutes before substrate elimination.

**III. SUBSTRATE ELIMINATION AND APPLICATION OF BLOCKING SOLUTION**

1. The remaining substrate solution was removed: Holding the pipette vertically and lightly pressing the tip of the pipette into the hole of the template. Carefully and progressively withdrawn the reagent.
2. Pipet 2 mL of blocking solution without introducing air bubbles.
3. Incubated at room temperature for 10 minutes, without the cover of the template carrier.

**IV. ELIMINATION OF BLOCKING SOLUTION AND FILTER PAPER APPLICATION**

1. Removed the blocking solution.
2. Removed the template: Grasping the handle of the template. Raising the template and removed it.
3. The gel was left on the plate of the carrier.
4. One thick filter paper was applied on the gel for 3 minutes at room temperature: Sloping the filter paper at about 45°. Aligning the lower side of the filter paper with the edge of the gel. Lowering the filter paper onto the gel. Pressing on the whole surface of the filter paper to ensure perfect adherence to the gel.
5. After 3 minutes, the filter paper was removed and dried the gel at 51°C.
6. The template was rinsed with distilled water or alcohol and dried it thoroughly with soft absorbent paper. Prior to reuse, ensuring that the template is completely dried.

**V. GEL SCANNING-Figure 5**

1. Cleaned the back side (the plastic support side) of the dried film with a damp soft paper.
2. If needed, take off any lint from the gel side with soft paper.
3. A densitometer/scanner was used to scan by selecting the appropriate scanning program.

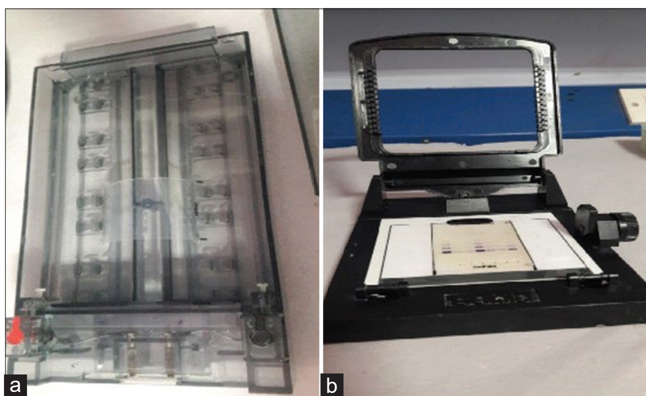

**Figure 4:** Sample applicator with Sebia K20 chamber with hydragel.j, (a) Sample appilicator was placed into position no.6 on the applicator carrier, (b) Sebia k20 chamber, with hydragel

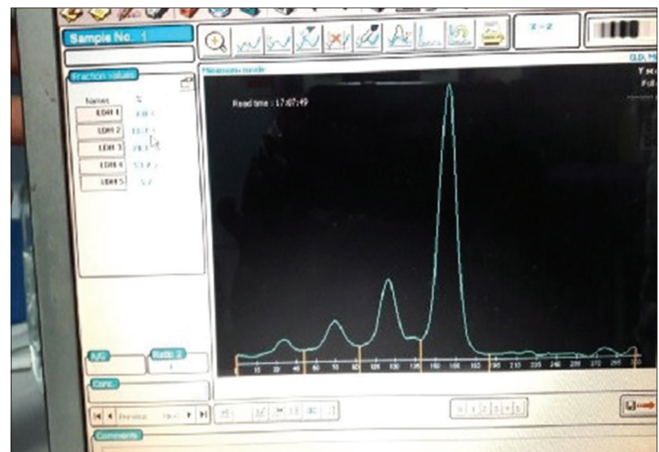

**Figure 5:** Graphs of scanned gel.j

**VI. WASH OF THE GEL:** After scanning, the gel was rinsed for a better preservation of the electrophoregrams (to prevent development of background stain).

1. The gel was immersed in the destaining solution for 10 minutes.
2. The gel was dried with hot 51°C air.

## RESULTS AND OBSERVATION

Detection of salivary LDH isoenzymes of smokers and gutkha chewers was estimated by SEBIA HYDRAGEL 20K KIT. The identification of oral epithelial changes was observed by cytological buccal smears [Figure 8] were stained and studied under light microscope. The study comprised of 70 subjects, of whom 20 individuals were smokers (group 1), 20 individuals were gutkha chewers (group 2), 20 individuals were smokers with the habit of gutkha chewing (group 3), and 10 healthy controls (group 4).

### Statistical analysis

All the analysis was done using MedCalc Version 14. A *P* value of <.05 was considered statistically significant. Comparison of mean values among the four study groups was done using Kruskal-Wallis analysis of variance with post-hoc Conover test. The individuals with habit of smoking and gutkha chewing showed *P* value <.001 with a significant increase in the isoenzyme LDH4. All the analysis was done using MedCalc Version 14. A *P* value of <.05 was considered statistically significant. Comparison of mean values among the four study groups was done using Kruskal-Wallis analysis of variance with post-hoc Conover test [Table 2]. The cytomorphometric changes in nucleolus among 4 groups, the *P*-value is significant in individuals with gutkha chewing and individuals with gutkha chewing and smoking [Table 3]. Chart 1 [Figure 9] Showing statistical increased LDH 4 isoenzyme in individuals with gutkha chewing and smoking. There is no significant increase in LDH 2 isoenzyme in 4 groups Chart 2 [Figure 10]. There is no significant increase in LDH 3 isoenzyme in 4 groups Chart 3 [Figure 11].There is significant increase in LDH 4 isoenzyme in 4 groups Chart 4 [Figure 12]. There is no significant increase in LDH 5 isoenzyme in 4 groups. Chart 5 [Figure 13] On comparison of nucleus among 4 groups, the karyorrhexis is not significant Chart 6 [Figure 14]. On comparison of nucleus among 4 groups, the karyolisis is not significant Chart 7 [Figure 15]. The Nucleus is showing significant pyknosis in the individuals with smoking and gutkha chewing Chart 8 [Figure 16]. The buccal micronuclei are significantly increased in the individuals with smoking and gutkha chewing Chart 9 [Figure 17].

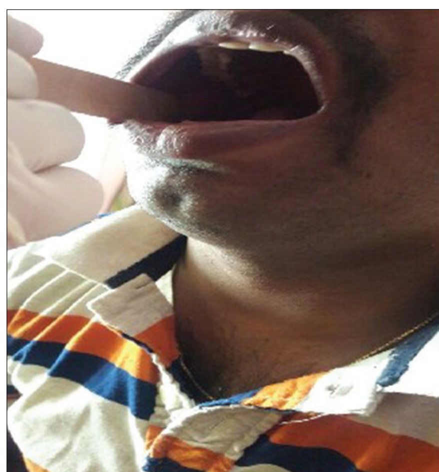

**Figure 6:** Collection of smear

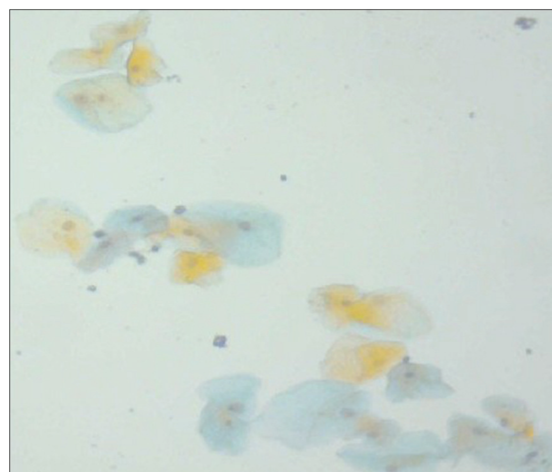

**Figure 7:** Oral epithelial cells stained PAP.jpg

**Table 2: On comparison of salivary LDH isoenzymes between smokers, gutkha chewers and healthy controls the P- value of LDH 4 statistically significant in individuals of smoking and gutkha chewing patients**

| | 2. Smoking | | 3. Gutka | | 4. Smoking + Gutka | | 5. Controls | | *P* | *Post-hoc* test |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | | |
| LDH-1 | 10.50 | 6.48 | 11.92 | 11.34 | 13.96 | 12.50 | 9.78 | 5.41 | 0.956; NS | - |
| LDH-2 | 12.47 | 8.96 | 13.15 | 6.24 | 13.44 | 6.30 | 12.42 | 5.01 | 0.9; NS | - |
| LDH-3 | 18.08 | 7.88 | 15.85 | 6.02 | 16.26 | 5.77 | 13.78 | 4.32 | 0.44; NS | - |
| LDH-4 | 30.73 | 16.96 | 37.03 | 20.28 | 42.14 | 16.34 | 11.49 | 2.05 | <0.001; Sig | 4>1,2,3 |
| LDH-5 | 28.23 | 25.89 | 22.05 | 20.18 | 14.21 | 8.81 | 14.23 | 5.36 | 0.84; NS | - |

Kruskal-Wallis ANOVA with *post-hoc* Conover test

**Table 3: Comparison of cytomorphometric changes in nucleus among 4 groups**

| | 1. Smoking | | 2. Gutka | | 3. Smoking + Gutka | | 4. Controls | | *P* | *Post-hoc* test |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | | |
| Karyorrhexis | 0.90 | 1.02 | 1.32 | 1.42 | 1.55 | 1.50 | 0.30 | 0.48 | 0.077; NS | - |
| Karyolysis | 0.75 | 0.91 | 1.00 | 1.25 | 1.05 | 1.10 | 0.20 | 0.42 | 0.189; NS | - |
| Pyknosis | 1.25 | 1.12 | 1.84 | 1.07 | 1.35 | 1.27 | 0.60 | 0.70 | 0.037; Sig | 2>4 |
| Buccalmicronuclei | 5.15 | 1.53 | 6.79 | 1.84 | 8.15 | 1.90 | 0.20 | 0.42 | <0.001; Sig | 3>2>1>4 |

Kruskal-Wallis ANOVA with *post-hoc* Conover test

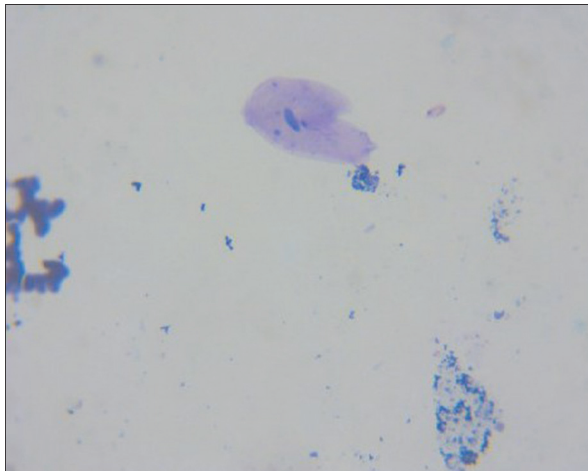

**Figure 8:** Epithelial cells showing buccal micronucleus

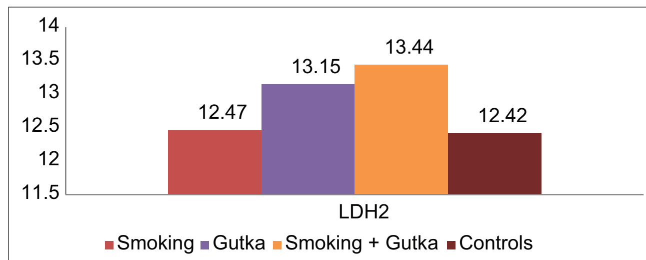


**Figure 10 Chart 2:** Salivary LDH 2 isoenzyme in 4 groups

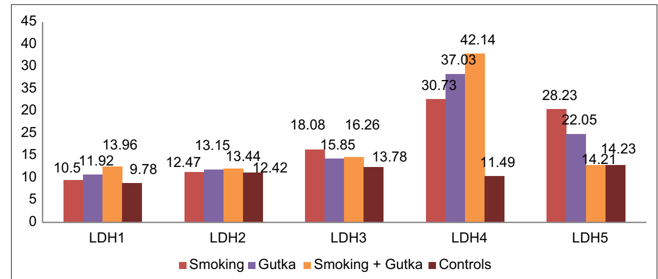


**Figure 9 Chart 1:** Comparison of salivary LDH isoenzymes among 4 groups

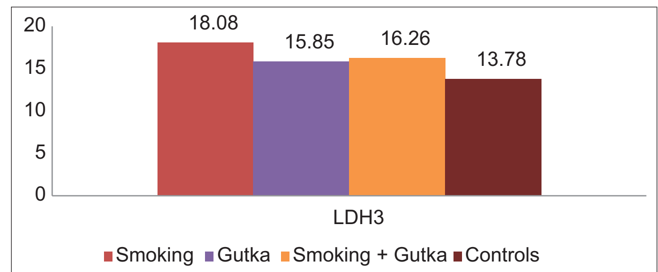


**Figure 11 Chart 3:** Salivary LDH 3 isoenzyme in 4 groups

## DISCUSSION

Saliva from patients has been used in a novel way to provide molecular biomarkers for OC detection. Saliva is a mirror of the body, reflecting virtually the entire spectrum of normal and disease states and its use as a diagnostic fluid meets the demands for an inexpensive, noninvasive, and accessible diagnostic tool.

Discovery of analyses in saliva of normal and diseased subjects suggests a very promising function of saliva as a local and systematic diagnostic tool. The ability to analyze saliva to monitor health and disease is a highly desirable goal for oral health promotion and research. So far, saliva has been used to detect caries risk, periodontitis, OC, breast cancer, salivary gland diseases, and systemic disorders such as human immunodeficiency virus and hepatitis C virus. However, due to lack of knowledge of disease markers and an overall low concentration of these markers in saliva when compared to serum, the diagnostic value of saliva has not been fully realized. However, nowadays, highly sensitive and high-throughput assays such as DNA microarray, mass spectrometry, and nanoscale sensors can measure protein and RNA markers at low concentrations in saliva, thus expanding the utility of saliva as a diagnostic tool.[10]

The development of cancer is associated with a high glycolytic activity with a shift from aerobic to anaerobic glycolysis. With the increase in the glycolytic activity, the concomitant increase in LDH enzyme may be reflected in certain tissues. Studies in the past have been carried out to evaluate the LDH isoenzymes in patients with leukemia, wherein an elevated enzyme level was observed. However, another study involving LDH isoenzymes ratio concluded that there was no significant alteration in ratio of patients with OC or precancer. When biopsy tissue LDH isoenzyme ratios of OC, OSMF, and oral leukoplakia with healthy oral tissues, it was found that the ratios were significantly altered in OC and OSMF.[11]

Saliva testing has been promoted as a noninvasive alternative to serum testing in diagnosis and prognosis of

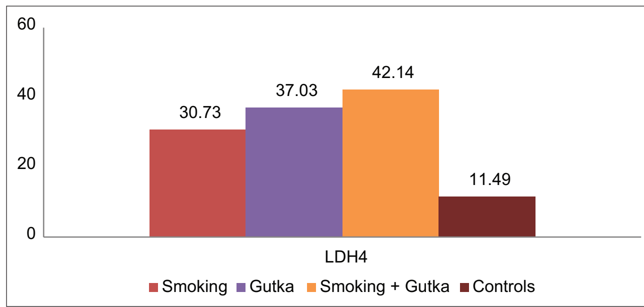


**Figure 12 Chart 4:** Salivary LDH 4 isoenzyme in 4 groups

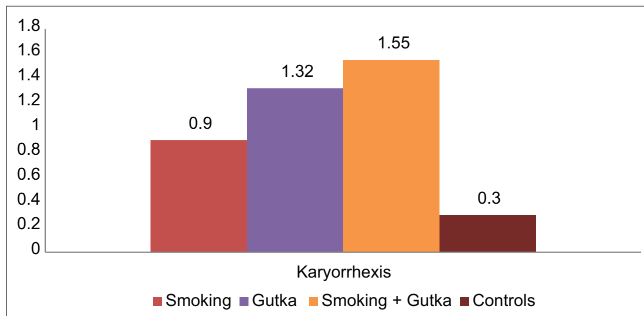


**Figure 14 Chart 6:** Comparison of karyorrhexis among 4 groups

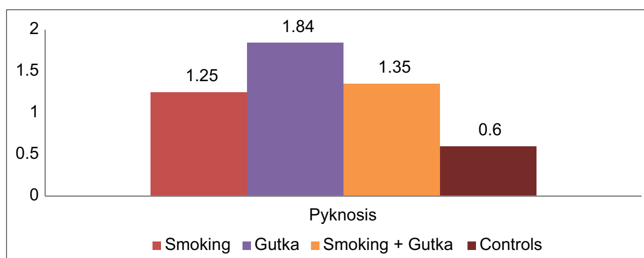


**Figure 16 Chart 8:** Comparison of pyknosis among 4 groups

OC that may be an effective modality for diagnosis and for prognosis prediction of OC.[11]

LDH is a cytoplasmic enzyme, which has the pivotal role in the clinical diagnosis of many disease processes. LDH is known to catalyze the oxidative conversion of the substrate pyruvate to lactate and has been used as an inflammatory marker. LDH is routinely present in the cytoplasm of the cell that gets released into the extracellular environment upon cellular lysis and cell death. Thus, LDH represents a marker to cell death and tissue breakdown and its raised level often signifies a disease process.[3]

The elevated salivary lactate dehydrogenase levels maybe also related to the hypoxic state seen in OSMF. Hypoxia triggers glycolytic pathways where the end product is lactate. This reaction is mediated by lactate dehydrogenase enzyme. Thus, in these conditions by reflex LDH levels are increased.[12]

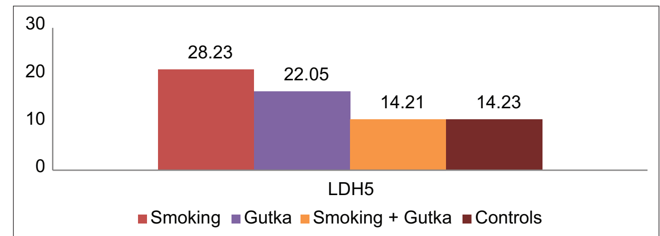


**Figure 13 Chart 5:** Salivary LDH 5 isoenzyme in 4 groups

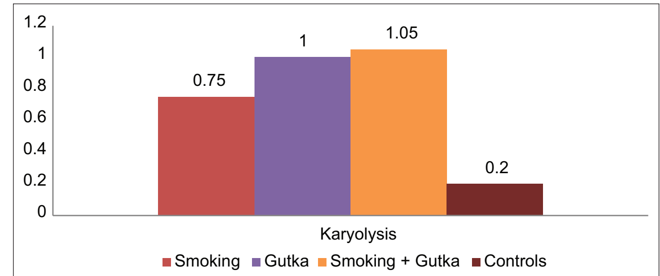


**Figure 15 Chart 7:** Comparison of karyolysis among 4 groups

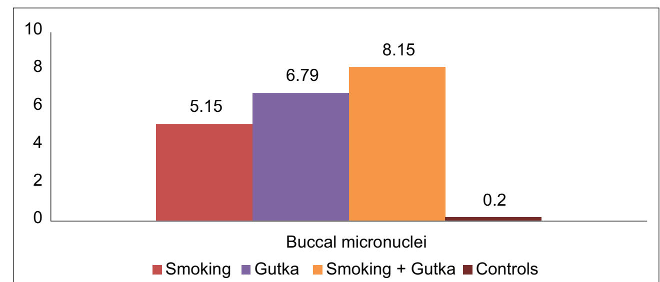


**Figure 17 Chart 9:** Comparison of buccal micronuclei among 4 groups [Figures 7 and 8]

In a study conducted by Joshi *et al*.[2] in 2012 concluded that LDH activity increased in saliva of patients with oral leukoplakia and oral squamous cell carcinoma (OSCC) in comparison to normal controls by agarose gel electrophoresis.

In the present study, agarose gel electrophoresis was used to detect the salivary LDH isoenzymes levels. The major source for whole saliva LDH is nonglandular. The oral epithelium is the major source for the nonsalivary glandular LDH in whole saliva. LDH has been described as a ubiquitous enzyme that plays a very important role in the diagnosis of the pathologic process.

Nagler *et al*.,[13] (2001) evaluated LDH isoenzyme profiles in both the whole saliva and oral epithelium, comparing them with the LDH plasma profile. The salivary LDH was examined in whole saliva, in parotid saliva, and in submandibular saliva under both resting and stimulated conditions. Exposure of whole saliva to CS *in vitro* resulted in a 41% reduction in LDH activity. However, CS exposure

had no effect on LDH activity in plasma. Whole saliva, in contrast to plasma, contains redox-active metal ions such as iron and copper that may enhance LDH loss of activity. Therefore, we conclude that whole saliva in the presence of CS becomes a potent protein-modifying agent that can destroy some of its endogenous components. In the present study showed that unstimulated whole saliva from group 1, group 2, and group 3 individuals, there was a significant decrease in isoenzymes LDH-1 and LDH-2 and there was no effect on LDH-3 isoenzyme.

Another study conducted by Joshi *et al*.[4] have been studied salivary isoenzymes in lichen planus. They have noted that salivary LDH-3, LDH-4, LDH-5, and M subunit were significantly increased in patients with oral lichen planus as compared to controls and no significant differences in levels of LDH isoenzymes in gender. As per them, increased levels of isoenzymes LDH in oral lichen planus may be due to carcinogen enhanced activity of LDH.

A study conducted by Nair *et al*.,[14] 1992, the main carcinogens in pan masala and gutkha are derived from their ingredients; areca nut, catechu, lime, and tobacco. Reactive oxygen species, implicated in multistage carcinogenesis, are generated in substantial amounts in the oral cavity during chewing. In the present study, there is a significant increase in salivary isoenzyme LDH-4. The result indicated that there is an effect of carcinogen (tobacco)–enhanced activity on salivary LDH.

Audrey M. D'Cruz and Varsha Pathiyil (2015) in a study on 60 volunteers reported a statistically significant correlation among various groups. Post-hoc test using Mann-Whitney U-test for pair-wise comparison of groups also showed statistical significance.[15] The present study showed 70 volunteers reported a statistical significant correlation among the groups. The individuals with habit of smoking and gutkha chewing showed $P$ value <.001, with a significant increase in the isoenzyme LDH-4. Dave *et al*.,[16] 1992 reported areca nut contains 40% polyphenols and several alkaloids including arecoline, arecaidine, guvacine, and guvacoline. Arecoline, the most important areca nut alkaloid, is present in 1% of the dry weight and has been shown to be genotoxic.

Patel and Metgud[17] 2015 have noted increased LDH in gastric cancer cells by immunohistochemistry. They are of the opinion that the increase is mainly due to increased LDH isoenzyme 5 and that the invasion and spread of gastric cancer cells could be indirectly promoted through the elevated activities of acid hydrolase due to the decrease of pH caused by the elevation of lactate. They feel that intestinal metaplasia and dysplasia might be a borderline lesion between normal gastric mucosa and gastric cancer.

The present study showed a decreased activity of LDH-1–specific and LDH-2–specific isoenzyme, therefore supporting previous studies connecting reduction in salivary enzyme activity such as amylase and unsaturated aldehyde content of CS. Thus, LDH enzyme modification by CS may serve as a model system for certain salivary protein dysfunction.

Nagler *et al*.[13] in 2001 concluded that major source for whole saliva LDH is nonglandular. The oral epithelium is the major source for the nonsalivary glandular LDH in whole saliva.

In another study carried out by Nagler *et al*.,[13] showed a profound increase in salivary LDH which is known to be mainly derived from the exfoliative oral epithelial cells (in this case OSCC cells) and as such may also be used as a general salivary marker for the diseased mucosa.

Nagler *et al*.[13] also stated about a future interesting prospective of salivary LDH study to examine the correlation between the level 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 may also be expected to increase.

Naderi *et al*. have reported the positive relation between micronuclei frequency and smoking intensity. The micronuclei frequency in buccal cells was higher in heavy smokers.[18] The changes in buccal mucosa of tobacco chewers and cigarette smokers were demonstrated by micronucleus assay. Cigarette smoking is one of the most important causes of OC. The number of micronuclei is a good indicator of chromosomal alterations in cytological samples. With increasing micronuclei number, the risk of chromosomal alterations will become higher.[18] Our study was done with Kruskal-Wallis analysis of variance with post-hoc Conover test which showed a significant increase in the buccal micronuclei of exfoliated cells in the oral mucosal cells of smokers and gutkha chewers. This shows a positive relationship between LDH isoenzymes and the individuals with the smoking and chewing of tobacco.

Exposure to cigarette smoke is associated with a high risk of developing cancers such as cancers of the upper aerodigestive tract and lung. *In vivo* and *in vitro* studies have shown that exposure to tobacco smoke, including mainstream and sidestream, or their mixture causes DNA single-strand breaks, aromatic adducts and

oxidative damage to DNA, chromosome aberrations, and micronuclei. Tobacco-induced oncogenic transformation was shown *in vitro* in various types of normal cells such as breast epithelial cells, lung epithelial type-II cells, and oral keratinocytes.[19]

Shetty *et al*.[11] in 2012 in their study stated that among 75 patients there was a very high significant difference between the mean salivary LDH levels in healthy controls (90.67 ± 4.06 IU/L) and in patients with oral potentially malignant disorders (OPMDs) (145.69 ± 3.72 IU/L) who also found that total salivary LDH is higher in oral leukoplakia and OSCC as compared to controls. They concluded that salivary LDH could be a reliable marker for OC and thus alteration in salivary LDH levels could be a crucial factor in pathogenesis of OC.

Further studies with more number of cases could throw a light in understanding the role of salivary LDH as a noninvasive technique in the diagnosis well before the onset of premalignant and malignant lesions.

## CONCLUSION

To conclude the present study was carried out to determine whether salivary LDH isoenzymes can be used as a biochemical marker in the individuals with habit of smoking and chewing gutkha. These LDH alterations in the saliva can stand as an early diagnostic tool. Moreover, since a noninvasive technique dramatically reduces anxiety, discomfort and simplified procurement of repeated samples for longitudinal monitoring over time. There was a significant correlation between the levels of salivary LDH isoenzymes and cytomorphometric analysis of oral epithelium in smokers and gutkha chewers. The changes in the saliva stands as a tool for monitoring time before the lesion is clinically evident.

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### Conflicts of interest
There are no conflicts of interest.

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