

Evaluation of salivary antioxidants – Superoxide Dismutase and Glutathione Peroxidase in OSMF patients and healthy individuals – A comparative study

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Type of Publication: Original Research Paper

Conflicts of Interest: Nil

Abstract

Background: Saliva is the first biological medium that is encountered during chewing. Saliva provides a natural defence against harmful substances and microorganisms. Research confirms that antioxidants are among the most important elements in saliva as they help in protecting against oral diseases like premalignant lesions, conditions and cancer. Hence, they are vital to tissue health and healing.

Aims and objectives: The aim of this study is to evaluate the two salivary antioxidants i.e superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and compare their levels in saliva of clinically diagnosed OSMF cases and healthy individuals.

Materials and methods: The study sample consists of 40 individuals.

Group 1: 20 healthy individuals without any habit.

Group 2: 20 individuals with clinically diagnosed OSMF cases.

Unstimulated saliva of individuals is collected. The saliva is centrifuged and the supernatant is taken and activity of

two salivary antioxidant enzymes is measured according to specific assay on the spectrophotometer.

Statistical analysis: The mean values of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the saliva of healthy and OSMF patients are analysed using Student 't' test.

Results: The mean values of the control group and OSMF for salivary superoxide dismutase (SOD) are 0.0413 and 0.0858 respectively. The mean values of the control group and OSMF for salivary glutathione peroxidase (GSH-Px) are 0.1 and 0.0024 respectively. Salivary superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels between the healthy and OSMF groups shows high a significant difference.

Keywords: Antioxidant enzymes, SOD, GSH-Px, saliva, OSMF.

Introduction

Saliva is the first biological fluid that is encountered during chewing or smoking. It provides a natural defence against bacteria and other substances like carbon monoxide, hydrogen cyanide, benzopyrene and cytotoxic

oxygen radicals that are harmful to health.¹ Oxidation is a chemical response that delivers free radicals, prompting chain reactions that may harm cells. An antioxidant is an agent that restrains the oxidation of other molecules. Antioxidants are available in all body fluids and tissues and protect against endogenously formed free radicals.² Research confirms that antioxidants are among the most important elements in saliva as they help in protecting against oral diseases like potentially malignant disorders and malignancies.³

Cigarette smoking and tobacco chewing are well known to be the major cause for various health disorders like respiratory disorders, cardiac diseases, potentially malignant disorders, carcinomas etc because tobacco contains carbon monoxide, hydrogen cyanide as their toxic components.¹

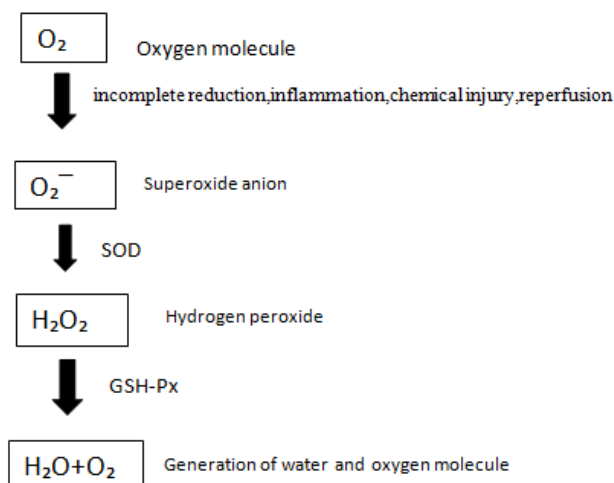
Reactive oxygen species assume an imperative part in cell signalling and metabolic process and add to pathogenic process in an assortment of inflammatory disorders. All organisms have a scope of enzymatic and non-enzymatic oxidant systems to shield them from harmful oxidative reactions. Under specific conditions, an increase in oxidants and decrease in antioxidants cannot be counteracted, and the oxidant and antioxidants balance shifts towards oxidative state (loss of electrons) O_2^- .^{2,3}

The levels of toxic components rely upon balance between the rates of production and the rates of clearance by endogenous antioxidant systems like superoxide dismutase, glutathione peroxidase, catalase, cycling enzymes, glutathione peroxidase and reductase and glutathione itself.¹ One of the principal reactive oxygen species produced in aerobic organism is oxidant O_2^- (superoxide anion) which is exceptionally cytotoxic. With the cytotoxicity of this oxidant, exposure to tobacco results in increased levels of antioxidant enzymes such as catalase [CAT], peroxidase [Pox], Cu/Zn superoxide

dismutase[SOD] and glutathione peroxidase [GSH-Px].¹ The higher reactive O_2^- is converted to H_2O_2 by SOD. SOD is an antioxidant enzyme that acts against superoxide oxygen radical discharged in inflammatory pathways whereas CAT, POx or GSH-Px are the enzymes that change over H_2O_2 to molecular oxygen and water.¹ These systems have a tendency to keep up stable state called redox homeostasis by scavenging the antioxidants under physiologic conditions, promoting immunity and hence, the antioxidants play an important role in preventing carcinogenesis.^{1,2}

The present study was taken up to measure and compare the effects and levels of SOD and GSH-Px in healthy and OSMF individuals.

Flow chart of reactions taking place due to tobacco



The higher reactive O_2^- (superoxide anion) is converted to H_2O_2 by SOD. SOD is an antioxidant enzyme that acts against superoxide oxygen radical released in inflammatory pathways Whereas GSH-Px is the enzymes that in turn convert H_2O_2 to molecular oxygen and water.¹ Superoxide dismutase converts $O_2^- \rightarrow H_2O_2$ Glutathione peroxidase converts $H_2O_2 \rightarrow H_2O + O_2$.

Materials and methods: 40 patients between the age group of 20-40 years were randomly selected for the study. Healthy individuals without any habit and systemic

disorders and clinically diagnosed OSMF cases [Ranganathan et al 2001 criteria] were included in the study. Group 1 comprised of 20 healthy individuals without any habit and without any systemic diseases as controls and group 2 comprised of 20 individuals with clinically diagnosed OSMF and no systemic disease. Patients with other potentially malignant disorders, established carcinoma cases, patients with any systemic disorders were excluded from the study.

Collection of saliva samples: Unstimulated whole saliva sample was used in the study. After rinsing the mouth with plain water to remove food debris or other particles and exfoliated cells, the patients were asked to pool the saliva in the bottom of mouth and to spit in sterile containers provided to them. Approximately 2ml of saliva was collected. The required assays were performed and the biochemical values were analysed and subjected to statistical analysis using Student 't' test. The values were estimated in spectrophotometer.

Salivary superoxide dismutase analysis: It was assayed by Fridovich and Misra method (1972).

1ml of unstimulated saliva was taken from sterile container and pipetted into Ependorff's tubes. Around 0.25 volume of chilled ethanol and 0.15 volume of ice cold chloroform was added to it. Mixture was shaken well for 1 min and centrifuged at 3000 rpm at 4⁰C for 5 min. Supernatant was taken for enzyme assay. To this 1ml of supernatant sample, 0.5 ml of sodium carbonate buffer [0.1M, pH 10.2] and 0.5ml of EDTA [0.1mM] were added. Distilled water was added to this mixture to make final volume of 2.5ml. Mixture was pipette into a cuvette, to which 0.5ml epinephrine [1mM] is added to initiate reaction. Reaction was monitored in UV spectrophotometer at 12 sec interval for 1 min at 480nm and 25⁰C. The change in absorbance is due to inhibition of conversion of epinephrine to adrenochrome.¹ The ability

of SOD to inhibit auto oxidation of adrenaline to adrenochrome at pH10.2 was the basis for this assay. The superoxide anion(O₂⁻), which is the substrate for the SOD enzyme, is generated indirectly by the oxidation of epinephrine by oxygen in an alkaline pH.¹ The SOD enzyme reacts with the O₂ formed during epinephrine oxidation, and therefore slows the rate and the adrenochrome formation.³

Salivary glutathione peroxidase analysis: It was assayed by Rotruck et al method (1973).

1ml of unstimulated saliva was taken from sterile container and pipetted in the Ependorff's tubes. Saliva was centrifuged at 2500 rpm for 5 min. Supernatant was stored at 4⁰C till the tests were performed. To 1ml of supernatant saliva, 0.5ml of Tris HCl buffer [0.01M, pH 7.4] was added. They were homogenised and then centrifuged at 10000 rpm for 10 min. The supernatant homogenate was used for further analysis. The reaction mixture consists of 0.2ml homogenate, 0.2ml EDTA [0.8mM], 0.2ml sodium phosphate buffer [0.4M, pH 7], 0.1 ml sodium azide [10mM], 0.2ml reduced glutathione [1mM] and 0.1ml hydrogen peroxide [2.5mM]. 1ml of distilled water was added and a total volume of 2ml mixture was prepared and incubated at 37⁰C for 10 min. 0.5ml of 10% Trichloroacetic acid [TCA] was added to stop the reaction. The mixture was centrifuged at 3200 rpm for 20 min. 1ml of the Supernatant was assayed for glutathione content by adding 1ml of Di-thiobis-nitro benzoic acid [(DTNB)/(ELLMAN's reagent)] [0.6mM] and 3ml of disodium hydrogen phosphate solution [0.3m] to it. The colour changes were read immediately on spectrophotometer at 420nm.¹

Statistical analysis: The data obtained were subjected to statistical analysis. The mean and standard deviation was determined using Descriptive statistics. The mean values of SOD and GSH-Px between saliva of healthy and OSMF

patients were analysed using Student 't' test . Test is significant when p value < 0.05.

Discussion: A great deal of research into detrimental effects of tobacco has inferred that it has a broad systemic effects many of which may provide mechanisms that increase an individual's susceptibility to diseases and affect their response to treatment by stimulating destructive/inflammatory responses and disabling protective/reparative responses. Tobacco constituents can exacerbate aspects of the respiratory burst and enhance reactive oxygen species(ROS)/ superoxide anion (O_2^-)oxidant production.³ Free radical scavengers may assume the form of enzymes (ex. superoxide dismutase and glutathione peroxidase) or low molecular weight free radical scavengers (ex. Vit E, total Thiol, glutathione etc.). A similar study was done by Saggu et al and he concluded that in smokers the level of SOD is increased compared to non-smokers and level of GSH-Px is decreased in smokers when compared with non-smokers.¹

Our study is also in accordance with a study by Kanehira et al in which a comparison of salivary antioxidant enzyme levels in elderly smokers and non-smokers which showed significant increase in levels of SOD and significant decrease in GSH-Px among smokers.⁴ Saliva, in addition to its cleansing and lubricating properties, constitute a first line of protection against free radical-mediated oxidative stress. Studies evaluating the effects of tobacco consumption on SOD and GSH-Px activity have suggested that tobacco consumption increases the SOD activity and decreases the GSH-Px activity.⁴

Our study is in accordance with Bray et al suggesting free radical particularly hydrogen peroxide are produced by direct interaction between smoke and saliva that will lead to significant change in antioxidant enzyme SOD.⁵

Work done on the status of antioxidants like glutathione peroxidase and reduced glutathione in saliva proved that

reduced glutathione is a scavenger of hydroxyl radicals and singlet oxygen. It works as a substrate for the hydrogen peroxide eliminating enzyme, glutathione peroxidase. Our present study revealed that a reduction of glutathione levels in OSMF patients was exhibited. This is in accordance with the study done by Tribble and Jones who reported that exposure to extensive flux of hydrogen peroxide and hydroxyl radicals results in imbalance in glutathione ratio.⁶

Work done by Guica et al showed a noteworthy reduction of GSH-Px activity in the smoking group, while the SOD action was comparable in the control and case groups. As per the sex, a noteworthy diminishing of GSH-Px activity was noted in males of the smoker group, while in the specimen of females no critical contrast of the enzymatic action was found. Also, among ex-smokers, there was huge contrast in the estimations of GSH-Px between the individuals who did not smoked for under ten years and the people who did not smoke for over ten years. Tobacco smoke may change the detoxification of hydrogen peroxide through a decreasing of GSH-Px action. The overproduction of H_2O_2 may prompt an oxidative stress that is incorporated in a large number of diseases, including precancerous and neoplastic injuries of the oral cavity. The impacts of tobacco smoke on salivary antioxidant enzymes diminish after withdrawal from smoking and the advantages turn out to be more clear with the progression of time.⁷

Greabu et al conducted a similar study and they found that there the exposure to Cigarette Smoke caused a factually noteworthy decline of salivary glutathione peroxidase in the body. They likewise discovered that Cigarette Smoke caused a critical decline of salivary total antioxidant status. Such reductions may have a unflinching part in the systems by which the harmful impacts of Cigarette Smoke initiate provocative or oral inflammatory diseases,

advances precancerous changes, and obliterate the oral cavity homeostasis.⁸

Farmand et al⁹ conducted a study on rats and they found that results obtained in their study showed significant upregulation of Cu/Zn SOD catalytic activity. This study is in concordance with Okamoto and Colepicolo¹⁰, who found that smoke exposure raised SOD activity.

All cells in eukaryotic organisms contains effective antioxidant enzymes. The three major classes of antioxidant enzymes are the superoxide dismutases, catalases and glutathione (GSH) peroxidases. Furthermore, there are various particular antioxidant enzymes responding with and, in general, detoxifying oxidant compounds.

According to Sies et al in OSMF patients, the obligatory utilization of reserve antioxidants to detoxify the overabundant free radicals, results in alteration of levels of various antioxidants.¹

Baharwand et al while considering the role of superoxide dismutase (SOD) as one of the most important salivary antioxidant enzymes, conducted study to analyse the level of this enzyme in smokers and non-smokers and he found that Cigarette smoke leads to a rise in salivary superoxide dismutase activity.¹²

Moore et al conducted a study on the antioxidant activity of saliva with diseases of periodontium due to smoking. The results showed that the major aqueous antioxidant component of whole saliva is uric acid, with lesser contributions from ascorbic acid and albumin. The total antioxidant activity (TAA) of saliva correlates with the concentration of uric acid, which contributes more than 70% of the TAA. Stimulation of salivary flow is related with increase in production of antioxidants. The antioxidant potential of saliva does not appear to be compromised in patients with periodontal disease

however, this may identify with the antioxidant flow from the gingival crevicular fluid because of smoking.¹³

In our present study

Salivary SOD: Mean levels of SOD were significantly higher in saliva of OSMF than healthy individuals. The increased level of SOD in our study confirms several findings in medical literature on oxidant – antioxidant imbalance. The elevated levels showed the protective defence mechanism of body occurring to scavenge and reduce the excessive free radicals produced by smoking and tobacco chewing.¹⁴

Salivary GSH-Px: It acts as a powerful antioxidant defence system which converts H₂O₂ produced by SOD into molecular oxygen and water. There is significant decrease in levels of GSH-Px in saliva of OSMF patients. Detoxification process of H₂O₂ is altered by tobacco through a decline in GSH-Px activity. The excess H₂O₂ production leads to increase consumption of reduced glutathione, thus sufficient amount of reduced glutathione was not available for detoxification of H₂O₂ leading to elevated oxidative stress which in turn leads to numerous diseases of oral cavity.¹⁵

Conclusion

Oxidative stress leads to numerous diseases including potentially malignant and malignant lesion of oral cavity which may be due to altered levels of salivary antioxidant systems that fail to cope up with altered level of oxidative stress originating due to tobacco consumption. Tobacco consumption induces a selective increase of antioxidant enzyme activity in tissues as defence mechanism. This increase is insufficient to protect tissues from the direct harmful effects of tobacco consumption. Excessive SOD produced as a defence mechanism have resulted in overproduction of hydrogen peroxide because of dismutation of the superoxide radical. The H₂O₂ must then be detoxified by the enzyme glutathione peroxidase.

It is possible that the GSH-Px level have been adversely affected by increased SOD level after person is exposed to tobacco. This accumulation of detoxified H₂O₂, which belong to ROS group acts as a constant source of oxidative stress.³ Estimation of antioxidant enzymes in saliva is valuable for evaluating level of oxidative stress caused by cigarette smoke and due to tobacco chewing. The purpose of this study was to measure the antioxidant levels in saliva of OSMF and healthy individuals and to analyse the relation between saliva and free radicals. The study showed high significant difference in salivary superoxide dismutase and salivary glutathione peroxidase enzyme levels among control and study group. Salivary analysis of antioxidant is simple, non-invasive procedure useful as diagnostic, prognostic and therapeutic marker in screening and experimental studies. Although number of samples are small, further studies with large sample size are required for determining its usefulness in this role.

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Sl.no	Age	Gender	SOD	GSH-Px
1	28	F	0.038	0.12
2	27	F	0.068	0.13
3	35	F	0.053	0.12
4	28	F	0.049	0.13
5	29	M	0.045	0.11
6	24	F	0.021	0.16
7	26	F	0.026	0.01
8	25	F	0.033	0.10
9	38	M	0.044	0.02
10	27	F	0.036	0.14

Sl.no	Age	Gender	SOD	GSH-Px
11	26	M	0.027	0.16
12	28	M	0.031	0.16
13	23	F	0.042	0.11
14	22	F	0.036	0.10
15	22	F	0.048	0.08
16	23	F	0.054	0.12
17	24	F	0.049	0.15
18	23	F	0.061	0.02
19	26	M	0.039	0.01
20	27	M	0.041	0.06

Optical Density[OD] of normal healthy individuals

Sl.no	age	gender	grading	SOD	GSH-Px
1	29	M	III	0.117	0
2	32	M	I	0.039	0.001
3	27	M	II	0.067	0.002
4	54	M	IV	0.6	0
5	24	M	II	0.073	0.002
6	24	M	I	0.056	0.003
7	25	M	I	0.055	0.002
8	23	M	II	0.069	0
9	31	M	III	0.127	0
10	48	M	IV	0.192	0

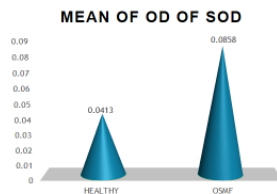
Sl.no	age	gender	grading	SOD	GSH-Px
11	34	M	III	0.127	0
12	27	M	II	0.098	0.002
13	26	M	II	0.089	0.003
14	29	M	II	0.093	0.002
15	29	M	III	0.125	0
16	36	M	III	0.113	0.001
17	38	M	III	0.129	0
18	42	M	III	0.136	0
19	26	M	III	0.145	0
20	29	M	IV	0.156	0

Optical Density[OD] of osmf patients

RESULTS

TABLE I
MEAN AND STANDARD DEVIATION (SD) OF
OPTICAL DENSITY (OD) OF SOD

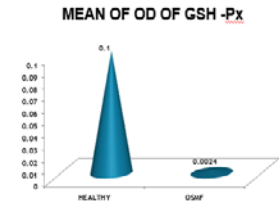
GROUP	MEAN	SD
HEALTHY	0.0413	0.0129
OSMF	0.0858	0.0341
T VALUE (P VALUE)	10.22(P<0.00001) SHOWS SIGNIFICANT DIFFERENCE	



*p-value (< 0.00001): Highly significant; SOD: superoxide dismutase
Mean levels of SOD are significantly higher in saliva of OSMF than healthy individuals

TABLE II
MEAN AND STANDARD DEVIATION (SD)
OF OD OF GSH-Px

GROUP	MEAN	SD
HEALTHY	0.1	0.0506
OSMF	0.0024	0.0022
T VALUE (P VALUE)	8.11(P<0.00001) SHOWS SIGNIFICANT DIFFERENCE	



*p-value (< 0.00001): Highly significant; GSH-Px: Glutathione peroxidase
There is significant decrease in mean levels of GSH-Px in saliva of OSMF patients when compared to healthy individuals