Evaluation of glutathione in oral squamous cell carcinoma

Sharma SM¹ · Muralee Mohan² ·
Suchetha Kumari³ · Shreyas J
Sorake⁴ ✉

¹ Professor and Head of Department

² Associate Professor

⁴ Postgraduate Student

Dept. of Oral and Maxillofacial
Surgery, A.B. Shetty Memorial Institute
of Dental Sciences, Mangalore

³ Professor, Dept. of Biochemistry,
A.B. Shetty Memorial Institute of Dental
Sciences, Mangalore

Received: 07 February 2008 / Accepted: 10 March 2009

© Association of Oral and Maxillofacial Surgeons of India 2009

Abstract

Purpose To estimate the RBC level of glutathione among oral squamous cell carcinoma cases and normal healthy individual. The study compares the pre and postoperative blood levels of glutathione in patients with oral squamous cell carcinoma.

Materials and method In the present study a total of 45, age matched subjects were recruited. The 45 subjects were split into 15 subjects in control, 30 clinically and histopathologically diagnosed cases of oral squamous cell carcinoma who had not received any prior treatment.

Results A statistically significant difference of mean value of glutathione levels in RBC of the control group was found to be higher than that of oral squamous cell carcinoma patient group. A noticeable rise in glutathione concentration in RBC of oral squamous cell carcinoma towards normal was seen postoperatively, however these variations were not statistically significant.

Conclusion Pre and postoperatively a marked variation in levels of glutathione was observed. In addition, it was noticed that the levels tended to shift closer to control/normal values postoperatively. The value of glutathione as a prognostic marker could be ratified if the study was carried out on a larger scale over a sustained period of time.

Keywords Glutathione · Antioxidant · Squamous cell carcinoma

Address for correspondence:

Shreyas J Sorake

Postgraduate Student

Dept. of Oral and Maxillofacial Surgery
A.B. Shetty Memorial Institute of Dental
Sciences
Mangalore, India

Introduction

Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, and immune system decline. Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases. Free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is when the availability of antioxidants is limited that this damage can become cumulative and debilitating. To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, that function interactively and synergistically to neutralize free radicals. These include antioxidants like glutathione, ceruloplasmin, lipoic acid, ascorbic acid (Vitamin C), tocopherols and tocotrienols (Vitamin E), carotenoids [2]

etc. Glutathione, an important water-soluble antioxidant and is synthesized from the amino acids glycine, glutamate and cysteine. When an individual is exposed to high levels of xenobiotics, more glutathione is utilized for conjugation (a key step in the body's detoxification process) making it less available to serve as an antioxidant. 4 oxidants are capable of stimulating cell division, which is a critical factor in mutagenesis. A mutation can arise which in turn is an important factor in carcinogenesis [5]. It is believed that antioxidants exert their protective effect by decreasing oxidative damage to DNA and by decreasing abnormal increases in cell division. Some research has indicated that people who have oral cancer like oral squamous cell carcinoma tend to have decreased serum levels of glutathione than healthy individuals [2]. In the present study, the concentration of glutathione (thiol) in blood samples from patients with oral squamous cell carcinoma were determined to gauge the response of

body's antioxidant status against oxidative stresses [6].

Materials and methods

The material for the present study comprised a total of 45 subjects. Individuals selected were in the range of 30–80 years. Institutional ethical clearance was obtained for the study and informed consent was obtained from each subject recruited in the study. The subjects were grouped into two groups; Group I-15 healthy subjects in control group without any habit and oral lesions. Group II-30 patients who were clinically and histologically diagnosed with squamous cell carcinoma of the oral cavity and who had not received any previous treatment. Readings of group II were again taken at postoperatively at 1st month and 6th month respectively from all these 30 patients, biopsies were taken from the deepest and most prominent part of the lesion and were subjected to histopathological evaluation of haematoxylin and eosin. These stained

sections were graded according to Bryne's invasive front grading system [2,3,4].

Method for estimation of glutathione in blood samples: – 4.0 ml of the venous blood from each subject was drawn from the medial cubital vein by using 24 gauge needles in a sterile disposable syringe set under aseptic measures. The blood was then transferred to 0.5 M-EDTA - containing bottles to prevent coagulation. 4ml of blood is added to a test tube containing 2.5 ml of histopaque-1077 (sigma) and then centrifuged for 20 minutes at 3000 rpm. Erythrocytes were separated out carefully and preserved in cryovials at -80°C in refrigerator.

Procedure

1. 0.1 ml of erythrocytes were taken into test tube and added to 1.9 ml of distilled water.
2. Then 3 ml of precipitating solution was added to it, centrifuged for 8 minutes and filtered thereafter.
3. 2 ml of filtrate was taken and mixed with 8 ml of phosphate solution.
4. Then, 1 ml of DTNB solution is added to the whole mixture. Yellowish color appeared.
5. Optical density of the solution was then measured within 10 minutes at 412 nm using a spectrophotometer.

This method is based upon the development of a relatively stable yellow color when 5, 5' - dithiobis (2-nitrobenzoic acid) is added to sulfhydryl compounds including glutathione. In this invasive front tumor five different histological features are graded and assigned points from 1 to 4 (refer annexure-2), the score for each variable is summed to provide a total malignancy score for each tumor. The scores from 4–9, 10–15, 16–20 would represent for well, moderately and poorly differentiated oral squamous cell carcinomas, respectively

Results

In the present study the mean of glutathione concentration values in erythrocytes for oral squamous cell carcinoma patients was 78.40050 and for control group was 90.87280 (µg/ml). There is a difference of 12.472 i.e., 14.44% between control and patient samples as shown in Table-2. Statistical analysis on comparing control vs patient samples was obtained by using T-test. The value for t is 6.086 and it is very significant as the p value is <0.001. In

Test & Control-Unpaired t-test

Table 1 Group statistics

	Group	N	Mean	Std. Deviation	Std. Error mean
Glutathione value (ug/ml)	Control	15	90.87280	5.10780	1.31883
	Oral squamous cell carcinoma	30	78.40050	7.04795	1.28677

Table 2 Independent samples test

t-test for equality of means						
	t	df	P-value	Mean difference	Std. Error difference	95% confidence interval of the difference Lower Upper
Glutathione value (ug/ml)	6.086	43	.000	12.47230	2.04927	8.430 16.605

Table 3 Gutathione value (ug/ml)

	Total no. of patients	Mean Glutathione value (ug/ml)
Control	15	90.87280
Well differentiated	9	80.55400
Moderately differentiated	18	77.63072
Poorly differentiated	3	76.55867

Table 4 Mean glutathione concentration

	Sex	Total no. of patients	Mean Glutathione value (ug/ml)
Control	Male	8	93.26938
	Female	7	88.13386

Table 5 Patient samples

	Sex	Total no. of patients	Mean glutathione value (ug/ml)
Oral squamous cell	Male	23	78.96035
Carcinoma patient	Female	7	76.56100

Paired T-Test

Table 6 Paired samples statistics

	Mean	N	Std. Deviation	Std. Error mean
Preoperative	78.4013	30	7.0479	1.2868
Postoperative 1 month	76.9763	30	7.9126	1.4446
Postoperative 6 months	80.0030	30	4.5878	.8376

Table 4, the mean glutathione concentrations are presented for males and females in the study. In control samples, there were 8 males and 7 females. The mean value for male and female group is 93.269 and 88.133 respectively. So the difference between two groups is of 5.136 i.e., 4.26%. In Table - 5 patient samples, there were 23 males and 7 females. The mean value for

male and female group is 78.960 and 76.561 respectively. The difference between two groups is of 2.399 i.e., 2.54%. But on statistical analysis the difference was found to be insignificant. So there is no gender predilection. Patients were scored in the range of 4–9, 10–15, >15 histopathologically according to the Bryne's invasive front grading system. Our

study has 9 patient samples with scores in the range of 4–9, 19 patient samples with scores in the range 10–15 and 2 patient sample with scores >15. So the mean glutathione concentration value for well differentiated (i.e., 4–9), moderately differentiated (i.e., 10–15) and poorly differentiated (i.e., >15) is 80.554, 77.630 and 76.558 respectively (Table 3). On statistical analysis the comparison is found to be insignificant. Post surgically proper antibiotic coverage and diet care was observed and were followed up at regular 1 month intervals. Patients were examined for any signs of recurrence post surgically their GSH levels were recorded at 1 month and 6 month intervals. At the end of one month there was a considerable fall in the level of GSH from 78.401 to 76.976 with a mean of 1.425, std deviation 6.5206, but statistically it was not to be significant as the *p* value remained at 0.241 patients received regular follow up and counselling regarding the habit breaking as well as psychological and nutritional advice so as to continue life normally as before. At the end of six months post operatively the blood GSH values were again assessed. It was observed that the GSH values post operatively had risen towards normal, with a mean value of 80.003. A difference in means 3.0267 was found compared to the 1 month postoperative and a mean difference of -1.6017 as compared to pre op values was observed as seen in Table 7. *P* value was 0.301 and 0.053 respectively which was statistically insignificant.

Discussion

The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins and carbohydrates for energy; however, it does not come without a cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules (ROS) commonly called free radicals. Free radicals are capable of attacking healthy cells of the body, causing them to lose their structure and function and may transform normal cells to malignant one. Cell damage caused by free radicals appears to be a major contributing factor for carcinogenesis. Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to deactivate themselves. Antioxidants such as glutathione, ceruloplasmin, alpha tocopherol etc are capable of stabilizing,

or deactivating, free radicals before they attack normal cells and prevent their conversion to cancer cells. In cancer there is an enormous production of free radicals in the system. Dormandy had proposed a close relationship between free radical activity and malignancy [9]. It must be noted that, Beutler and Kelly's method measures the total thiols including GSH.

However it has been recognized that the levels of total thiols fairly well correlate with levels of GSH [5]. The values of total thiols was assumed to represent glutathione. In the present study the mean glutathione concentration in erythrocytes was estimated and the value was found to be higher in control group than patient samples as shown in Table 3.

Table 7 Paired samples test

	Paired differences					t	df	P-value
	Mean	Std. Deviation	Std. Error mean	95% confidence interval of the difference				
				Lower	Upper			
Preoperative - Postoperative 1 month	1.4250	6.5206	1.1905	-1.010	3.8598	1.197	29	.241
Preoperative - Postoperative 6 months	-1.6017	8.3222	1.5194	-4.709	1.5059	-1.054	29	.301
Postoperative 1 month - Postoperative 6 months	-3.0267	8.2060	1.4982	-6.091	.0375	-2.020	29	.053

The OSCC patients were graded histopathologically as well differentiated, moderately differentiated and poorly differentiated. The mean erythrocyte glutathione concentration was found to be 80.554 in well differentiated (9 patients), 77.630 in moderately differentiated (18 patients) and 76.558 in poorly differentiated (3 patients). Irrespective of the grades of OSCC, the values of GSH for control were found to be higher than the patient samples. The values for moderately and poorly differentiated squamous cell carcinoma were less than well differentiated [14]. This indicates that with progression of the disease, there is corresponding decrease in GSH concentration from well differentiated to poorly differentiated OSCC. In two patients of poorly differentiated OSCC that were studied, the levels were found to be more or less similar to that of moderately, so it can either be stated that the levels reached a steady state with prolonged illness or it may be the sample size is less. No definite conclusion can be drawn with regard to difference in GSH level concentration from moderately to poorly differentiated oral squamous cell carcinoma [15].

Sometimes problem lies with the histopathological grading, as the repeated biopsy of the same patient may lead to higher grade than the previous one because invasion may vary from site to site and depth of tissue. Sometimes within the same block, grades might vary among superficial and deep sections. However in the present study we tried to take deeper sections and the deeper biopsy from the most prominent part of the lesion. The decrease in the GSH levels may be due to increased turnover of GSH for preventing oxidative damage in these patients. They have observed that GSH levels were mainly reduced in poorly, differentiated tumors than in well and moderately differentiated tumors. Reduced antioxidant levels in oral cancer were reported in various stages of oral cancer.

Lower levels of GSH causes overproduction of free radicals and lipid peroxides which in turn may induce damage to the cell membrane and cellular molecules (DNA, RNA) leading to neoplasia. Similarly lower erythrocyte GSH levels in cervical cancer patients were reported by [7]. In contrast to decreased lipid peroxidation in tumor tissues, enhanced lipid peroxidation was associated with antioxidant depletion in the venous blood of OSCC patients [8,10,12]. Erythrocytes are exposed continuously to oxidative stress. Although the reducing capacity of the normal erythrocytes is greater than its oxidizing potential, lack of antioxidant defense lead to an increase in membrane lipid peroxidation. In particular, the erythrocytes are susceptible to ROS-induced lipid peroxidation due to continuous exposure to oxygen tension, and high content of PUFA, a target for peroxidation as well as iron, a potent catalyst for the lipid peroxidation reaction. Antioxidant depletion in blood may be due to increased scavenging of lipid peroxides as well as sequestration by tumor cells. Tumor cells have been reported to sequester essential antioxidants such as GSH to meet the increased demands of a growing tumor

[8]. The low levels of antioxidants in the blood of oral cancer patients may be due to their increased utilization to scavenge lipid peroxides as well as their sequestration by tumor cells. The enhanced antioxidant [17] capacities in tumor tissues can make them less susceptible to oxidative stress, conferring a selective growth advantage on tumor cells. These finding suggest that normalization of the levels of these antioxidants might be used to reduce oral tumor malignancy [10].

Summary and Conclusions

There are three main modes of treatment that for cancer i.e. surgery, radiation and chemotherapy. All these treatment procedures involve production of free radicals at cancer site and killing cancer cells. However these treatment modalities tend to have side effects on normal cells, their antioxidant level including GSH level get lowered for the normal cells to do their function normally.

From the present study following conclusions can be derived:

1. The mean glutathione concentration in RBC of control group is found to be

higher than that of OSCC patient group and is statistically significant.

2. The mean glutathione concentration difference in RBC among males and females in both control and patient group is found to be statistically insignificant.
3. The mean glutathione concentration in RBC of histopathologically diagnosed well differentiated squamous cell carcinoma patients is found to be more than that of moderately and poorly differentiated oral squamous cell carcinoma patients. So as the oxidative stress increases the GSH level decreases.
4. The mean glutathione levels 1 month post surgery is reduced, this marginal difference could be due to the stresses experienced by the patient post surgery. However the difference is not statistically significant.
5. The mean glutathione levels 6 month post surgery showed an upward rise towards normal. However the difference was found to be not statistically significant.

It must be noted that the patients recruited here were drug naive, and received no treatment. The levels of GSH are due to the cancerous growth and this cannot be attributed to treatment procedures. The lowered GSH levels seen in RBC has to be attributed to the growth of OSCC and sequestration of GSH in the cancer cells. A marked variation in the levels of glutathione was observed preoperative and postoperatively. These tendencies could be further ratified by carrying out studies on a larger sample size over sustained period of time.

References

1. Acworth IN, Bailey B: The handbook of oxidative metabolism. ESA, Inc. chelmsford; 1995
2. Sawair FA, Irwin CR, Gordon DJ, Leonard AG, Stephenson M, Napier SS (2003) Invasive front grading: reliability and usefulness in management of oral squamous cell carcinoma. *J Oral Pathol Med* 32(1): 1–9
3. Bryne M, Koppang HS, Lilleng R, Kjaerheim A (1992) Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. *J Pathol* 166(4): 375–381
4. Bryne M, Nielsen K, Koppang HS, Dabelsteen E (1991) Reproducibility of two malignancy grading systems with reportedly prognostic value for oral cancer patients. *J Oral Pathol Med* 20(8): 369–372
5. Jaswal S, Mehta HC, Sood AK, Kaur J (2003) Antioxidant status in rheumatoid arthritis and role of antioxidant therapy. *Clin Chim Acta* 338(1–2): 123–129
6. Uikay AK, Hazarey VK, Vaidhya SM (2003) Estimation of serum antioxidant enzymes superoxide dismutase and glutathione peroxidase in oral submucous fibrosis: a biochemical study. *J Oral Maxillofac Pathol* 7(2): 44–45
7. Upadhyaya S, Upadhyaya S, Krishna Mohan et al. (2004) Oxidant antioxidant status in colorectal cancer patients-before and after treatment. *Indian J Clin Biochem* 19(2): 80–83
8. Subapriya R, Kumaraguruparan R, Ramachandran CR, Nagini S (2002) Oxidant-antioxidant status in patients with oral squamous cell carcinoma at different intraoral sites. *Clin biochem* 35(6): 489–493
9. Dormandy TL (1983) An approach to free radicals. *Lancet* 2(8357): 1010–1014
10. Fiaschi AI, Cozzolino A, Ruggiero G, Giorgi G (2005) Glutathione, ascorbic acid and antioxidant enzymes in the tumor tissue and blood of patients with oral squamous cell carcinoma. *Eur Rev Med Pharmacol Sci* 9(6): 361–367
11. Yilmaz YF, Akbiyik F, Tuncel U, Unal A (2007) Lipid peroxidation and antioxidant levels in patients with laryngeal carcinoma. *Kulak Burun Bogaz Ihtis Derg* 17(2): 81–84
12. Sharma A, Rajappa M, Saxena A, Sharma M (2007) Antioxidant status in advanced cervical cancer patients undergoing neoadjuvant chemoradiation. *Br J Biomed Sci* 64(1): 23–27
13. Slater TF (1984) Free radical mechanisms in tissue injury. *Biochem J* 222(1): 1–15
14. Krishnamurthy S, Jaya S (1986) Serum-tocopherol, lipo-peroxides, and ceruloplasmin and red cell glutathione and antioxidant enzymes in patients of oral cancer. *Indian J Cancer* 23(1): 36–42
15. Ketterer B (1988) Protective role of glutathione and glutathione transferase in mutagenesis and carcinogenesis. *Mutat Res* 202(2): 343–361
16. Todd R, Donoff RB, Wong DT (1997) The molecular biology of oral carcinogenesis: Toward a tumor progression model. *J Oral Maxillofac Surg* 55(6): 613–623
17. Sabitha KE, Shyamaladevi CS (1999) Oxidant and antioxidant activity changes in patients with oral cancer and treated with radiotherapy. *Oral Oncol* 35(3): 273–277
18. Navarro J, Obrador E, Carretero J, Petschen I, Avino J, Perez P, Estrela JM (1999) Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth in vivo. *Free Radic Biol Med* 26(3–4): 410–418
19. Beutler E, Duron O, Kelly BM (1963) Improved method for determination of blood glutathione. *J Lab Clin Med* 61: 882–888
20. Sarah Goodwin (2000) Emory university health sciences centre Oral exposure to antioxidant glutathione could help prevent flu infections. This article comes from Science Blog.

Source of Support: Nil, **Conflict of interest:** None declared.