Salivary Lactate Dehydrogenase (LDH) as a Marker for Radiation-induced Mucositis in Head and Neck Cancers: A Preliminary Study

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Abstract

Background: In this study, we sought to understand the usefulness of salivary lactate dehydrogenase as a predictive marker for the development of radiation-induced mucositis.

Methods: This was a prospective study with head and neck cancer patients who required curative radiotherapy (>60Gy). We collected patients' saliva before the onset of radiation and after 2 Gy of radiation to assess lactate dehydrogenase levels. The patients received the stipulated oral and dental care. Data on incidence and severity of mucositis was collected using a preform sheet and oral mucositis assessment scale published by the Radiation Therapy Oncology Group throughout the 7-week treatment period.

Results: Salivary lactate dehydrogenase increased with exposure to radiation ($P<0.0001$) and there was an observed association with mucositis severity ($P<0.0001$; r = 0.515).

Conclusion: The present results have established, for the first time, that salivary lactate dehydrogenase could be a useful predictive marker to understand the development of radiation-induced mucositis in patients with head and neck cancer. The proximity of the oral cavity for regular observation and saliva collection is an added advantage.

Keywords: Head and neck cancer, Salivary lactate dehydrogenase, Mucositis

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Introduction

Reports suggest that nearly 60% of all people afflicted with cancers of the head and neck (H&N) region need radiation therapy during the course of their treatment.[1] However, the use of radiation is associated with side effects and the development of

mucositis, which is characterized by inflammation and pain of the mucosal surface membrane in the radiosensitive oral mucosa that necessitates continuous medical care.[2-4] Oral mucositis is classified as tolerable (grades 1 and 2) and intolerable (grades 3 and 4) depending on the severity.[3] While tolerable mucositis is clinically manageable, intolerable mucositis impairs diet, affects quality of life, increases morbidity, and loss of weight.[2-4]

From a cellular perspective, mucositis affects the integrity of the mucosal barrier. It increases the chances for microbial growth and may lead to systemic infections that are difficult to treat in immunocompromised cancer patients.[2-4] The development of mucositis is a very complex process. Studies have shown that the incidence and severity of mucositis depend on patient related factors like age, gender, body mass index, alcohol and tobacco use, oral hygiene, stage of disease, white blood cell count, salivary rate, and normal flora of the mouth in addition to extrinsic factors like dose per fraction, total dose, overall treatment duration, use of chemo-irradiation, and fractionation schedule.[5,6] However, none of these factors have a predictive value and cannot predict if the patient will develop mucositis and, more importantly, its severity during the course of the radiation treatment.

Research in the area of diagnostics suggests that the biochemical endpoint from the site of treatment and/or body fluid is beneficial.[7] As H&N cancers pertain to the early part of the digestive tract and encompass the easily accessible oral cavity, the saliva produced in this region is a useful body fluid, especially in ailments of the oral cavity.[8] Saliva collection does not need skilled personnel or special equipment and is non-invasive.

Lactate dehydrogenase (LDH), a crucial enzyme in metabolism, is involved in catalyzing the reversible conversion of pyruvate and lactate during glycolysis and gluconeogenesis.[9] Lactate dehydrogenase is present in the cell cytoplasm in normal healthy conditions. However, exposure of the cell to a cytotoxic agent leads to cell rupture and release of LDH into the extracellular environment.[8] Reports suggest that changes occur to salivary LDH levels in various oral pathogeneses like gingivitis, periodontitis, and cancer. Hence, LDH is a useful marker in ascertaining the oral health of the individual.[10-15]

Because ionizing radiation is cytotoxic, we hypothesize that increased levels of LDH will be observed in cases with increased severity of mucositis. We have conducted this study to ascertain the role of salivary LDH in predicting radiation-induced mucositis in H&N cancer patients who undergo curative cisplatin based chemo-irradiation.

Materials and Methods

We conducted this study from January 2012 to July 2013 in the Departments of Radiation Oncology and Biochemistry at Father Muller Medical College, Mangalore, Karnataka, India. The subjects consisted of histopathologically confirmed adult H&N cancer patients scheduled to receive curative chemoradiotherapy (60-70 Gy). Table 1 lists the exclusion and inclusion criteria. The Institutional Ethics Committee approved this study.

Radiation treatment

The participants of the study were scheduled to receive external irradiation at an average energy level of 6 MeV from a linear accelerator (Varian Medical Systems, Unique 2012, Palo Alto, CA, USA). Planned treatment included a curative target dose of 60-70 Gy, 5 days per week without any intended gap, and no more than one fraction per day of 2 Gy for 6-7 consecutive weeks. Patients received their cisplatin infusion (50 mg/m^2/day; IV) as per standard guidelines.[16,17]

Saliva collection

During the first visit, we explained the nature and purpose of the study to eligible patients who satisfied the inclusion criteria. The explanations were provided in either English or the patients' mother tongue (Kannada, Tulu or Malayalam) by one of the investigators. The subjects were informed that they had the right to withdraw from

the study at any time during the study and non-willingness to participant in the study would not deprive them of the necessary planned treatment. The patients who consented were then included in the study after they provided a written informed consent to participate.

Unstimulated saliva was collected in accordance with the method suggested by Navazesh at two time points: i) before the start of radiation treatment (day 0) before exposure to the first 2 Gy fraction and ii) 24 h after administration of the first 2 Gy fraction and prior to the second 2 Gy fraction.

Each patient was asked to thoroughly rinse the mouth area with distilled water to remove any food debris. After 10 min, the patients were requested to salivate into a sterile plastic cup. Salivary flow rate (mL/min) was measured by the following formula:[18]

$$\frac{\text{Weight of container with saliva (g) -Weight of container without saliva (g)}}{\text{Duration of saliva collection}}$$

The collected saliva was immediately transported to the Biochemistry Laboratory in an ice box. The collected saliva was centrifuged at 3000 rpm for 10 minutes, and the supernatants were stored in a freezer (-20ºC).

LDH in saliva

The stored saliva was removed from the freezer, thawed, and analyzed using appropriate blanks, controls, and standards in a UV-visible spectrophotometer (Shimadzu, Japan). The LDH assay was performed according to the kinetic spectrophotometric method described by Demetriou et al.[19] using a reagent kit obtained from Roche Diagnostics. The assay was based on an LDH-catalyzed reduction of pyruvate with NADH to form NAD+. The rate of oxidation of NADH to NAD+ was measured as a decrease in absorbance at 340 nm and expressed in terms of units/mg of protein. We used the internal and external quality control program from Biorad to ensure accuracy and precision of the LDH values.

Clinical evaluation for mucositis
Mucositis grading

One of the investigators (RT) assessed the degree of mucositis before the start of treatment and weekly during the radiation treatments in accordance with Radiation Therapy Oncology Group (RTOG) guidelines. The severity of oral mucositis was graded from 0 to 4 based on the RTOG guidelines.[3] Grades 0, 1 and 2 were 'tolerable' and grades 3 and 4 were 'intolerable' forms of mucositis.[3] Calibration of assessment was not required because only one researcher evaluated the patients throughout the study period.

All patients were provided with the standard oral, dental, and medical supportive care. Patients were provided with a 1:100 povidone-iodine solution (1 mL of Betadine and 100 mL of water) as recommended by Madan and co-workers.[20] Patients were requested to perform oral cleanings
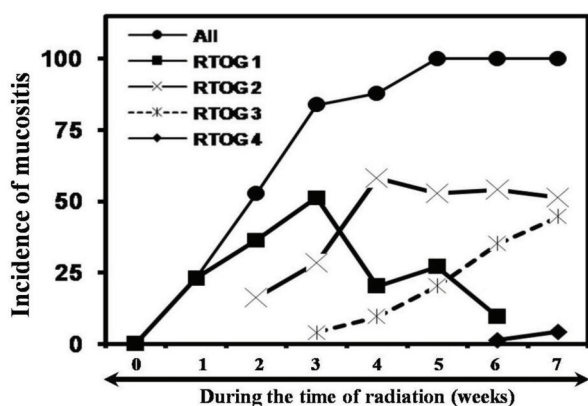
Figure 1. Incidence of different grades of mucositis during the 7-week treatment course for head and neck (H&N) cancers treated with radiation (>60 Gy). RTOG: Radiation Therapy Oncology Group.
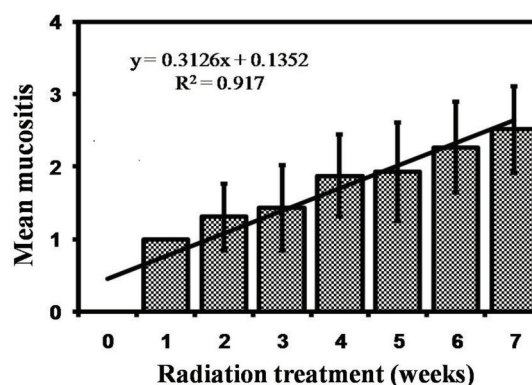
Figure 2. Average mucositis during the 7-week treatment course for head and neck (H&N) cancers treated with radiation (>60 Gy). RTOG: Radiation Therapy Oncology Group.

Table 1. Inclusion and exclusion criteria for study patient selection.

Inclusion criteria	Exclusion criteria
1. Age >18 years 2. Recently diagnosed and have not received radiotherapy/chemo-irradiation, or chemotherapy for the cancer.	1. Unwilling to be a part of the study. 2. Pregnant patients. 3. Patients who previously received chemotherapy or radiation treatments. 4. Current use of high doses of NSAIDs. 5. Presence of co-morbid conditions such as poorly controlled diabetes mellitus or hypertension. 6. Presence of mental illnesses, including schizophrenia and bipolar disorders. 7. Metastatic cancer.

thrice daily (early morning, after lunch, and before retiring for the day) using a soft toothbrush. Patients with spontaneous gum bleeding received cleaning solutions. The patients were asked to eat at least 30 min after rinsing their mouths. All patients were hospitalized during the treatment period; therefore, it was easy to monitor their adherence to diet, medications, practice of oral hygiene and the use of mouthwash.

Statistical analysis

The values were expressed as mean with standard deviation. Significance of the difference of the values between the groups was evaluated by analysis of variance (ANOVA), Bonferroni multiple comparison. We used the paired t-test to determine the presence of any statistical significance for the difference in LDH levels. The correlation between the differences in the

salivary LDH (days 1 and 0) with the cumulative mucositis score (obtained by adding the mucositis grades from each week) was analyzed by Pearson's correlation analysis. Statistical analyses were performed with SPSS software (SPSS Inc., Chicago, IL). $P<0.05$ was considered significant.

Results

A total of 83 patients required curative radiotherapy and enrolled in this study. Of these, 3 succumbed to the disease while 6 discontinued for various reasons. Hence, there were 74 evaluable cases throughout the course of the study. The mean age of the evaluable patients was 53.17 ± 9.83 years and consisted of 57 males and 17 females. Table 2 summarizes the tumor characteristics, TNM staging, treatment fraction, and dose.

Radiation exposure caused an increase in the
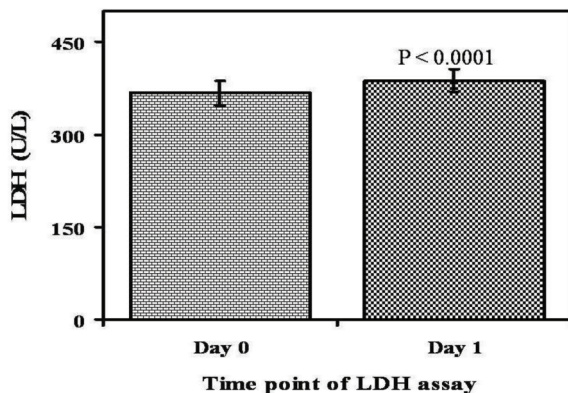
Figure 3. Levels of salivary lactate dehydrogenase (LDH) on days 0 and 1 (24 hafter the first radiation fraction of 2 Gy).
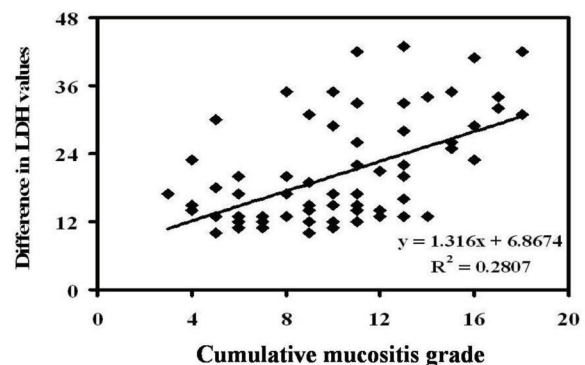
Figure 4. Association between the cumulative mucositis grade with the difference in the lactate dehydrogenase (LDH) values obtained y deduction of the value of day 0 from day 1.

incidence and degree of mucositis throughout the study period (Figures 1, 2). Since initiation of the treatment, radiation exposure caused grade 1 mucositis in 22.97% (17/74) of the patients at the end of week 1 (Figure1). The increased exposure dose led to an exponential increase in the number of patients with mucositis, such that by the end of week 5 all patients had mucositis (Figure 1). Grade 2 mucositis was observed for the first time at the end of week 2 in 16.21% (12/74) of patients. The highest incidence of grade 2 mucositis was seen in 58.10% (43/74) of patients at the end of week 4 (Figure 1).

Grade 3 mucositis was observed towards the end of week 3 (4.05%) with a peak incidence in 44.59% (33/74) of patients at the end of treatment on week 7 (Figure 1). Grade 4 mucositis was observed in 1 patient (1.35%) at the end of 6 weeks. By the end of the treatment a total of 3 (4.05%) patients developed grade 4 mucositis (Figure 1).The incidence of tolerable (grades 1 and 2) and intolerable (grades 3 and 4) mucositis followed an inverse pattern (Figure 1).

Analysis of the salivary LDH levels showed a statistically significant increase on day 1 (387.61±18.97 U/L) compared to day 0 (367.24±19.68; $P<0.0001$; Figure 3). Pearson correlation, by considering the difference in salivary LDH levels (days 1-0) with the relative sum of mucositis (cumulative mucositis from weeks 0 to 7), also showed a significant difference ($P<0.0001$, r=0.515; Figure 4).

Discussion

Radiation-induced mucositis is a dose-limiting toxicity in H&N cancers. Its development and degree of severity has a consequential role on the uninterrupted completion of the proposed curative therapy.[5] In lieu of these observations, any prognostic assay which can predict the severity of mucositis would be beneficial in planning a possible preventive intervention. Studies are underway to ascertain the utility of any prognostic marker that is non-invasive, easy to perform, and affordable. In consideration, assays that use saliva are preferred because, unlike blood, saliva is easy

Table 2. Patient, tumor, and treatment characteristics.	
Age (years)	53.17±9.83
Male: female	57:17
Tumor site (Frequency and percentage)	
Alveolus	2 (2.70)
Buccal mucosa	13 (17.6)
Floor of the mouth	3 (4.05)
Gingivobuccal sulcus	4 (5.40)
Maxilla	1 (1.35)
Hypopharynx	1 (1.35)
Parotid	1 (1.35)
Post pharyngeal wall	1 (1.35)
Pyriform sinus	3 (4.05)
Posterior cricoid	1 (1.35)
Retromolar trigone	2 (2.70)
Mandible	1 (1.35)
Supraglottis	4 (5.40)
Tongue/Base of tongue	29 (39.18)
Tonsil	5 (6.75)
Vallecula	3 (4.05)
Tumor details (TNM stage) (Frequency and percentage)	
Primary	
T1	4 (5.40)
T2	40 (54.05)
T3	20 (27.03)
T4	10 (13.51)
Regional nodes	
N0	22 (29.73)
N1	12 (16.21)
N2	36 (48.64)
N3	2 (2.70)
NX	2 (2.70)
Radiation treatment	
Dose (Gy)	68.03±1.99
Fraction	33.82±1.59

to obtain and does not need the use of trained personnel. Additionally, saliva is also considered to be useful in understanding oral biology and its pathogenesis.[8,10,21]

Lactate dehydrogenase is a tetramer that consists of 2 major subunits (A and B), and exists in 5 isozyme forms: A4 (LDH-5), A3B1 (LDH-4), A2B2 (LDH-3), A1B3 (LDH-2), and B4 (LDH-1). Numerous studies suggest that LDH is an important marker in various human pathological conditions.[8,22] In a healthy tissue/unicellular milieu, LDH is present in the cell cytoplasm. When the cell is subjected to injury,

necrosis, hypoxia, or hemolysis, LDH is released into the extracellular environment.[8]

From a clinical perspective LDH is an important marker in cardiology, hepatology, hematology, and oncology.[23] It is an important prognostic marker in the diagnosis of myocardial infarction (late detection), hemolytic anemia, ovarian dysgerminoma, and testicular germ cell tumor.[23] In oncology, LDH is an important marker to ascertain disease progression and is elevated in people with germ cell tumors, lymphoma, melanoma, and renal cell carcinoma.[23,24] Hospital-based observations have shown that enhanced serum LDH levels are a prognostic factor for poor survival in nasopharyngeal,[25-28] non-small cell lung cancer,[29] osteosarcoma,[30] renal cell carcinoma,[31,32] biliary tract cancer,[33] thymic carcinoma[34], multiple myeloma,[35] pancreatic ductal adenocarcinoma,[36] malignant mesothelioma,[37] breast cancer,[38] gastric cancer,[39] thymic carcinoma,[40] and urologic cancers.[41]

In the present research, we hypothesize that LDH is released after cell exposure to cytolytic effects. Ionizing radiation, as a potent cytotoxic agent, can cause changes in salivary LDH levels. In support of this hypothesis, the treated H&N site is principally exposed to radiation; therefore, saliva would be an ideal and easy to collect body fluid for the end point assay. We have investigated the benefit of salivary LDH as a possible marker to ascertain the impending development and severity of mucositis.[5]

The results indicated that exposure to radiation caused an increase in the incidence and development of mucositis with higher treatment fractions and increased dose; these results agreed with earlier reports.[3,5,42,43] Our observations that the differences in LDH levels during the time point assays correlated with the cumulative degree of mucositis, which indicated that salivary LDH could be a useful body fluid to assay the preeminent side effects of radiation-induced mucositis during the treatment of H&N cancers. In support, previous studies carried out with cultured cells showed that exposure to radiation increased LDH levels proportionate to the quantity of cell death and inversely to cell survival as evaluated by the clonogenic assay.[44-46]

These observations clearly indicated that increased cell death resulted in increased release of LDH. Together, these observations clearly indicated that LDH could be an important marker for mucositis. From a biochemical perspective, the salivary LDH profile has been reported to differ from plasma, which suggested that the oral milieu contributed to the total salivary LDH and not the plasma.[8,21] Research has also shown that salivary LDH originates from various sources and the combination of secretions from both major and minor salivary glands, fluids diffused through the oral epithelium and gingiva, material that originates from gastrointestinal reflux, and cellular and other debris contribute to the total level of LDH.[10]

Conclusion

The present study showed, for the first time, that salivary LDH could be an important biochemical marker for mucositis. The limitation of this study was that we considered only one early time point post-irradiation, 24 h after the first 2 Gy irradiation dose. Studies should ascertain the most effective time point post-irradiation. This would enable researchers to determine the optimal harvesting time for the assay to be performed in order to develop a predictive assay that uses salivary LDH.

Acknowledgement

Conflict of Interest

None declared.

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