Original Research Article

Oral Mucositis and Salivary Nitric Oxide Levels in Patients on Radiotherapy for Head and Neck Tumors

.

ABSTRACT

Introduction: Mucositis has been a complication of great importance in antineoplastic treatments of head and neck tumors because when not treated properly it can lead to the interruption of radiotherapy or chemotherapy. Knowing that mucositis is a common inflammatory condition in patients undergoing radiotherapy and that nitric oxide (NO) can be a marker of inflammation.
Aim: to seek an association between mucositis, pain and NO levels in patients diagnosed with squamous cell carcinoma (SCC) in different periods of radiotherapy.
Methodology: Clinical examination was performed weekly to investigate presence of mucositis, in which the degree and intensity of pain were evaluated by using the visual analogue scale (VAS) and mouthwash samples were collected from twenty patients. In the collected samples, the concentrations of NO were measured by using the Griess method.
Results: of the twenty patients, two were excluded due to worsening of the clinical picture. Of the 18 patients who had their treatment finished, ten had some degree of mucositis and pain, with the highest levels being observed in the last week of treatment. As for the levels of NO, it was observed that low values varied widely among the patients and weeks studied. There was a statistically significant positive correlation between mucositis degrees and pain intensities, although the NO levels were correlated neither with mucositis nor with pain intensity throughout the experimental weeks.
Conclusion: although NO is an inflammatory mediator involved in diseases of the oral cavity, its presence cannot be associated with mucositis and pain in patients with head and neck cancer who are on radiotherapy.

Keywords: Radiotherapy, Nitric oxide, Mucositis

1. INTRODUCTION

Oral mucositis (OM) is a common complication in the head and neck region during antineoplastic treatments, being capable of causing great impact on them and even their interruption as it can lead to ulceration of the mucosa with intense pain, which in turn makes feeding, speech and oral hygiene difficult. Therefore, its development is related to either chemotherapy or radiotherapy as well as to the association of both forms of anticancer treatment [1].

The pathophysiology of mucositis is divided into four phases: inflammatory, epithelial, ulcerative, and curative. In the inflammatory phase, the epithelial tissue releases interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α), all causing an increase in local vascularization [2]. Recently, it was suggested that NO plays an important role in the pathology of intestinal mucositis was suggested [3].

NO is an important molecule with multiple functions in various tissues, including the oral one [4], produced mainly by activated macrophages. It is a regulatory molecule of extreme importance in the processes of immune response, inflammation, bone metabolism, and apoptosis. This gaseous molecule may have beneficial effects, such as antimicrobial activity and modulation of the immune response. On the other hand, at high concentrations, NO can act as an influential cytotoxic molecule by triggering damage to adjacent tissues, including alveolar bone [5, 6].

Since mucositis is characterized by an inflammatory process involving different cytokines and inflammation mediators, the present study aimed to investigate the possible role of NO in these conditions by associating its levels in the saliva of patients on radiotherapy treatment of head and neck with the degree of mucositis. The intensity of pain reported by patients was also verified with the visual analog scale (VAS).).

2. MATERIAL AND METHODS

Patients were informed about the objective and methods of the study and signed an informed consent form prior to approval by the Research Ethics Committee of the University of Taubaté (UNITAU) University Hospital according to protocol numbers 64672117.8.0000.5501 and approval number #12030180. All methods were carried out in accordance with relevant guidelines and regulations in the Ethics approval and consent to participate.

2.1 Study Group

We studied 20 patients of both genders aged over 18 years old who underwent three-dimensional radiotherapy (3DRT) for the treatment of squamous cell carcinoma (SCC) in the head and neck region.

The included patients were diagnosed with SCC and received a radiotherapy dose greater than or equal to 60 Gy.

According to the radiotherapy treatment protocol for head and neck tumors, these patients fall into two groups: patients undergoing postoperative radiotherapy at a total dose of 60 Gy, which corresponds to approximately 30 fractions (sessions) of RT daily with breaks on weekends for radiation recovery, thus lasting an average of six weeks; and patients undergoing radical radiotherapy at a total dose of 60 to 80 Gy, which corresponds to 38 fractions of RT daily with breaks on weekends, thus lasting an average of seven weeks.

All patients were treated in the same 2D radiotherapy equipment (Siemens Mevatron MXE2; 6MV photon energy), and irradiated in the same cervicofacial field. The protocol which includes oral cavity and salivary glands. All received the same dose of radiation during treatment, so it is not study bias.

The following data were collected from each patient: age, gender, previous medical and dental history, history of oral and perioral lesions before radiotherapy treatment, medications in use at the time of the interview, smoking and drinking habits, diagnosis of tumor lesion, site and stage of the lesion, oncological treatment, time of diagnosis, total radiation dose (cGy) and fractionation schemes (cGy/session).

All patients received prophylactic laser therapy on the first day of radiotherapy, which was applied daily during the radiotherapy treatment according to a laser therapy protocol established by the ICESP Dental Service. This protocol employs low-level laser therapy

associated with hygiene guidance, use of artificial saliva, and administration of analgesic medication when necessary.

2.2 Collection and Storage of Samples

Mouthwash samples were collected from each patient during seven consecutive weeks of radiotherapy treatment. For each collection, the patients were instructed to rinse their mouths with 10 mL of distilled water for 30 seconds and deposit the rinsed liquid into a Falcon® tube (50 mL).

The first sample collection was performed before the beginning of the first radiotherapy session, whereas the others were performed weekly until the end of the radiotherapy treatment. The samples were stored at -20ºC until the material was processed.

2.3 Assessment of Oral Mucositis and Pain

At all times when saliva samples were collected, the oral mucosa of the patients was examined by an oral specialist.

Mucositis was classified according to the WHO classification, in which a 0–4 staging system was used depending on the following clinical manifestations: no mucositis (grade 0); presence of erythema and/or mucosal ulceration in the oral cavity, hard and soft palates, and oropharynx without pain (grade 1); erythema or painful ulceration not interfering with food consumption (grade 2); painful ulceration interfering with food consumption (grade 3); and severe symptoms needing enteral or parenteral feeding support (grade 4) [2].

In addition to the clinical examination, the pain intensity of each patient was assessed by using the visual analog scale (VAS). This scale consists of a 10-cm line along which the patient records the intensity of pain being experienced, where the left end of the scale indicates “absence of pain” and the right side of the scale indicates “unbearable pain” [7].

2.4 Analysis of NO Levels

Nitric oxide levels were determined indirectly by using the concentration of nitrite detected with Griess reagent, consisting of equal volumes of three solutions (A, B, and C). Solution A was produced from 0.6 g of sulphanilic acid dissolved in 70 mL of hot distilled water. After cooling, the solution was added to 20 mL of concentrated hydrochloric acid and distilled water to a volume of 100 mL. Solution B was produced from 0.6 g of alpha-naphthylamine dissolved in 20 mL of distilled water and 1 mL of hydrochloric acid. The volume was made up to 100 mL with distilled water. Solution C was produced from 16.4 g of CH3COONa 3H2O dissolved in distilled water in a volume of 100 mL.

After mixing equal parts of the three solutions, Griess reagent was added to the wells of a 96-well plate. Then, the same volume of samples was added. Reading was performed by using an ELISA reader at a wavelength of 520 nm. Standard samples of nitrate at concentrations ranging from 100 mM to 0.32 mM were used to calculate its concentration, thus constituting a standard curve.

2.5 Statistical Analysis

Data were stored in a database and later submitted to statistical analysis by using SPSS software, version 26.0. The Kruskal-Wallis and Dunn's multiple comparison tests were used

to compare the variables. All statistical analyses were carried out at a 0.05% significance level of 0.05% and 95% confidence interval.

3. RESULTS AND DISCUSSION

Of the 20 patients studied, 17 were male and three were female, with their age ranging from 47 to 80 years old (mean ages of 64.38 to 8.40 years, respectively). Two patients discontinued the treatment due to worsening of their general clinical condition, resulting in hospitalization.
The primary tumors found in the patients were mainly on the tongue (n = 5), parotid glands (n = 4), buccal mucosa (n = 3), maxillary sinus (n = 3), cheek region mucosa (n = 2), oropharynx (n = 2), and nasal cavity (n = 1).

Of all participants, one was a smoker and 19 reported to be former smokers, whereas 13 were former alcoholics and seven reported consuming alcohol. During the radiotherapy phase, nine patients had to extract some teeth and one had to undergo curative procedures.

Of the 18 patients evaluated, eight (44.44%) had no mucositis or pain during radiotherapy and the others had different degrees of mucositis and pain, with the highest ones being observed in the last week of treatment. The grades of mucositis and pain intensity from the first to the seventh week are shown in Figure 1A and 1B. Despite these increases, there was statistical significance in the grades of mucositis only between the first and seventh week (Kruskal-Wallis and Dunn's multiple comparison test).

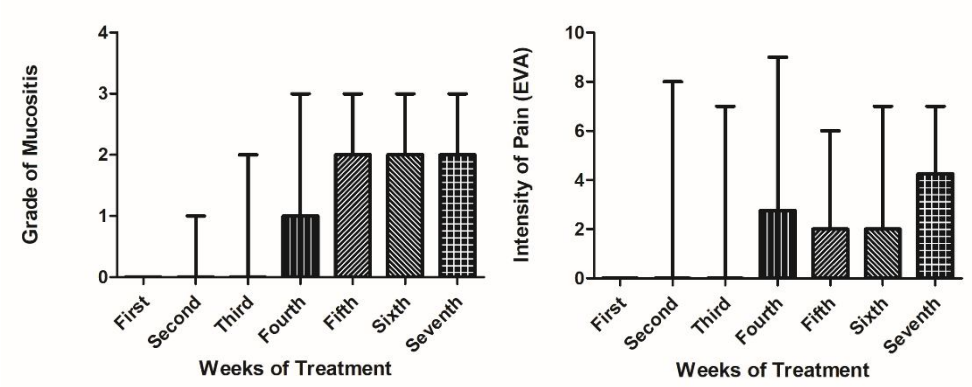

Fig 1: A) Mean degree of mucositis observed in patients undergoing three-dimensional radiotherapy (3DRT) for the treatment of squamous cell carcinoma (SCC) in the head and neck region over seven weeks of treatment; B) Mean degree of pain intensity reported by patients undergoing three-dimensional radiotherapy (3DRT) for the treatment of squamous cell carcinoma (SCC) in the head and neck region over seven weeks of treatment.

Low levels of nitric oxide were found in the mouthwash samples of the patients, which varied among them and between the weeks studied, but without statistical significance (Kruskal-Wallis and Dunn's multiple comparison test). Figure 2 shows the means of indirect concentration of NO from the first to the seventh week of treatment.

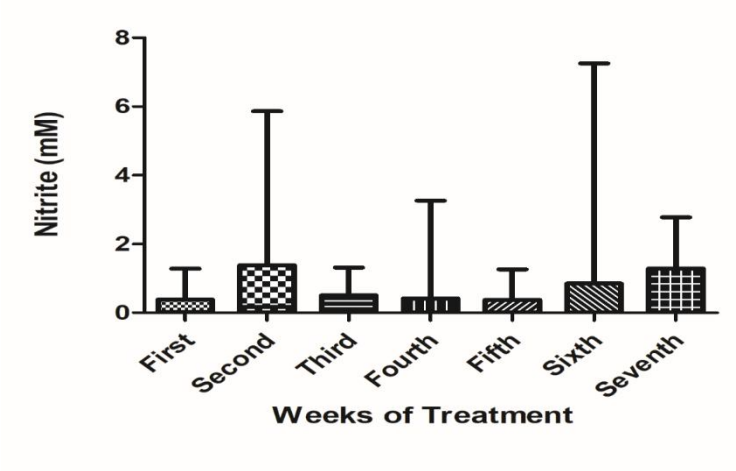

Fig 2: Mean of indirect concentrations of nitric oxide (NO) present in the saliva of patients undergoing three-dimensional radiotherapy (3DRT) for the treatment of squamous cell carcinoma (SCC) in the head and neck region over seven weeks of treatment.

After analyzing the correlation between degree of mucositis, pain intensities, and NO concentration by using the Spearman's test, a statistically significant positive correlation was observed between degree of mucositis and pain intensity in every week studied, except the first one, when the patients had not yet been submitted to any treatment and had neither mucositis nor pain. On the other hand, the levels of NO could not be correlated with either mucositis or pain intensity in any of the weeks (Table 1).

Table 1 – Spearman's correlation coefficients between degree of mucositis, pain intensities, and nitric oxide concentrations observed in patients undergoing three-dimensional radiotherapy (3DRT) for the treatment of squamous cell carcinoma (SCC] in the head and neck region in the weeks of treatment; **P* < .0001.

Week 2			
	Mucositis	**Pain**	**NO**
Mucositis		1*	0.07337471
Pain	1*		0.07337471
NO	0.07337471	0.07337471	
Week 3			
	Mucositis	**Pain**	**NO**
Mucositis		1*	0.1861032
Pain	1*		0.1861032
NO	0.1861032	0.1861032	
Week 4			
	Mucositis	**Pain**	**NO**
Mucositis		0.9859158*	-0.06695961

Pain	0.9859158*		-0.0401425
NO	-0.06695961	-0.0401425	

Week 5

	Mucositis	**Pain**	**NO**
Mucositis		0.9276993*	-0.05088634
Pain	0.9276993*		-0.04526374
NO	-0.05088634	-0.04526374	

Week 6

	Mucositis	**Pain**	**NO**
Mucositis		0.8207243*	0.2071341
Pain	0.8207243*		0.1383832
NO	0.2071341	0.1383832	

Week 7

	Mucositis	**Pain**	**NO**
Mucositis		0.8675604*	-0.1928335
Pain	0.8675604*		-0.000062
NO	-0.1928335	-0.2000062	

4. CONCLUSION

Head and neck cancer treatment is often interrupted by a complication called oral mucositis (OM). The appearance of this condition is related to the treatment with radiotherapy and/or chemotherapy and their dosages [1, 8-10].

Several authors have demonstrated that the degree of OM is related to the dosage and duration of treatment, generally appearing between 5 and 7 days after the start of chemo-radiotherapy treatment and after the second week of radiotherapy [1]. Therefore, its most severe manifestations appear in the last weeks of treatment with the presence of erythema and white scaly plaques, which may lead to the exposure of connective tissue as a result of loss of epithelial cells [11]. In fact, in the present study, a significant increase in the presence and severity of mucositis was observed between the first and last week of the study. However, a few patients presented higher degrees of condition, and none reached grade 4. It is also important to note that eight patients did not even develop mucositis.

The pain usually caused by mucositis causes the patient to have limitations in speaking and intaking food, which can result in malnutrition, anorexia, and predisposition to opportunistic infections [11]. In the present study, the pain reported by the patients was correlated with the presence and severity of mucositis, being more frequent and intense after the fourth week of treatment. Furthermore, the eight patients who did not develop mucositis also did not report any painful sensations. The low frequency and severity of mucositis, and consequently of pain, in all patients can be justified using the prophylactic laser therapy protocol, in association with oral hygiene guidance, use of artificial saliva and administration of analgesic medication when necessary, starting on the first day of radiotherapy.

Prophylactic laser therapy is a widely used measure to alleviate the patient's discomfort and decrease the degree of inflammation [9], in which the laser acts by stimulating cellular activity. This, in turn, leads to the release of growth factors by macrophages, proliferation of keratinocytes, increase in the population and granulation of mast cells, and angiogenesis. These effects accelerate the wound-healing process as it also decreases inflammation, resulting in faster tissue repair [12].

The NO present in saliva is part of a nonspecific defense mechanism against periodonto-pathogenic bacteria [13] and plays a stimulatory role [14]. However, the chemical mechanisms of NO are complex processes involving numerous possible reactions with direct and indirect effects depending on different concentrations [15].

Surprisingly, in the present study, low and variable levels of NO were found to be not correlated with mucositis and pain.

A study carried out in rats demonstrated that radiation induced a decrease in the sublingual gland activity and in the salivary expression of inducible NOS. Therefore, the radiotherapy treatment itself may have interfered with the presence of NO in the samples collected [16].

Another factor that may have influenced the low levels of NO was the use of samples of mouthwash instead of saliva, which diluted the saliva components approximately tenfold. Mouth rinsing was used precisely because these patients usually have xerostomia, thus making it difficult to collect saliva. Although NO is a molecule involved in inflammatory processes, including in the oral cavity, it was not possible to correlate it with the development of mucositis in patients with head and neck tumors undergoing radiotherapy and prophylactic laser therapy. New studies using other populations and other methodologies should be carried out to increase the knowledge on the role of NO in the development of diseases in the oral cavity.

CONSENT

Written informed consent was obtained from all individual participants included in the study.

ETHICAL APPROVAL

The study protocol was approved by the central ethics committee of University of Taubaté (UNITAU) and University Hospital according to protocol numbers 64672117.8.0000.5501 and approval number #12030180.

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki."

ACKNOWLEDGEMENTS

This study was supported by grants from the São Paulo research foundation (FAPESP) - grant number: 2020/15533-8

REFERENCES

1. Antunes HS, Herchenhorn D, Small IA, Araújo CM, Viégas CM, Cabral E, et al. Phase III trial of low-level laser therapy to prevent oral mucositis in head and neck cancer patients treated with concurrent chemoradiation. Radiother Oncol 2013, 109(2):297-302. doi: 10.1016/j.radonc.2013.08.010.

2.Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, et al. Mucositis Study Section of the Multinational Association for Supportive Care in Cancer; International Society for Oral Oncology. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer 2004, 100 (9 Suppl):1995-2025. doi: 10.1002/cncr.20162.

3.Leitão RF, Brito GA, Oriá RB, Braga-Neto MB, Bellaguarda EA, Silva JV, et al. Role of inducible nitric oxide synthase pathway on methotrexate-induced intestinal mucositis in rodents. BMC Gastroenterol 2011;11:90. doi: 10.1186/1471-230X-11-90.

4.Toda N, Ayajiki K, Okamura T. Neurogenic and endothelial nitric oxide regulates blood circulation in lingual and other oral tissues. J Cardiovasc Pharmacol. 2012;60(1):100-8. doi: 10.1097/FJC.0b013e318252452a.

5.Kendall HK, Marshall RI, Bartold PM. Nitric oxide and tissue destruction. Oral Dis 2001;7(1):2-10.

6.Pallos D, Leão MV, Togeiro FC, Alegre L, Ricardo LH, Perozini C, Ruivo GF. Salivary markers in patients with chronic renal failure. Arch Oral Biol. 2015;60(12):1784-8. doi: 10.1016/j.archoralbio.2015.09.008.

7.Jensen MP, Karoly P, Braver S. The measurement of clinical pain intensity: a comparison of six methods. Pain 1986;27(1):117-126. doi: 10.1016/0304-3959(86)90228-9.

8.Carvalho CG, Medeiros-Filho JB, Ferreira MC. Guide for health professionals addressing oral care for individuals in oncological treatment based on scientific evidence. Support Care Cancer 2018;26(8):2651-2661. doi: 10.1007/s00520-018-4111-7. Epub 2018 Feb 22.

9.Zecha JA, Raber-Durlacher JE, Nair RG, Epstein JB, Elad S, Hamblin MR, et al. Low-level laser therapy/photobiomodulation in the management of side effects of chemoradiation therapy in head and neck cancer: part 2: proposed applications and treatment protocols. Support Care Cancer 2016;24(6):2793-805. doi: 10.1007/s00520-016-3153-y.

10.Palmieri M, Sarmento DJS, Falcão AP, Martins VAO, Brandão TB, Morais-Faria K, et al. Frequency and Evolution of Acute Oral Complications in Patients Undergoing Radiochemotherapy Treatment for Head and Neck Squamous Cell Carcinoma. Ear Nose Throat J 2021;100(5_suppl):449S-455S. doi: 10.1177/0145561319879245.

11.Volpato LE, Silva TC, Oliveira TM, Sakai VT, Machado MA. Radiation therapy and chemotherapy-induced oral mucositis. Braz J Otorhinolaryngol 2007;73(4):562-8. doi: 10.1016/s1808-8694(15)30110-5.

12.Zadik Y, Arany PR, Fregnani ER, Bossi P, Antunes HS, Bensadoun RJ, et al. Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO). Systematic review of photobiomodul ation for the management of oral mucositis in cancer patients and clinical practice guidelines. Support Care Cancer 2019;27(10):3969-3983. doi: 10.1007/s00520-019-04890-2.

13.Brennan PA, Thomas GJ, Langdon JD .The role of nitric oxide in oral diseases. Arch Oral Biol. 2003;48(2):93-100. doi: 10.1016/s0003-9969(02)00183-8.

14.Rettori V, Lomniczi A, Elverdin JC, Suburo AM, Faletti A, Franchi A, McCann SM. Control of salivary secretion by nitric oxide and its role in neuroimmunomodulation. Ann NY Acad Sci 2000;917:258–267.

15.An S. Nitric Oxide in Dental Pulp Tissue: From Molecular Understanding to Clinical Application in Regenerative Endodontic Procedures. Tissue Eng Part B Rev 2020;26(4):327-347. Doi: 10.1089/ten.TEB.2019.0316.

16.de la Cal C, Lomniczi A, Mohn CE, De Laurentiis A, Casal M, Chiarenza A, et al. Decrease in salivary secretion by radiation mediated by nitric oxide and prostaglandins. Neuroimmunomodulation. 2006;13(1):19-27. doi: 10.1159/000093194.