Elevated tissue nitric oxide in oral squamous cell carcinoma

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INTRODUCTION

Nitric oxide (NO) is a short-lived, endogenously produced free radical that acts as a signaling molecule and plays an important role in many regulatory functions in vivo. It is generated as a reaction product of the enzymatic conversion of L-arginine to L-citrulline by three isotypes of NO synthase (NOS): endothelial NOS, neuronal NOS and macrophage or inducible (iNOS). NO is also a key signaling molecule involved in many functions such as neurotransmission, vasodilation, inflammation and immunity. It is also well known for its role in antitumor and antipathogen host response. However, under suitable conditions, NO interacts with oxygen or other free radicals, thus generating a potent oxidant, i.e., peroxynitrite (reactive nitrogen species [RNS]).

Context: Nitric oxide (NO) is a diatomic molecule that has been implicated in tumor progression of oral squamous cell carcinoma (OSCC). However, the mechanism of NO affecting tumor growth and progression remains unknown. Tumor progression has been recently received considerable attention, and there is increasing evidence of correlation of tumor biology and behavior.

Aims: We aim to evaluate tissue NO of OSCC patients and correlate these findings with grade and stage of the OSCC patients.

Materials and Methods: To count tissue nitric oxide in normal and OSCC cases. To compare the findings of tissue nitric oxide between normal and OSCC cases.

Subjects and Methods: Forty-two fresh tissue specimens from the excisional biopsy of OSCC patients and 42 tissue samples of normal healthy mucosa using ultraviolet visible spectrophotometer.

Statistical Analysis Used: Statistical analysis was done using Student’s unpaired t-test and Tukey’s post hoc analysis.

Results: Tissue NO level was higher in OSCC compared with control group (P < 0.01). There was an increase in NO levels with advanced clinical staging and with decreased differentiation of tumor.

Conclusions: Increased tissue NO levels in OSCC patients along with an increase in the clinical stage of the tumor and decreased differentiation of tumor indicates the association of NO with tumor growth and with staging and grading of is well recognized.

Keywords: Nitric oxide, oral cancer, oral squamous cell carcinoma

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Peroxynitrite and its degradation products have been linked to interaction with a range of compounds including DNA and thus considered to be involved in pathogenesis of cancer.\[^{[5]}\] NO seems to play a part in various stages of carcinogenesis from initiation to progression.\[^{[2]}\]

First study to identify NOS in oral squamous cell carcinoma (OSCC) was carried out by Rosbe et al.\[^{[6]}\] suggesting that iNOS may play a significant role in tumor growth. Numerous studies have reported significantly higher expression of iNOS or high plasma or salivary levels of NO or its products in OSCC, relating it with oral cancer pathogenesis.\[^{[4]}\] According to Connelly et al.,\[^{[1]}\] increased NO may result from increased NO synthesis perhaps due to the upregulation of iNOS. Release of tumor necrosis factor-alpha and interleukin-1 in consequence of tumor cells-macrophage interaction may activate NO synthesis.\[^{[7]}\] Rasheed et al.\[^{[9]}\] suggested potential involvement of reactive oxygen species (ROS) and RNS in the pathogenesis of head and neck squamous cell carcinoma (HNSCC) furthermore illustrated the risk of ROS-/RNS-induced damage; healthy tobacco users are exposed to, implicating their higher risk for upper aerodigestive tract cancer. After that Gokul et al.\[^{[9]}\] concluded oxidant–antioxidant imbalance possibly considered as one of the factors responsible for pathogenesis of cancer, and Korede et al.\[^{[9]}\] found increased total NO levels in oral precancer and cancer patients. Moreover, study suggested that oxidative DNA damage, a vital phenomenon in oral carcinogenesis, may occur due to interplay of RNS and ROS.\[^{[8]}\] Whereas Avci et al.\[^{[10]}\] found decreased NOS activity and NO levels in malignant oral cancer tissues as compared to benign. NO induces apoptosis and its decreased production in malignant tissues and therefore results in increased proliferation in cancer.\[^{[10]}\]

Expression of NO has also been found to be correlated with advanced grading of human mammary tumors.\[^{[11]}\] There have been numerous studies in the past related with the role of NO in understanding its role in cancer biology much to arriving at the controversy rather than a conclusion.\[^{[12]}\] Hence, the study was conducted to evaluate the levels of tissue NO of OSCC patients as compare with normal healthy controls and correlate these findings with grade and stage of the OSCC patients.

### SUBJECTS AND METHODS

Ethical clearance was obtained from the institute before commencing the study. The study comprised of 84 patients, of which 42 were histopathologically diagnosed OSCC cases and the remaining 42 were controls (pericoronal tissue or excess tissue obtained during extraction). Informed consent was obtained from all the patients before the study. After thorough clinical examination of OSCC patients according to tumor node metastasis staging system (TNM classification system), TNM staging was noted.\[^{[13]}\] The biopsy specimens from OSCC were graded histopathologically using Anneroth’s multifactorial grading system\[^{[14]}\] [Table 1]. Tissue samples of each case of histopathologically proven OSCC patients undergoing excision of oral tumors were taken for the study. The control group included 42 tissue samples of healthy normal mucosa obtained from the patients undergoing routine dental surgical procedures such as pericoronal flap surgery.

Inclusion criteria include clinically diagnosed and histopathologically confirmed cases of oral OSCC. Approximately 1 g of lesional tissue specimen was taken from the excisional biopsy.

Exclusion criteria: participants with other systemic diseases. Furthermore, participants taking any medications/antioxidant supplementation were not included in the study.

### Collection of samples

Forty-two tumor tissue samples were included in the study. Sixteen cases were well-differentiated OSCC, 15 cases were moderately differentiated OSCC and 11 were poorly differentiated OSCC. Fresh tissue samples were collected at the time of surgery and kept in deep freezer. The tissue samples were thoroughly washed to remove blood stains with deionized water and then blotted dry and weighted. One gram of the sample was homogenized in 10 ml of 0.1 M cold phosphate buffer of pH 7.4 with 1 mM

### Table 1: Clinical characteristics of oral squamous cell carcinoma patients participated in the study

| Serial number | Clinical characteristics of patients | Number of patients |
|---------------|-------------------------------------|--------------------|
| 1             | Healthy patients (control group)     | 42                 |
| 2             | OSCC patients (study group)          | 42                 |
| 3             | Male/female                          | 27/15              |
| 4             | Age (years)                          | Mean (range)       | 57.61±11.05 (31-77) |
| 5             | Tumor location                       | Buccal mucosa      | 21                 |
|               |                                    | Alveolus            | 12                 |
|               |                                    | Tongue             | 3                  |
|               |                                    | Retromolar area     | 2                  |
|               |                                    | Soft palate         | 1                  |
|               |                                    | Lip                | 3                  |
| 6             | Stage                                | Stage-I            | 2                  |
|               |                                    | Stage-II           | 4                  |
|               |                                    | Stage-III          | 19                 |
|               |                                    | Stage-IV           | 17                 |
| 7             | Grade                                | Well-differentiated OSCC | 16        |
|               |                                    | Moderately differentiated OSCC | 15        |
|               |                                    | Poorly differentiated OSCC | 11        |

OSCC: Oral squamous cell carcinoma
EDTA for 10 min, and the obtained homogenate was centrifuged at 3000 rpm for 15 min. The clear supernatant was used for analysis which was performed using ultraviolet (UV) visible spectrophotometer (Thermo scientific Instruments, Spectrascan UV 2700).

**Biochemical measurements**

Estimation of NO was performed using the Miranda et al. method by measuring the end product, namely nitrite and nitrate. The sample was deproteinized by ethanol (1:2 V/V). In this analysis, reduction of nitrate and measurement of nitrite is performed in a single step. Reduction is achieved by vanadium (III). Nitrite is immediately trapped by Griess reagent (naphthyl ethylenediamine dihydrochloride + sulfanilic acid) to give a pink-colored complex which is measured at 540 nm. To 1 ml of protein-free filtrate, 1 ml of saturated vanadium chloride (VCl3) (400 mg dissolved in 50 ml of 1 M HCL), 0.5 ml of 0.1% (W/V) NEDD and 0.5 ml of 2% (W/V) sulfanilic acid in 5% H3PO4 were added. Blank, standard and sample blank were taken separately. All tubes were incubated at 37°C for 45 min, and optical density was read at 540 nm using UV visible spectrophotometer. The results were expressed as µmol/g protein for tissue samples. Statistical analysis was done using Student’s unpaired *t*-test and Tukey’s *post hoc* analysis.

**RESULTS**

The results obtained from the assessed parameters in OSCC patients and healthy controls are shown in Tables 2-4. Significant elevation of tissue NO was found in OSCC tissue as compared with the values of the control group [Table 2]. Not only the mean calculation is more in the study group (44.67) than in the control group (14.62) but also there is a highly significant difference between mean values of calculation of both the groups (i.e., *P* < 0.01).

When the tissue NO levels of OSCC were correlated with the TNM staging, following observations were recorded. T1N0M0 (*n* = 2) – 18.4 ± 0; T1N1M0 (*n* = 5) – 32.55 ± 8; T1N2M0 (*n* = 3) – 40.1 ± 9.42; T2N0M0 (*n* = 4) – 44.01 ± 11.25; T2N1M0 (*n* = 14) – 46.76 ± 8.97; T2N2M0 (*n* = 10) – 50.15 ± 10.96 and T3N2M0 (*n* = 4) – 66.65 ± 9.64. It was noted that, as the TNM stage progresses, NO level increased [Table 3].

Tissue NO level of OSCC was compared between well, moderately and poorly differentiated OSCC and it was seen that the tissue NO increased as the histopathological grading increased. The findings were recorded as well-differentiated (30.07 ± 9.12), moderately differentiated (48.07 ± 10.66) and poorly differentiated (70.75 ± 11.45) [Table 4].

**DISCUSSION**

The role of NO is multidimensional because of its functions as an intracellular messenger and it is also implicated as a deleterious agent in various pathological conditions including cancer. Chronic inflammation can lead to the production of NO, which in turn has the potential to mediate DNA damage, directly or indirectly through the generation of more persistent RNS and ROS, thereby resulting in carcinogenesis. Consequently, RNS is found to be involved in both initiation and promotion of multistep carcinogenesis [17].

The results obtained from the study revealed a significant elevation of tissue NO in OSCC when compared to the control group (i.e., *P* < 0.01). These findings can be correlated with several studies like Gokul et al.,[8] who found significantly elevated NO in the tissue samples of OSCC, thereby indicating its association with tumor progression. Beevi et al.[17] and Rasheed et al.[13] showed increased plasma levels of NO in OSCC group. Increase in the level of NO and iNOS is seen in carcinomas other than OSCC such as breast cancer, cervical cancer, brain cancer, lung cancer, head and neck cancer and gastric cancer.[18,19]

According to the Beevi et al.[17] the antioxidant defenses are compromised in patients with oral cancer and hence the oxidative stress is increased as evidenced by elevated levels of NO products. A weak antioxidant defense system makes the mucosal cells more vulnerable to the genotoxic effect of ROS. This creates an intracellular environment more favorable for DNA damage and disease progression.[17]

Accordingly, this study found significantly higher NO in the tissues of OSCC patients compared to control group. This can be explained as at lower concentration, NO acts as an antioxidant by scavenging superoxide anion (O2−) and terminating lipid peroxidation. At high local concentration, NO also forms more persistent peroxynitrite (RNS)
through interacting with equal flux of O2⁻.3 Peroxynitrite can react with large variety of compounds including DNA, cellular lipids and proteins and thus are thought to be involved in the pathogenesis of oral cancer.[20]

The study results showed as the TNM staging of the tumor increased, tissue NO level also increased. High concentrations of NO in the tissue of patients with OSCC in the advanced stage of the disease were also obtained by Gokul et al.[8] Similar results were obtained by Korde et al.,[9] who observed a high level total NO in serum of SCC patients with clinical Stage III/IV. One of the causes of high concentrations of total NO in the tumor tissue of the advanced stage is due to the secretion of NO by cells of the immune system such as macrophages.[21] In the advanced stage, stimulated macrophages and neutrophils express iNOS and generate high amounts of NO and therefore RNOS, which can nitrosate amines.[22,23] These nitrosamines can be formed by chemical intermediates associated with nitrosative stress and are potentially carcinogenic.[24] These observations led to the proposal that nitrosamines could be formed under conditions of chronic inflammation, which in turn can lead to cancer.[16,25]

The most interesting and novel finding of the current study was that NO level increases with increasing histopathological grading of the tumor [Table 3]. Patel et al.[26] found the plasma NO level comparable in well to moderately differentiated OSCC but not comparable in poorly differentiated OSCC. The study results showed categorical increase in NO levels with decreased differentiation of tumor. This can be supported by studies carried out by Brennan et al.[27] who showed that iNOS expression correlated with severity of dysplasia. Rajendran and Varkey[27,28] immunohistochemically investigated the expression of iNOS in thirty OSMF samples and correlated it with different grades of epithelial dysplasia associated with the disease. Korde et al.[9] found increased total NO levels in oral precancer and cancer patients. Biopsies of human mammary tumors showed that there is a greater expression of iNOS in higher tumor grades which tend to be more invasive.[29] These data support the hypothesis that NO may play a critical role in the growth and spread of tumors.[10] This study is unique in itself, as this is the first study, according to the best of authors’ knowledge, ever being conducted to evaluate the NO levels in different histopathological grading of the tumor.

To summarize, the study found significantly elevated levels of tissue NO in OSCC patients, so this indicates that increased NO level is involved in carcinogenesis and tumor progression.

**CONCLUSION**

The data obtained from the study showed that tissue NO level is higher in OSCC when compared to normal healthy mucosa, thereby sustaining the fact that NO plays an important role in carcinogenesis. Furthermore, we obtained increase in NO levels with advanced clinical stage of the tumor and with decreased differentiation of tumor. Hence, the association of NO with tumor growth and the grading and staging of OSCC is well recognized. The regulation of tumor growth by NO represents as important new dimension in cancer biology. Hence, a futuristic treatment strategy can be designed to reduce tumor growth and can add a possible therapeutic benefit in the treatment of oral cancer. Further longitudinal studies on larger scale are necessary to establish the exact relationship between NO and cancer progression.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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**Table 3: Correlation between NO levels and clinical stage**

| Clinical stage | Calculations NO (µmol/g protein) | Mean±SD |
|----------------|----------------------------------|---------|
| T1N0M0 (n=2)  | 18.4±0                           |         |
| T1N1M0 (n=5)  | 32.55±8.02                       |         |
| T1N2M0 (n=3)  | 40.1±9.42                        |         |
| T2N0M0 (n=4)  | 44.01±11.25                      |         |
| T2N1M0 (n=14) | 46.76±8.97                       |         |
| T2N2M0 (n=10) | 50.15±10.96                      |         |
| T3N2M0 (n=4)  | 66.65±9.64                       |         |
| Total (n=42)  | 49.00±11.57                      |         |

n: Number of patients, NO: Nitric oxide, SD: Standard deviation

**Table 4: Comparison of mean nitric oxide levels in patients with various histopathological grading**

| Histopathological grading | NO level, (mean±SD) | F ratio | P | Post hoc analysis | P |
|---------------------------|---------------------|---------|---|--------------------|---|
| Well (n=16)               | 30.62±8.56          | 35.89   | 0.001 | Well versus moderate | <0.01 |
| Moderate (n=15)           | 51.08±7.46          |         |     | Well versus poor   | <0.01 |
| Poorly (n=11)             | 70.75±1.78          |         |     | Moderate versus poor | <0.01 |

n: Number of patients, NO: Nitric oxide, SD: Standard deviation
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