

## SALIVARY ANTIOXIDANT STATUS FOLLOWING RADIOTHERAPY IN PATIENTS WITH HEAD AND NECK CANCER

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### ABSTRACT

**Background:** Head and neck cancer (HNC) represents 3% of all the malignant tumours. It is the sixth most common human malignancy. Patients with head and neck cancer receiving radiotherapy are exposed to ionizing radiation which may damage normal tissue located in the field of radiation. The present study aims to determine the changes in antioxidants status of pre and post RT saliva samples of HNC patients

**Materials and Methods:** The pre and post radiotherapy saliva samples were collected from every patient with head and neck cancer. The changes in the antioxidant levels of saliva in pre and post radiotherapy saliva samples of head and neck cancer patients was determined.

**Observation:** The three biochemical parameters levels Malondealdehyde, Glutathione peroxidase, Myeloperoxidase decreased following radiotherapy. But the decrease was not statistically significant for Glutathione peroxidase and Myeloperoxidase and highly significant for Malondealdehyde.

**Conclusion:** The Total Antioxidant Capacity increased following radiotherapy and it was observed that after radiotherapy their increased levels were statistically not significant.

**KEY WORDS:** Saliva, Antioxidants, Radiotherapy, Head and neck cancer.

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### INTRODUCTION

Head and neck cancer (HNC) represents 3% of all the malignant tumours. It is the sixth most common human malignancy [1]. About 48% of the tumors cases are within the oral cavity, and 90% of these cases are squamous cell carcinoma (SCC), which affects the lips, mouth, tongue, nasopharynx, oropharynx, hypopharynx, larynx and paranasal sinuses [2,3]. Annually, more than 5 lakhs new cases of SCC are diagnosed worldwide [4]. High rates of morbidity and mortality are observed. With the advancement in the use of chemotherapy & RT the survival has substantially improved [5].

RT is usually recommended as the primary treatment or as an adjunct to surgery, in combination with or without chemotherapy, or as a palliative measure in the management of HNC. The dose of radiation necessary for cancer treatment depends upon the location, the type of malignancy and whether or not RT is the sole treatment or is to be given in combination with other modalities. Majority of the patients receive a total dose between 50 and 70 Gy, usually given over a 5 to 7 week period~ treatment being given once a day, five days a week, with 2 Gy per fraction [6].

In addition to the antitumor effects of ionizing

radiation it may also damage normal tissue located in the field of radiation. The oral cavity has many complex areas with dissimilar structures that respond differently on exposure to ionizing radiation, e.g., mucosal lining, skin covering, submucosal connective tissue, salivary gland tissue, teeth, and bone/cartilage. The RT produces early changes in the oral mucosa (oral mucositis), skin (erythema, desquamation), salivary glands (hyposalivation), taste buds (decrease acuity) and teeth (radiation caries) [7-11]. Changes is also observed in all tissues at a later stage [12,13], especially of gingival and periodontal changes, including loss of attachment at the radiation sites are also observed [14,15].

The ionizing radiation damages DNA, including single & double-strand breaks, base damage, and DNA-protein cross links. As a consequence, a second tumor may develop immediately or years after the primary tumor treatment [16-18]. Attempts have been made to evaluate the changes in the total antioxidants (TAC) in the saliva of patients with HNC before and after RT. The present study aims to determine the changes in antioxidants status of pre and post RT saliva samples of HNC patients.

### **MATERIALS AND METHODS**

The study population included a group of HNC patients without any prior treatment (RT and/or CT) scheduled to receive RT at the department of RT. The study was performed after approval from institutional ethical committee. A signed informed consent was obtained from each subject. The details about the age, gender, occupation & the history regarding tobacco and alcohol consumption were collected from each subject. The Inclusion criteria was

1. Patient above the age of 18 years.
2. Patient having HNC of stage II to stage IV, according to TNM classification.
3. The radiation dose received by patient should be greater than or equal to 60 Gy, delivered in 30 fractions, over a period of 7 weeks.
4. At least one third of the oral cavity mucosa should be included in the RT field.

The Exclusion criteria were

1. Patients having open mouth sores before RT.

2. Patients who had undergone prior RT or CT.
3. Patients with HIV infections, diabetes mellitus or hyperthyroidism.
4. Those patients who died during the treatment period or moved to another RT centre.

**Collection of Saliva sample for analysis:** The subjects were advised to rinse their mouth with normal saline, then about 5ml of saliva were collected in a sterile container from each patient and labeled.

The Pre Radiotherapy (RT) samples were collected before initiation of RT. All the patients received a megavoltage therapy (4 MeV) using a source to skin distance of 100 cm. The doses ranged from 45 to 70.4Gy, delivered in daily fractions of 2.0Gy, 5 days per week for 5–7 weeks. At the end of 7<sup>th</sup> week a Post RT sample was collected.

The saliva samples collected were examined for Total Antioxidant capacity (TAC), Malondialdehyde (MDA), Glutathione peroxidase (GPx) and Myeloperoxidase (MPO).

### **Estimation of Total antioxidants capacity (TAC) by Phosphomolybdenum method:**

This is a based on the principle of conversion of Molybdenum by reducing agents like antioxidants to molybdenum, which further reacts with phosphate under acidic pH resulting in the formation of green coloured complex. The intensity of the green coloured complex is read spectrophotometrically at 695nm [19].

**Calculations:** The concentration of the total antioxidants in the saliva was obtained by plotting the absorbance of the test against the standard graph, and the concentration is expressed as µg/mL.

### **Estimation of Malondialdehyde (MDA):**

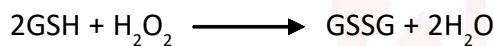
About 250µL of saliva is diluted to 500µL with distilled water. To this diluted sample 1mL of TCA- TBA-HCl reagent was added. The sample was kept in boiling water bath for 15 minutes. The reaction mixture was cooled and centrifuged. The supernatant was taken and the optical density of the pink colour formed was read at 535nm. The concentration of malondialdehyde in the sample was obtained by plotting the obtained absorbance against the standard graph. The optical density of the pink colour

formed is directly proportional to the concentration of malondialdehyde in the given sample [20].

**Calculation:** The optical densities of the test samples is directly proportional to the concentration of MDA in the sample and calculated by the plotting against the standard graph and multiplied by the respective dilution factors. The final concentration is expressed as  $\mu\text{M/L}$ .

**Estimation of Glutathione peroxidase (GPx):** The free radicals are continuously produced in vivo and there are a number of protective antioxidant enzymes for dealing with these toxic substances. GPx is one of the free radical scavenger enzymes, which is reduced progressively due to exposure to heavy metals. Therefore, this enzyme is an indicator of such contamination.

**Principle:** This procedure is based on the reaction between leftover glutathione in the following reaction with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) to form a compound, which absorbs maximally at 412nm



**Reagents:**

1. 0.4M sodium phosphate buffer (pH 7.0)
2. 10mM sodium azide
3. 4mM reduced glutathione
4. 2.5mM hydrogen peroxide (Prepare fresh solution from the stock bottle, stored refrigerated)
5. 10% TCA
6. 0.3M disodium hydrogen phosphate solution
7. DTNB: Dissolve 40mg in 100ml of 0.3M disodium phosphate solution
8. Reduced glutathione standard (100 $\mu\text{g}/\text{mL}$ ): 5mg of reduced glutathione is dissolved in 50 mL of distilled water.
9. Enzyme extract: Prepare 10% tissue homogenate in 0.4M sodium phosphate buffer (pH 7.0). Centrifuge at 10,000rpm for 15 minutes at 4°C. Use the supernatant for the assay.

**Standardisation**

Sl.No.	GSH(mL)	Distilled water (mL)	Conc. Of Std. ( $\mu\text{g}$ )	Phosphate solution (mL)	DTNB (mL)	O.D. at 412nm
Blank	0	0.5	0	2	0.25	0
1	0.1	0.4	10			0.042
2	0.2	0.3	20			0.156
3	0.3	0.2	30			0.262
4	0.4	0.1	40			0.357
5	0.5	0	50			0.463

**Procedure:** About 0.4ml of buffer, 0.1 ml Sodium azide, 0.2 ml of Reduced Glutathione, 0.5ml of enzyme extract and 0.1ml of hydrogen peroxide are pipetted into a test tube. With the distilled water the final volume is made upto 2 ml with distilled water. Then the tube is incubated at 37°C for 30 minutes. About 0.5ml of 10% TCA is added to stop the reaction. To determine the unused/residual glutathione content, centrifuge the content, save the supernatant and 0.5ml of this, add 2ml of disodium hydrogen phosphate and 0.25ml of DTNB reagent and read the absorbance at 412nm. Set a blank with only disodium hydrogen phosphate and DTNB reagent. The activity is expressed as  $\mu\text{g}$  of glutathione consumed/min/mg protein [21].

**Estimation of Myeloperoxidase (MPO):**

**Principle:** MPO, a heme containing peroxidase abundantly found in neutrophils enzymatically produces powerful antioxidant (hypochlorous acid) and show antimicrobial activity in tissues. Myeloperoxidase activity is estimated by the Malheston et al. method and is measured spectrophotometrically at 510nm using 4-aminoantipyrene as hydrogen donor [22].

**Reagents:**

- 50mmol/l of sodium phosphate buffer (pH 6.7)
- 4-aminoantipyrene solution: 0.5mg of 4-aminoantipyrene/ml solution in 0.17M phenol.

**Procedure:**

- Pipette out following into the cuvette

  1. 4 aminoantipyrene / phenol solution - 1500 $\mu\text{l}$
  2. sodium phosphate buffer - 400 $\mu\text{l}$
  3. 1-7 mmoles/L hydrogen peroxide - 100 $\mu\text{l}$

- o 100 $\mu\text{l}$  of the enzyme extract was added and the increasing absorbance at 510nm for 30 seconds intervals for 5 minutes was recorded using kinetic mode of spectrophotometer.
- o Increase in absorbance by each lysate sample was recorded
- o For calculation the molar extinction coefficient of 6580 for quinomiamine at 510nm was considered.
- o The unit of MPO activity of blood was expressed in units per pico moles /dl of the blood and in units per ml of blood.
- o The units of the MPO activity of the tissues were expressed in units per milligrams of weight.

**Statistical analysis:** All the data were expressed in mean and standard deviation. The results obtained in all the above parameters were statistically analyzed to obtain a P value that concludes the difference between the means of population involved in the study. Statistical analysis is done using student t test. P (probability) < 0.05 was considered significant.

**RESULTS**

The mean TAC was higher in saliva of patients post RT when compared to that of pre radiotherapy samples. The mean TAC was 0.66±0.43mM/L in saliva of pre RT samples and in post RT the mean TAC was 0.75±0.39mM/L. The p value was not significant (p value 0.44) (Table 1).

The mean MDA was higher in pre RT saliva samples than post RT saliva sample. The mean MDA in pre RT was 0.008±0.0018uM/L and post RT samples was 0.006±0.015uM/L. The p value was < 0.0001) and statistically highly significant (p value <0.0001) (Table 1).

The mean GPx was higher in pre RT samples of saliva than in post RT sample of saliva. The mean GPx was 2.85±0.63U/ml in pre RT saliva sample and 2.70± 0.65 U/ml. The p value (0.41) was not statistically significant (Table 1).

The mean MPO of pre RT saliva sample was less when compared to that of post RT saliva sample. The mean MPO was 27.75±8.49 pico/L in pre RT and 26.40±7.06 picoM/L in post RT saliva sample. The p value was 0.54 and was not statistically significant (Table 1).

**Table 1:** Showing the statistical calculations for the various parameters.

			MINIMUM	MAXIMUM	MEAN	SD	P Value
1	TAC (µM/L)	Pre Radiotherapy	0.24	1.81	0.66	0.43	0.44 Not significant
		Post Radiotherapy	0.26	1.98	0.75	0.389	
2	MDA (µM/L)	Pre Radiotherapy	0	0.09	0.008	0.018	<0.0001 Highly significant
		Post Radiotherapy	0	0.07	0.006	0.015	
3	GPx (U/ml)	Pre Radiotherapy	0	3.13	2.85	0.63	0.41 Not significant
		Post Radiotherapy	0	3.21	2.7	0.65	
4	MPO (picoM/L)	Pre Radiotherapy	11.89	36.83	27.75	8.49	0.54 Not significant
		Post Radiotherapy	14.08	41.01	26.4	7.06	

**DISCUSSION**

**MNA:** The micronucleus (MN) represents chromosome fragments or whole chromosomes which fail to get incorporated into main nuclei at mitosis. The MN consequently appear only in cells that undergo nuclear division [23].

The MNA in buccal mucosal cells is an innovative genotoxicity technique, which holds promise for the study of epithelial carcinogens. MN are suitable internal dosimeters for revealing tissue specific genotoxic damage in individuals exposed to carcinogenic mixtures.

Casartelli et al. observed the frequency of MN in buccal mucosal cells of normal mucosa, precancerous lesions and squamous cell carcinoma. They observed a gradual increase in MN counts

from normal mucosal to precancerous lesions to carcinoma suggested a link of this biomarker with neoplastic progression [24].

RT plays an important role in the treatment of many cancers, but it also produces genetic damage. Many studies have been done on MN in buccal cells of patients undergoing RT for HNC treatment. The most striking increase in cytogenetic damage (150-300 MN/1000 cells) was observed in an early study of three patients exposed to a cumulative dose of 3400-4000cGy [25].

Some authors reported 68 MN/1000 cells after 2000 cGy [9] and 16 MN/1000 cells after treatment with 1000 cGy for 3 weeks [26].

Moore et al., [27] observed more than 16 fold



increase in MN frequency shortly after the initiation of RT, followed by return to baseline 12 weeks later and 3 weeks after cessation of the RT.

Many studies have shown that there is a statistically significant increase in the MN frequency in buccal mucosal cells after exposure to genotoxic agents. The MN frequency decreases after micronutrient supplementation or chemoprevention, but the magnitude of changes is usually relatively small [28].

In the present study, the frequency of MN increased from 9.96 in pre RT samples to 22.72 MN/100cells in post RT samples which was statistically significant. The extent of DNA damage evaluation by the comet assay in peripheral blood cells is perfectly reflected by the MNA on oral exfoliated epithelial cells, and MN frequency can be used with the same effectiveness and greater efficiency in early detection of oral premalignant conditions.

**TAC:** The measurement of TAC in human saliva helps in assessing the oxidative stress during disease states [29]. Oral carcinoma is more prevalent in old aged people due to the reduction in the salivary antioxidant activity or due to an increase in reactive oxygen species or reactive nitrogen species causing DNA aberrations [30]. Soheila M et al., [31] observed that the TAC was significantly low in pre RT samples, than in post RT and control group. There was statistically significant increase in TAC in post RT samples compared with that of pre RT samples.

The present study also showed an increase in the TAC levels in post RT saliva samples than in pre RT saliva samples. But the increase in TAC was not statistically significant. RT caused a reduction in lipid peroxidation and an improvement in the TAC in HNC patients. The post RT level of TAC did not reach the TAC level as among groups but the increase in TAC level was vital for survival and for improved quality of life [32].

The results of TAC may be markedly different depending on the assay performed [33].

**MDA:** RT induces lipid peroxidation by inactivating the antioxidant enzymes, thereby rendering the system inefficient in management of the free radical attack, the degree of radiation

affects the extent of the depression of the antioxidant enzyme activities and increases lipid peroxidation. MDA is formed only by fatty acids with three or more double bonds and is used as a measure of lipid peroxidation. MDA produced by peroxidation can cause cross linking and polymerization of membrane components [34].

MDA is produced by peroxidation can cause cross linking and polymerization of membrane components [34,35]. This can alter intrinsic membrane properties, such as deformability, iron transport, enzyme activity and the aggregation state of cell surface determinants. Because MDA is diffusible, it will also react with nitrogenous bases of DNA. All of these effects may explain why MDA is mutagenic and carcinogenic [36]. The mean level of serum MDA was 0.008  $\mu$ M/L in Pre RT saliva samples with standard deviation of  $\pm$  0.018  $\mu$ M/L. The mean level of serum MDA was 0.006  $\mu$ M/L in Post RT saliva samples with standard deviation of  $\pm$  0.015  $\mu$ M/L. This decrease in level of MDA in Post RT samples were statistically highly when compared with Pre RT samples. This result was similar to studies done by Lobo et al.,<sup>37</sup> Oral cancer patients showed significantly higher MDA level in saliva when compared to controls. One week after RT, there was significant increase in MDA in oral cancer patients. After the completion of RT of six weeks, MDA level decreased restoring the values near-to-controls. The pattern of change in MDA was similar between blood and saliva [37].

But in the study done by Mahendra RP et al., the mean serum MDA level was high in the oral squamous cell carcinoma patients as compared to the healthy individuals. This level further increased after the RT indicating more damage to the cellular structure from free radicals which lead to oxidative stress [38].

**GPx:** GPx is a selenium-containing antioxidant enzyme that effectively reduces  $H_2O_2$  & lipid peroxides to water & lipid alcohols, respectively, & in turn oxidizes glutathione to glutathione disulfide. Reduced glutathione plays a major role in the regulation of the intracellular redox state of vascular cells by providing reducing equivalents for many biochemical pathways. In the absence of adequate GPx activity or glutathione levels, hydrogen peroxide and lipid peroxides are

not detoxified and may be converted to OH-radicals and lipid peroxyl radicals, respectively, by transition metals ( $Fe^{2+}$ ). The GPx/glutathione system is thought to be a major defense in low-level oxidative stress. GPx an enzyme dependent on the micronutrient selenium (Se), plays a critical role in the reduction of lipid and hydrogen peroxides [39].

If GPx activity is decreased, more hydrogen peroxide is present, which leads to direct tissue damage and activation of nuclear factor-kB-related inflammatory pathways [40,41]. A study was done by Manasaveena V et al., [42] in 2014, to evaluate the magnitude of oxidative stress and levels of enzymatic antioxidants in Oral Squamous cell carcinoma (OSCC) patients receiving RT and chemo radiotherapy (CRT). The GPx levels were found to be 65.713  $\mu$ g/dl in healthy control group, 13.8  $\mu$ g/dl in disease control patients, 16.49  $\mu$ g/dl in the group which received RT, 34.2  $\mu$ g/dl in chemo and RT received group.

In the study done by Demirciet al., on antioxidant status in 35 cervical cancer patients, there was no significant differences in the levels of GPx pre-RT and post-RT [43]. The mean GPx was higher in pre RT samples of saliva than in post RT sample of saliva. The mean GPx was  $2.85 \pm 0.63$  U/ml in pre RT saliva sample and  $2.70 \pm 0.65$  U/ml. The p value (0.41) was not statistically significant.

**MPO:** MPO is a peroxidase enzyme most abundantly present in azurophilic granules of the neutrophils and released by a degradation process. MPO reacts with hydrogen peroxide converted from the extra oxygen consumed in the respiratory burst to form hypochlorous acid - a complex that can oxidize a large variety of substances [44].

This oxidative damage to biomolecules such as DNA, proteins and lipids may form the first step in carcinogenesis. MPO has also been implicated in the activation of carcinogens present in tobacco smoke, one of the major etiological factors in oral cancer.

## CONCLUSION

The three biochemical parameters levels Malonaldehyde (MDA), Glutathione peroxidase (GPx), Myeloperoxidase (MPO) decreased

following RT. But the decrease was not statistically significant for GPx and MPO and highly significant for MDA. The TAC increased following RT and it was observed that after RT their increased levels were statistically not significant. However the profile can also be influenced by the patients by their history of smoking, drinking and others factors. Further investigation is necessary to confirm and expand these findings.

**Conflicts of Interests: None**

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