

EVALUATION OF SALIVARY OXIDATIVE STRESS IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA UNDERGOING HEAD AND NECK RADIOTHERAPY

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ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms in the head and neck region. The standard therapeutic approach typically includes surgical intervention followed by adjuvant radiotherapy, which, although necessary, may significantly impair salivary gland function and disrupt redox homeostasis in the oral cavity.

Aim: The aim of this study was to evaluate the effect of radiotherapy on the quantity and quality of saliva, assessed through salivary biomarkers FRAS, MDA, and SOD, in patients with oral squamous cell carcinoma who had undergone surgical treatment and subsequent head and neck radiotherapy.

Materials and Methods: The study included 20 patients of both sexes, aged 41 to 76 years, who were surgically treated and subsequently underwent head and neck radiotherapy. Unstimulated saliva samples were collected at two time points: before and after completion of radiotherapy. Salivary flow rate was measured immediately after collection, and following sample processing, ferric reducing ability of saliva (FRAS), malondialdehyde (MDA) concentration, and superoxide dismutase (SOD) activity were determined spectrophotometrically.

Results: The salivary flow rate was significantly reduced after radiotherapy compared to the pre-treatment values. The post-radiotherapy FRAS levels were significantly lower, whereas MDA levels were significantly higher. SOD activity was slightly higher before radiotherapy compared to the post-radiotherapy values.

Conclusion: Our findings confirm that radiotherapy is associated with increased oxidative stress and reduced antioxidant capacity in saliva. Although antioxidants may potentially mitigate radiotherapy-induced complications, their clinical application requires caution due to the possibility of protecting the tumor cells, thereby potentially compromising the oncologic treatment outcomes.

Keywords: OSCC, radiotherapy, oxidative stress, saliva

INTRODUCTION

Head and neck cancer (HNC) represents the seventh most common malignancy worldwide [1]. Approximately 48% of head and neck cancers oc-

cur in the oral cavity, and 90% of these cases are oral squamous cell carcinoma (OSCC) [2]. Despite the advances in diagnostic techniques and the in-

roduction of novel therapeutic approaches in the recent decades, the prognosis for patients with head and neck squamous cell carcinoma remains poor, with a five-year survival rate below 50% [3].

Radiotherapy (RT) is a widely used modality in the management of head and neck cancer. Although it is an effective therapeutic approach for tumour cell destruction, it simultaneously promotes the generation of reactive oxygen species (ROS). These highly reactive molecules can damage the key cellular macromolecules, including DNA, RNA, microRNA, and proteins, through multiple pathophysiological mechanisms [4]. Oxidative stress occurs when there is an imbalance between the ROS production and the body's capacity to neutralize or detoxify their harmful effects via antioxidant mechanisms. Free radicals are molecules containing an unpaired electron in their outer orbital, which renders them highly reactive and capable of interacting with various biomolecules such as proteins, lipids, and lipoproteins, ultimately leading to structural and functional cellular damage [5,6]. Free radicals arise from both endogenous and exogenous sources, including radiotherapy. The human body possesses a broad spectrum of antioxidants whose primary role is to counteract oxidative agents. Functionally, these antioxidant systems can be classified into two main categories: enzymatic and non-enzymatic antioxidants [7].

The aim of the present study was to evaluate the effect of radiotherapy on the quantity and quality of saliva, assessed through the salivary biomarkers FRAS, MDA, and SOD, in patients with oral squamous cell carcinoma who had undergone surgical treatment followed by head and neck radiotherapy.

MATERIALS AND METHODS

The study was conducted at the University Clinic for Maxillofacial Surgery – Skopje “Academician Ilija Vaskov,” as well as at the Research Centre – Biochemical and Microbiological Laboratory, Faculty of Dentistry – Skopje, Ss. Cyril and Methodius University in Skopje. The study included twenty patients with histopathologically confirmed oral squamous cell carcinoma (OSCC) who had undergone surgical treatment followed by head and neck radiotherapy. All participants provided written informed consent prior to enrolment.

Inclusion criteria were: patients with histopathologically confirmed OSCC and patients undergoing radiotherapy following surgical treatment. Exclusion criteria included: previous radiotherapy, death during treatment, and the presence of oral or systemic diseases that could influence salivary oxidative stress.

Unstimulated saliva was collected from all participants at two time points: before radiotherapy and one week after completion of radiotherapy. Saliva collection was performed using the spitting method according to Navazesh's recommendations [8], over a 10-minute period. Salivary flow rate was determined by direct visual measurement of the collected volume in graduated plastic tubes and expressed as millilitres per minute (ml/min).

The collected saliva samples were vortexed using a vortex mixer (Dragon Lab MX-S, China). Aliquots were then transferred using a micropipette into 2 mL plastic microcentrifuge (Eppendorf) tubes for storage. The samples were frozen at -20°C for no longer than 45 days. Prior to the spectrophotometric analysis, the frozen samples were rapidly thawed in warm water and subsequently centrifuged in a refrigerated centrifuge (BIOBASE – High Speed Refrigerated Centrifuge) at $+4^{\circ}\text{C}$ for 10 minutes at 4000 rcf.

On the day of analysis, the supernatant obtained after centrifugation was used for spectrophotometric determination (SPECORD 50 PLUS, Analytik Jena, Germany) of the following salivary parameters: ferric reducing ability of saliva (FRAS) using the method of Benzie and Strain [9], malondialdehyde (MDA) concentration using the method of Buege and Aust [10], and superoxide dismutase (SOD) activity using the method of Marklund and Marklund [11]. Statistical analysis was performed using IBM SPSS Statistics 24 software.

RESULTS

According to the predefined inclusion and exclusion criteria, a total of 20 participants (100%) were enrolled in the study, of whom 11 (55%) were male and 9 (45%) were female. The mean age of the participants was 62.8 ± 10.2 years, ranging from 44 to 76 years. In the intervention group, the salivary flow rate was 0.49 ± 0.15 ml/min before RT (median [IQR] 0.50 [0.40–0.55], range 0.25–0.90) and 0.26 ± 0.11

ml/min after RT (median [IQR] 0.25 [0.20–0.30], range 0.08–0.60) (Table 1). Statistically significant difference was observed in the salivary flow rate between the pre-RT and post-RT measurements in the intervention group (Wilcoxon test: $Z = -3.859$; $p < 0.001$) (Table 1). The salivary flow rate was significantly lower after radiotherapy compared to the pre-radiotherapy values. In the intervention group, FRAS levels decreased from $407.1 \pm 102.07 \mu\text{mol AAE/L}$ before RT (median [IQR] 433.88 [371.93–463.71], range 133.68–578.98) to $210.59 \pm 98.95 \mu\text{mol AAE/L}$ after RT (median [IQR] 187.30 [158.97–234.21], range 60.45–450.08) (Table 2). Statistically significant difference was observed in FRAS levels before and after radiotherapy (Wilcoxon test: $Z = -3.659$; $p < 0.001$). FRAS values were significantly lower after radiotherapy compared to the pre-radiotherapy measurements (Table 2). In the intervention group, MDA concentration increased from $0.41 \pm 0.21 \text{ nmol/ml}$ before RT (median [IQR] 0.34 [0.27–0.50], range 0.22–0.99) to $2.13 \pm 1.33 \text{ nmol/ml}$ after RT (median [IQR] 1.78 [1.40–2.44], range 0.85–6.90) (Table 3). Statistically significant difference was also found in the salivary MDA concentration before and after radiotherapy (Wilcoxon test: $Z = -3.920$; $p < 0.001$). MDA levels were significantly higher following radiotherapy compared to pre-treatment values (Table 3). In the intervention group, SOD activity decreased from $1.11 \pm 0.28 \text{ U/ml}$ before RT (median [IQR] 1.05 [0.89–1.36], range 0.75–1.67) to $0.96 \pm 0.31 \text{ U/}$

Table 3. Analysis of MDA Levels in the Intervention Group before and after Radiotherapy

nmol/ml	N	Mean	Std. Dev.	Min	Max	Percentiles		
						25th	50th	75th
IG – pre-RT	20	0.41	0.21	0.22	0.99	0.27	0.34	0.50
IG – post-RT	20	2.13	1.33	0.85	6.9	1.40	1.78	2.44
(Wilcoxon test: $Z=-3.920$; $p<0.001$)								

Table 4. Analysis of SOD Activity in the Intervention Group before and after Radiotherapy

U/ml	N	Mean	Std. Dev.	Min	Max	Percentiles		
						25 th	50 th	75 th
IG – pre-RT	20	1.11	0.28	0.75	1.67	0.89	1.05	1.36
IG – post-RT	20	0.96	0.31	0.47	1.46	0.71	0.95	1.25
(Wilcoxon test: $Z=-1.792$; $p=0.073$)								

tion and quantity of the secreted saliva [12, 13]. In our study, a statistically significant reduction in salivary flow rate was observed after radiotherapy compared to the pre-treatment measurements. Similar findings have been reported in the majority of previous studies, concluding that radiotherapy directly damages salivary glands, resulting in persistent hyposalivation and clinically manifest xerostomia [14–16].

The inclusion of FRAS as a study parameter underscores the importance of the disrupted balance between the antioxidant defences and oxidative processes in the pathogenesis of numerous oral and systemic diseases. FRAS values were significantly lower after radiotherapy compared to the pre-radiotherapy levels. This finding, also reported in previous studies, is expected and reflects enhanced oxidative stress induced by radiotherapy, which damages the surrounding healthy tissues and generates ROS, thereby reducing the total antioxidant capacity as evidenced by lower FRAS values [17, 18].

However, conflicting data have been reported in the literature, indicating increased antioxidant levels following radiotherapy. For example, Pang et al. [19] analysed oxidative stress markers and antioxidant capacity in patients undergoing radiotherapy for nasopharyngeal carcinoma and demonstrated that T-AOC

and GSH-PX increased during the early phases of treatment (T1, T2), but significantly declined at the end of therapy (T3) in patients with mucositis, while AOPP remained elevated throughout treatment. These findings suggest that the timing of measurement is critical for interpreting oxidative stress markers and may explain discrepancies among studies regarding antioxidant levels after radiotherapy.

Malondialdehyde (MDA) is one of the most widely studied biomarkers of oxidative stress, formed as a terminal product of lipid peroxidation in cellular membranes. Due to its relative stability and ease of detection, MDA is commonly used as an indicator of lipid peroxidation and oxidative damage in various biological fluids. In saliva, MDA concentration is frequently assessed to estimate the local oxidative stress, and numerous studies have demonstrated altered MDA levels in conditions associated with chronic inflammation and oxidative damage [20].

The statistically significant increase in salivary MDA observed after radiotherapy in our study is consistent with the known biological effects of radiation. Radiotherapy induces direct damage to cellular membranes and mitochondria through increased ROS production, leading to intensified lipid peroxidation and accumulation of MDA. Špiljak et al. [21] emphasized that the

effects of radiotherapy are not solely due to the direct ionization but also to the tumour microenvironment responses, where hypoxia and metabolic reprogramming modify tissue reactions to radiation.

Several studies have confirmed that radiotherapy in patients with head and neck squamous cell carcinoma (HNSCC) leads to a significant increase in the oxidative stress markers, particularly in the saliva and serum [4, 22, 23]. Nevertheless, some investigations have reported decreased MDA levels following radiotherapy [24, 25]. Divergent findings regarding MDA after radiotherapy are likely attributable to the methodological differences, particularly the timing of measurement. When assessed during the treatment or immediately thereafter, MDA is often elevated due to the acute oxidative insult. However, if measured after a longer post-treatment interval, MDA levels may decline if tumour burden is substantially reduced or eliminated [26]. Thus, although discrepancies exist in the literature, the prevailing consensus indicates that radiotherapy increases MDA during the acute treatment phase, while subsequent normalization or reduction may occur following the successful tumour control and recovery of antioxidant mechanisms.

Superoxide dismutase (SOD), one of the most important enzymatic antioxidants, neutralizes superoxide anions and protects tissues from oxidative damage [27]. The absence of a statistically significant difference between pre- and post-radiotherapy SOD values in our study, despite slightly higher activity prior to radiotherapy, suggests that the effect of radiation on this enzyme may be variable. In some cases, radiation induces SOD depletion, whereas in others it activates adaptive regulation via NRF2 signaling pathways, potentially leading to stable or mildly increased levels. Such variability may depend on the timing of assessment, individual biological response, and the extent of radiation exposure [23, 28].

STUDY LIMITATIONS

This study has several limitations, including a relatively small sample size, the limited specificity of MDA as a biomarker due to potential analytical interferences and its formation

through multiple oxidative pathways, as well as the absence of stratification according to the applied radiation dose and number of radiotherapy sessions, which may have influenced the precision of result interpretation.

CONCLUSION

Reduced antioxidant capacity and increased lipid peroxidation following radiotherapy are associated with pronounced oxidative stress and impaired salivary gland function. These findings confirm the impact of radiotherapy on the salivary flow rate while also suggesting potential alterations in saliva quality and providing further insight into radiation-induced changes in its biochemical composition.

Given the elevated levels of oxidative stress markers and the decreased antioxidant capacity observed, further research is warranted to explore the potential application of selective antioxidant strategies aimed at mitigating treatment-related complications. However, such approaches must be carefully evaluated to ensure selective protection of surrounding healthy tissues without shielding the tumour cells or compromising the effectiveness of oncologic therapy.

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Резиме

ЕВАЛУАЦИЈА НА ОКСИДАТИВНИОТ СТРЕС ВО ПЛУНКАТА КАЈ ПАЦИЕНТИ СО ОРАЛЕН ПЛАНОЦЕЛУЛАРЕН КАРЦИНОМ ПОДЛОЖЕНИ НА РАДИОТЕРАПИЈА НА ГЛАВАТА И НА ВРАТОТ

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Вовед: Оралниот планоцелуларен карцином (OSCC) е една од најчестите малигни неоплазми во пределот на главата и на вратот. Терапевтскиот пристап најчесто вклучува комбинација од хируршка интервенција и адјувантна радиотерапија, која, иако неопходна, може значајно да ја наруши функцијата на плунковните жлезди и редокс рамнотежата во усната празнина.

Цел: Целта на нашето истражување беше да се процени ефектот на радиотерапијата врз квантитетот и врз квалитетот на плунката, евалуирани преку саливациските биомаркери FRAS, MDA и SOD, кај пациенти со орален планоцелуларен карцином, хируршки третирани и подложени на радиотерапија на главата и на вратот.

Материјал и методи: Во истражувањето беа вклучени 20 испитаници од двата пола, на возраст од 41 до 76 години, хируршки третирани и подложени на радиотерапија на главата и на вратот. Од испитаниците беше собрана нестимулирана плунка во два временски интервала: пред радиотерапијата и по нејзиното завршување. По собирањето беше одредена количината на излачената плунка, а по обработката на примероците, спектрофотометриски беа одредувани капацитетот на редукција на железото во плунката (FRAS), концентрацијата на малондиалдехид (MDA) и активноста на ензимот супероксид-дисмутаза (SOD).

Резултати: Количината излачена плунка беше статистички значително пониска по спроведената радиотерапија во споредба со вредностите пред радиотерапијата. По радиотерапијата вредностите на FRAS беа статистички значително пониски, додека вредностите на MDA беа статистички значително повисоки. Активноста на SOD беше нешто повисока пред радиотерапијата во споредба со вредностите по неа.

Заклучок: Резултатите од нашата студија потврдуваат дека радиотерапијата се поврзува со зголемување на оксидативниот стрес и намалување на антиоксидативниот капацитет на плунката. Иако антиоксидансите потенцијално би можеле да ги ублажат компликациите од радиотерапијата, нивната примена бара особена претпазливост поради можноста за заштита и на туморските клетки, што може да доведе до компромитирање на онколошкиот третман.

Клучни зборови: OSCC, радиотерапија, оксидативен стрес, плунка