Estimation of Serum Malondialdehyde before and after Radiotherapy in Oral Squamous Cell Carcinoma Patients Undergoing Antioxidant Therapy

1Mahendra R Patait, 2RN Mody

1Professor, Department of Oral Diagnosis and Maxillofacial Radiology, SMBT Dental College and Hospital, Sangamner, Maharashtra, India
2Professor and Head, Department of Oral Diagnosis and Maxillofacial Radiology, Hitkarini Dental College, Jabalpur, Madhya Pradesh, India

Correspondence: Mahendra R Patait, Professor, Department of Oral Diagnosis and Maxillofacial Radiology, Ujwal, Dwarka Nagari Housing Society, Kathe Lane, Poona Road, Nashik, Maharashtra-422001, India, e-mail: reetpat123@yahoo.com

ABSTRACT

Many researchers have shown that lipid peroxides (Malondialdehyde) are the by-products of the chemical damage done by-oxygen free radicals (ROS), to the lipid components of cell membrane, which showed changes in its serum values in radiation treated malignant cases and antioxidants mediate anticancer activity, by modifying the cellular metabolic balance. Present study was conducted with the aim to estimate and compare the serum malondialdehyde (MDA) level in normal individuals, oral cancer patients before and after radiotherapy undergoing antioxidant therapy.

Keywords: Malondialdehyde, Lipid peroxidation, Oral squamous cell carcinoma, Radiotherapy, Antioxidants.

INTRODUCTION

The etiology of oral cancer is voluminous but few firm conclusions can be drawn as the incidence of oral squamous cell carcinoma appears to be directly related to the use of tobacco in any form, i.e. smokeless tobacco and smoking tobacco, quantity smoked and the duration of the exposures.1 The role of factors, such as immunological susceptibility and enzymatic induction in promoting the cancer remains unclear. Radiotherapy2 has been used successfully in the treatment of oral cancers for many years, due to rapid development in medical radiotherapy instrumentation and technology. The mode of radiotherapy has generated additional free radicals during the irradiation process, and less defended by poor antioxidant system in these individuals. The major antioxidant defense system appear to be the scavenging of free radicals and signet oxygen in conjugation with vitamin E and superoxide dismutase and the reduction of hydroperoxide by the activities of catalase and glutathione peroxidase. Antioxidants are considered to be agents suitable for chemoprevention trial because they block oxidative damage to DNA resulting from the carcinogen-induced generation of oxygen free radicals during radiotherapy.

CHAIN REACTION OF LIPID PEROXIDATION

One of the first step in the initiation of lipid peroxidation is the generation of a superoxide radical or its protonated molecule, the perhydroxyl radical. The latter could directly initiate polyunsaturated fatty acids (PUFA) peroxidation. Hydrogen peroxide which is produced by superoxide dismutation or by direct enzymatic production has a very crucial role in the initiation of lipid peroxidation. Hydrogen peroxide reduction by reduced transition metal generates hydroxyl radicals which oxidize every biological molecule. Complexed iron could be activated by O2 or H2O2 to ferryl iron compound, which is supposed to initiate PUFA peroxidation. The presence of H2O2 especially hydroperoxidase activates enzymes, such as cyclooxygenase and lipoxygenase. Lipid peroxidation could also be initiated by other free radicals.3 Propagation results in the rapid formation of thiobarbituric acid reactive substance (TBARS) and lipid hydroperoxides.4 Malondialdehyde is formed only by fatty acids with three or more double bonds and is used as a measure of lipid peroxidation. The cyclic peroxide radical are the precursors of cyclic endoperoxide and malondialdehyde.

Malondialdehyde produced by peroxidation can cause cross linking and polymerization of membrane components.5,6 This can alter intrinsic membrane properties, such as deformability, iron transport, enzyme activity and the aggregation state of cell surface determinants. Because malondialdehyde is diffusible, it will also react with nitrogenous bases of DNA.7 All of these effects may explain why malondialdehyde is mutagenic,8 genotoxic to cultured cells9 and carcinogenic.10
REVIEW OF LITERATURE

Suha A et al (1989)\(^1\) observed that before vitamin E therapy, lipid peroxidation of chronic hemodialysis patients were significantly higher than those of healthy controls. Treatment with vitamin E (300 mg/day) for 1 month results in a significant decrease of lipid peroxidation value. Punnonen R et al (1993)\(^2\) analyzed lipid peroxidation in normal tissues as well as endometrial cancer tissue from Finnish and Japanese patients and found that lipid peroxidation was slightly higher in endometrial cancer patients as compared with normal, both in Finns and Japanese people. Balasubramanyan N et al (1994)\(^3\) found that the tissue levels of lipid peroxides was significantly higher in carcinoma of the uterine cervix patients than that of normal individuals (p < 0.001) and suggested impaired antioxidant status in carcinoma of uterine cervix. Arivashagan S et al (1997)\(^4\) analyzed the relationship between lipid peroxidation and antioxidant status in erythrocytes from 24 adult gastric cancer patients and found that erythrocyte lipid peroxidation was markedly increased, while both enzymatic and nonenzymatic antioxidants were decreased in erythrocytes of gastric ulcer patients. Oberley TD et al (1997)\(^5\) demonstrated biochemically that the antioxidant enzyme levels are low in most animals and human cancer. Kong O et al (1998)\(^6\) reviewed the role of patients built in cellular defense mechanisms in a tumor’s protection against nonsurgical antineoplastic therapies. They found the overexpression of antioxidants like superoxide dismutase (SOD) to be the most important. Oxygen radicals are highly toxic and have been important in various diseases including carcinogenesis. They produce a variety of pathological changes through lipid peroxidation and DNA damage. They therefore thought of treating free radical induced diseases with antioxidants but found that the underlying mechanism that most chemotherapeutic agents and ionizing radiation exerts on tumor cells is not increased antioxidation, but rather the production of more free radicals, leading to irreversible tissue injury. A small increase in reactive oxygen species (ROS) following nonsurgical antineoplastic therapies induce the expression of antioxidant, such as SOD but overproduction of ROS exhausts the production of SOD and other adaptive antioxidant defenses. Based on these consideration they hypothesized that the appropriate administration of antioxidant inhibitors may be useful strategy in the treatment of solid tumors. Huang et al (1999)\(^7\) determined the serum malondialdehyde (MDA) levels in breast cancer patients (n = 35) and control (n = 35) using high performance liquid chromatography (HPLC) and found that there was significant increased lipid peroxidation in the serum of the breast cancer patients. Chiuo JF et al (1999)\(^8\) measured lipid peroxidation in plasma and erythrocyte of cervicitis patients and leiomyoma patients in comparison with matched controls. Plasma thiobarbituric acid reacting substances (TBARS) in both groups were found to be significantly higher than in controls (p < 0.05). Ahmed MI et al (1999)\(^9\) studied the levels of lipid peroxides in patients of carcinoma of cervix and compared the values with those of normal women. They found the levels of lipid peroxides to be significantly higher in malignant than in normal tissue samples and their levels were correlated with advanced clinical stage (p < 0.001). Sabitha KE et al (1999)\(^10\) studied the effect of radiation on oral cancer patients using activities of superoxide dismutase (SOD), catalase, glutathione peroxides (GPX), glutathione reductase (GS), glutathione-s-transferase (GST) and levels of MDA. The level of MDA showed a significant increase in untreated and oral cancer patients who had undergone radiation therapy when compared with normal patients. Radiation 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. Shariff et al (2009)\(^11\) evidenced an oral antioxidant supplementation to malignant patients undergoing radiotherapy had a beneficial effect in lowering the serum MDA levels by improving the antioxidant status, which directly reflects, reduction in the oxidative stress and lipid peroxidation in these subjects.

MATERIALS AND METHODS

For the present study, 60 patients were selected at random from Outpatient Department of Oral Medicine and Radiology, Government Dental College and Hospital and Department of Radiotherapy, Government Medical College, and Rashtra Sant Tukdoji Cancer Hospital, Nagpur, and were divided into two groups as follows:

**Group I:** Control group consisted of 20 healthy, not having any oral lesions or known systemic diseases in the age group of 40 to 75 years.

**Group II:** Study group consisted of 40 patients in the age group of 40 to 75 years, who had histopathologically confirmed oral squamous cell carcinoma and were further divided into group A consisting of 20 oral squamous cell carcinomas patients and group B consisting of 20 oral squamous cell carcinomas patients planned to undergo antioxidant therapy during radiation therapy. [The antioxidant capsules used in the study (1 BD for 5 weeks) had the following composition: \(\beta\)-carotene (as 30% dispersion)—10 mg, vitamin C—100 mg, vitamin E acetate—25 mg, selenium (as selenium dioxide)—150 mcg, copper (as copper sulfate)—1 mg, zinc (as zinc sulfate)—7.5, manganese (as manganese sulfate)—1.5]. The criteria for selection of study group:

- Patients who were planned to be given only radical radiotherapy of total 50 Gy radiation dose over a period of 5 weeks with a daily dose of 2 Gy using a cobalt—60 unit (Patients receiving any other mode of treatment were not included in the study).
- Patients who were without any known systemic diseases or disorder.

The relative history of each patient from both the groups was recorded. Intraoral and extraoral examinations were carried
out with the help of mouth mirror, probe/explorer in good light and squamous cell carcinoma was clinically classified on the basis of American Joint Commission of Cancer (AJCC) – 1997 tumor staging system with TNM grading. They were also classified on the basis of histopathology and divided into three groups; poorly differentiated, moderately differentiated and well differentiated. The complete data of the patients were filled in the case history performa (Graph 1).

Under aseptic conditions, 5 ml of blood was drawn from each patient using sterile disposable 22 gauge needle and 5 ml syringe. In patients with oral squamous cell carcinoma it was drawn twice:

i. Before onset of radiotherapy.
ii. After completion of radiotherapy.

**DETERMINATION OF LIPID PEROXIDES IN SERUM (KEI SATOH METHOD)**

**Principle**

The principle of this method is to determine the level of serum lipid peroxides using malondialdehyde as a marker, thiobarbituric acid as a main reagent and measuring these values on photoelectric colorimeter at 530 nm of light under green filter.

**Reagents**

- 1,1,3,3-tetramethoxypropane—10M (E-Mark’s, Germany)
- Thiobarbituric acid reagent (TBA) (E-Mark’s, Germany)
- Trichloroacetic acid—20 mg/dl in distilled water
- Sulfuric acid—0.05 m
- Sodium sulfate solution—(2M solution).

**EXPERIMENTAL PROCEDURE**

Blood was collected in a sterile plain bulb and kept standing for 20 minutes at room temperature. The serum was separated by centrifugation at 3000 rpm for 15 minutes. Around 0.5 ml serum was mixed thoroughly with 2.5 ml of trichloroacetic acid (TCA) and mixture was allowed to stand for 10 minutes at room temperature. This was further centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The precipitate collected was washed twice with dilute sulfuric acid. About 2.5 ml of dilute sulfuric acid was added to the tube containing precipitate and was mixed well with 3 ml of thiobarbituric acid reagent (TBAR). Reaction mixture was heated in boiling water bath for 30 minutes and allowed to cool to room temperature. Then 4 ml of n-butyl alcohol was added and give vigorous shaking chromogen was extracted in organic phase. Color intensity of the chromogen was measured at 530 nm in photoelectric colorimeter. Results were compared using standard solution of 1,1,3,3-tetramethoxypropane and expressed as µmol per liter.

**STATISTICAL ANALYSIS**

- Student paired t-test was used for comparing the sample from control vs preradiotherapy.
- Students unpaired t-test was used for comparing the samples from control vs postradiotherapy.

**DISCUSSION**

Oral cancer is a multifocal disease and experimental studies have shown that such lesions develop in several steps, according to Goldhaber (1957).22

The phases in the development of malignant neoplasm of the oral cavity are initiation and promotion. The increasing incidence of oral cancer is clearly age related, which may reflect the declining immune surveillance with age and duration of exposure to initiators and promoters. These include exposure to chemical irritants, physical irritants, viral infections, hormonal effects, cellular ageing and immunological surveillance.23,24

Radiation therapy is one of the clinical means by which oral cancer can be treated. Many biochemical complications, such as damage to cellular DNA25 and membrane structure and alterations in the immune system,26 arise as a result of a radiation treatment. The biological effects they produce are thought mainly to be caused by the production of free radicals from the interaction with the cell constituents, especially water.27 Oxygen, mainly present in most biological systems, aggravates the damage done by radiation. Much of the initial damage done is due to the formation of hydroxyl radical, which can react with other cellular components to produce organic radicals.28 One of the important underlying phenomena is an oxidative/change in the membrane lipids, which may be triggered by free radicals lipid peroxidation is initiated by very potent free radicals, which include super peroxide (O2–) and hydroxyl radical (OH–) and the reactive molecule hydrogen peroxide (H2O2). Hydrogen peroxide and O2 may be directly damaging or more often interact to form a highly reactive species that can attack almost every molecule in the living cells.29 The well-characterized product of the lipid peroxidation is MDA, a three carbon dialdehyde (OCH—CH2—CHO) produced during the oxidative decomposition of PUFA and is formed during the metabolism of certain hydrocarbon carcinogens.30 It is also reported to initiate skin carcinogenesis.31

The mean level of serum MDA in control group was found to be 0.3084 µmol/l with standard deviation of ± 0.10167 µmol/l and the mean level of serum MDA in study group carcinoma patients was 0.598 µmol/l with standard deviation of ± 0.1609 µmol/l. This increase in level of MDA was statistically highly significant as compared to control group (p < 0.001) (Table 1). The mean level of serum MDA in study group patients after radiotherapy was 0.792 µmol/l with standard deviation of ± 0.1157 µmol/l. As compared to preradiotherapy level of MDA this increase in level was statistically highly significant (p < 0.001) (Table 1 and Graph 2).

The above findings are similar to the study done by Sabitha KE et al (1993),20 they studied the malondialdehyde levels in pre- and postradiotherapy oral cancer patients. Balasubramanyan N et al (1994)13 estimated the circulating lipid peroxide, antioxidant component and the activities of defense...
enzymes in uterine cervical carcinoma patients before and after radiotherapy and radiotherapy combined with chemotherapy and compared it with control. They found that lipid peroxides levels increased in all the stages of uterine cervical carcinoma compared to that of normal. Bhuvarashamurthy V et al (1999)32 studied tissue lipid peroxides in patients who had carcinoma of uterine cervix and the values were compared with those of normal. The tissue level of lipid peroxides and activity of glutathione-S-transferase were found to be significantly higher than that of normal from stage II onwards. Huang et al (1999)17 determined the serum MDA level in breast cancer patients (n = 35) and controls (n = 35). They found that there was significant increased lipid peroxidation in the serum of the breast cancer patients as compared with the control group. Almost similar results were obtained in two separate studies by Arivashagan S et al (1997)14 and Ahmed MI et al (1999).19 Punnonen R et al (1993)12 also analyzed lipid peroxidation in normal as well as endometrial cancer patients from Finnish and Japanese patients and found that lipid peroxidation was slightly higher in endometrial cancer patients as compared with normal, both in Finnish and Japanese patients, these findings were consistent with the findings of Chious JF et al (1999)18 and with the findings of Ray S et al (1999)15 who studied the lipid peroxidation in cervicitis patients and in human uterine tumors. In the recent study when the study group was divided into well-differentiated squamous cell carcinomas (WDSC), moderately-differentiated squamous cell carcinoma (MDSC) and poorly-differentiated squamous cell carcinoma (PDSC) and findings were correlated with each other the following results were seen.

In study the mean level of serum MDA in oral squamous cell carcinoma patients after radiotherapy without antioxidant therapy was 0.792 µmol/l with standard deviation of ± 0.1157 and in patients with antioxidant therapy it was 0.763 µmol/l with standard deviation of ± 0.0953, when compared with each other using unpaired t-test the difference was found to be statistically nonsignificant (p > 0.05) (Tables 3, 4 and Graph 3).

Gitanjali G et al (1999)34 studied the correlation of lipid peroxidation and α-tocopherol supplementation in patients with cervical carcinoma, receiving radical radiotherapy and concluded that vitamin E acts as a antipromotor of carcinogenesis and MDA is a by-product of lipid peroxidation inherent in carcinogenesis. They also found that serum vitamin E levels in the carcinoma patients did not correlate with oral supplementation of vitamin E.

Antioxidants are considered to be agents suitable for chemoprevention because they block oxidative damage to DNA resulting from the carcinogen-induced generation of oxygen-free radicals. According to JJ De Cosse (1988)35 vitamins C and E have been shown to inhibit nitrosamine formation and have a beneficial effect in several experimental colon cancer patients.

JJ De Cosse at al (1989)36 and G McKeowaessen et al (1988)37 carried out two prospective controlled clinical trials, which involved randomized daily dietary supplementation of tocopherol (50 mg /d) and/or carotene (20 mg/d) versus placebo to male smokers, aged between 50 to 69 years (mean age 61 years), over a period of 5 to 8 years. A 16% reduction in the occurrence of colorectal cancer was observed (and a 34% reduction in the occurrence of prostate cancer) in those males who received vitamin E, compared with those who did not. This finding alone is not sufficient to recommend systemic use of vitamin E as findings are neither consistent nor ideally tested. In the recent study when the study group was divided into well-differentiated squamous cell carcinomas, moderately-differentiated squamous cell carcinoma and poorly-differentiated squamous cell carcinoma and findings were correlated with each other the following results were seen.

In oral squamous cell carcinoma patients without antioxidant therapy before radiotherapy the mean MDA level in WDSC (0.57 ± 0.09), MDSC (0.590 ± 0.01), and PDSC carcinoma (0.84 ± 0.08) was statistically highly significant compare to the after radiotherapy mean MDA level in WDSC (0.72 ± 0.09), MDSC (0.77 ± 0.11) and PDSC (0.80 ± 0.05) simultaneously (Table 2).

In oral squamous cell carcinoma patients with antioxidant therapy before radiotherapy the mean MDA level in WDSC (0.56 ± 0.17), MDSC (0.595 ± 0.10), and PDSC carcinoma (0.64 ± 0.08) was statistically significant compare to the after radiotherapy mean MDA level in WDSC (0.80 ± 0.05), MDSC (0.78 ± 0.18) and PDSC (0.77 ± 0.11) simultaneously (Table 2).

The t-values in oral carcinoma patients before radiotherapy in WD vs MD (0.3564), MD vs PD (0.4236), WD vs PD (1.338) and after radiotherapy in WD vs MD (0.9441), MD vs PD (1.8554), WD vs PD (0.6365) without antioxidant therapy was statistically was nonsignificant (Table 4).

The t-values in oral carcinoma patients before radiotherapy in WD vs MD (0.3588), MD vs PD (0.30), WD vs PD (0.60) and after radiotherapy in WD vs MD (0.2769), MD vs PD (0.4399), WD vs PD (0.026) with antioxidant therapy was statistically nonsignificant (Table 4).

**SUMMARY AND CONCLUSION**

Thus, it can be concluded that the mean serum MDA level increases in the oral squamous cell carcinoma patients as compared to the healthy individuals. This level further increases after the radiotherapy which indicates more damage to the cellular structure from free radicals lead to oxidative stress. This oxidative stress (increase serum MDA) again suggests the requirement of an adjunct therapy21 to treat the oral squamous cell carcinoma along with radiotherapy to reduce the chances of secondary cancer. No definite conclusion about a causal connection between vitamin E and the occurrence of cancer can be drawn until the final result on large scale investigation are done. So a clinical trial on large sample size is required to study the effect of antioxidant on lipid peroxides level in oral squamous cell carcinoma patients, undergoing radiation therapy.
OBSERVATIONS AND RESULTS

Table 1: Serum malondialdehyde (MDA) level in control group and oral squamous cell carcinoma patients before and after radiotherapy without antioxidant therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Mean malondialdehyde level ± standard deviation µmol/l</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>0.3084 ± 0.1016</td>
<td></td>
</tr>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>20</td>
<td>0.598 ± 0.1609</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>20</td>
<td>0.792 ± 0.1157</td>
<td></td>
</tr>
</tbody>
</table>

Where, Group I = control group; Group II = study group consisting of, Group A<sub>1</sub> = oral squamous cell carcinoma patients planned to undergo radiotherapy without antioxidant therapy, Group A<sub>2</sub> = oral squamous cell carcinoma patients after radiotherapy without antioxidant therapy

Graph 1: Site distribution in oral squamous cell carcinoma patients

Table 2: Serum malondialdehyde (MDA) level in control group and oral squamous cell carcinoma patients before and after radiotherapy with antioxidant therapy

<table>
<thead>
<tr>
<th>Group</th>
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<tbody>
<tr>
<td>I</td>
<td>20</td>
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<tr>
<td>B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>20</td>
<td>0.596 ± 0.0952</td>
<td>p &lt; 0.001</td>
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<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>20</td>
<td>0.763 ± 0.0953</td>
<td></td>
</tr>
</tbody>
</table>

Where, Group I = control group; Group II = study group consisting of, Group B<sub>1</sub> = oral squamous cell carcinoma patients before radiotherapy to undergo antioxidant therapy, Group B<sub>2</sub> = oral squamous cell carcinoma patients after radiotherapy with antioxidant therapy

Graph 2: Serum malondialdehyde (MDA) level in control group and oral squamous cell carcinoma patients before and after radiotherapy without antioxidant therapy

Graph 3: Serum malondialdehyde (MDA) level in control group and oral squamous cell carcinoma patients before and after radiotherapy with antioxidant therapy

Table 3: Serum malondialdehyde (MDA) level in oral squamous cell carcinoma patients after radiotherapy with and without antioxidant therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Mean malondialdehyde level ± standard deviation µmol/l</th>
<th>p-value</th>
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<tr>
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<td>20</td>
<td>0.763 ± 0.0953</td>
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Where, Group A<sub>2</sub> = oral squamous cell carcinoma patients after radiotherapy without antioxidant therapy; Group B<sub>2</sub> = oral squamous cell carcinoma patients after radiotherapy with antioxidant therapy

Graph 4: Serum malondialdehyde (MDA) level in oral squamous cell carcinoma patients after radiotherapy with and without antioxidant therapy

Table 4: A t-value in control and study groups

<table>
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<tr>
<th>Test used</th>
<th>GI&lt;sub&gt;V&lt;/sub&gt;A&lt;sub&gt;1&lt;/sub&gt;</th>
<th>GI&lt;sub&gt;V&lt;/sub&gt;B&lt;sub&gt;1&lt;/sub&gt;</th>
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<th>A&lt;sub&gt;2&lt;/sub&gt;V&lt;sub&gt;A&lt;/sub&gt; − B&lt;sub&gt;2&lt;/sub&gt;</th>
<th>GI&lt;sub&gt;V&lt;/sub&gt;A&lt;sub&gt;2&lt;/sub&gt;</th>
<th>GI&lt;sub&gt;V&lt;/sub&gt;B&lt;sub&gt;2&lt;/sub&gt;</th>
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<td>t-value</td>
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<td>0.95</td>
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<td>14.78</td>
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<tr>
<td>p-value</td>
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<td>HS</td>
</tr>
</tbody>
</table>

Where, GI = control group; A<sub>1</sub> = oral squamous cell carcinoma patients before radiotherapy; A<sub>2</sub> = oral squamous cell carcinoma patients after radiotherapy without antioxidant therapy; B<sub>1</sub> = oral squamous cell carcinoma patients before radiotherapy to undergo antioxidant therapy; B<sub>2</sub> = oral squamous cell carcinoma patients after radiotherapy with antioxidant therapy; P-t = paired student’s test; U-t = unpaired student’s test; HS = highly significant; NS = nonsignificant
ACKNOWLEDGMENT

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