

Characterization of Salivary Glutathione reductase in Normal Individuals and its Implications on Smokers

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Abstract: The assay of saliva is an increasing area of research with implications for basic and clinical purposes. Although this biological fluid is easy to manipulate and collect, careful attention must be directed to limit variation in specimen integrity. In this study, glutathione reductase (GR) activity of saliva obtained from smokers and non smokers of both the sex of various age groups were assessed. The investigation of salivary GR from non smokers revealed a pH optimum of 6.8, temperature optimum as 37°C and a low K_m of 0.058 mM for the substrate (Oxidized glutathione, GSSG). A significant reduction in the salivary GR activity has been observed from smokers of both acute and chronic than the non smokers. A drastic decrease in the GR activity was noticed in chronic smokers than the acute smokers, proving the possibility of utilizing the enzyme as a diagnostic biomarker for detecting the oral, throat and neck cancers. This optimized developed protocol was also found to be simple and cost effective. [Researcher 2010;2(4):74-81]. (ISSN: 1553-9865).

Key words: Glutathione reductase, oral cancer, saliva.

1. Introduction

O₂ is essential for the survival of all in this earth (Mazumder *et al.*, 2006). Approximately 5% of O₂ involved with normal processes like metabolic respiration, strenuous exercise and biotransformation of xenobiotics is responsible for the generation of free radicals or reactive oxygen species (ROS) (Peter Møller and Steffen Loft, 2006). A free radical is a molecule containing one or more unpaired electrons in atomic or molecular orbitals that includes superoxide ions (O₂⁻), hydroxyl radicals (OH[•]) and H₂O₂ (Barry Halliwell, 2009). These ROS may induce oxidative damage to various macromolecules like polyunsaturated fatty acids in cell membranes, carbohydrates, proteins and DNA (Stadtman, 1992), which results in homeostatic imbalance (Bonfont *et al.*, 2000). These events lead to cardiovascular, neurodegenerative diseases, inflammation (Akhilesh Kumar and Sharmila Chattopadhyay, 2007), aging, cancer (Wiseman and Halliwell, 1996), diabetes (Singh *et al.*, 2009) and others. Therefore, the factors that shift the physiological process, to normal homeostatic balance are of considerable interest.

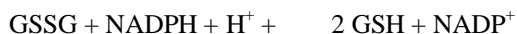
Cigarette smoke (CS) is a complex mixture of over 4700 identified constituents and four hundred of them have been proven to be carcinogens (Maurizio Battino

et al., 2007). Enormous numbers of free radicals or ROS are produced during cigarette smoking (Fatma Fidan *et al.*, 2006). Free radicals in the particulate (tar) of cigarette smoke appear to be a relatively stable semiquinone (Freischlag *et al.*, 1999) and those in the gas phase that contains more than 10¹⁴ low-molecular weight compounds are short-lived carbon, oxygen-centered organic radicals and a high concentration of nitrogen oxides per puff (Pryor, 1997; Fatma Fidan *et al.*, 2006). Among several harmful substances in CS, the most important compound is nicotine that may be acquired through active or passive smoking and is also responsible for the compulsive use of tobacco (Ross Cooper, 2006; Marcela Fu *et al.*, 2009). Nicotine, when smoked in cigarettes is absorbed across buccal and nasal membranes. The drug has a fast onset of action with a half-life of 2 h and can be detected in blood, saliva and urine (Robson *et al.*, 2010). CS, which contains several carcinogens are also known to initiate, promote and metastasis of oral cancer (Nagaraj and Zacharias, 2007). Oral squamous cell carcinoma (SCC) is the most common malignancy of the head and neck, with a worldwide incidence of over 300,000 new cases annually and the major inducers of SCC is the exposure to cigarette smoke, responsible for 50–90% of the cases (Maurizio Battino *et al.*, 2007).

The recent literature has revealed an increasing incidence of SCC of tongue in young adults (Randhawa *et al.*, 2008).

Saliva in humans is a mouth fluid possessing several functions involved in oral health and homeostasis with an active protective role in maintaining oral healthiness. It is a complex secretion whose components exert a well documented role in health and disease (Johan Aps and Luc Martens, 2005). Similar to other biological systems, the salivary antioxidant system includes various molecules and enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), etc., which can be used as biomarkers for diagnosis of various periodontal diseases (Nurdan Ozmeric, 2004). The anticarcinogenic capability of saliva was shown to significantly inhibit the initiation and progression of oral cancer in an animal model. Reports are there for both increased and reduced activities of GPx and glutathione reductase (GR), one of the most important antioxidant defense systems in the body, in different tissues of diabetic animals. Elevated GPx activity was reported in the parotid glands of diabetic rats, whereas reduced activity of the enzyme was found in submandibular glands (Carlos Arana *et al.*, 2006). Sathishkumar *et al.* (2008) has proved that saliva possess a significant antioxidant activity in the non smokers than the smokers of both types (acute and chronic), through various *in vitro* antioxidant assays.

Glutathione reductase (EC 1.6.4.2) is an enzyme responsible for keeping glutathione in its reduced state i.e., it converts the oxidized glutathione (GSSG) into reduced glutathione (GSH).



GR is a member of a group of enzymes known as flavo disulphide oxidoreductases which also contains thioredoxin reductase, mercuric ion reductase and trypanothione reductase (Sylke Müller, 2004). This enzyme is critical in preventing high levels of oxidative stress because its activity can counteract oxidation. GR is also important in the synthesis of DNA precursors as well as proton transport across membranes (Mauro Serafini, 2006).

To our knowledge, among various antioxidant enzymes, salivary GR is an important, least studied enzyme and so far, there is no scientific documentation about its activity in normal individuals and fluctuations in smokers. No reports are available for its limits of detection (LOD). i.e., activity range. In this regard, the present study carried out in our laboratory has been focused in evaluating the various kinetic properties (pH, temperature and K_m) of salivary GR in the non smokers and its activity in smokers of various age groups and to correlate the possibility of occurrence/ incidence of oral cancer among the smokers.

2. Materials and methods

2.1 Preparation of salivary sample

Mixed saliva (about 5 ml) was collected 2–3 h after breakfast from both the smokers and non smokers with the age groups ranging from 15yrs to 60yrs. The sample was centrifuged for about 15 minutes at 16,000 rpm for 5 minutes to remove the cellular components (Sathishkumar *et al.*, 2008). The supernatant obtained was used for GR assay. The sample can be stored at -80°C until further analysis. The activity of GR was measured in both smokers and non smokers (15 yrs to 65 yrs of both genders). The sample size for both categories was fixed as 40.

2.2 Glutathione reductase (GR) assay

A method proposed by Carlberg and Mannervik (1975) and modified by Mohandas *et al.* (1984) was adopted to investigate GR. To 1.65ml of phosphate buffer, added 0.05ml of 1mM GSSG, 0.1ml of 1mM -NADPH and 0.1ml of 0.5mM EDTA. To this reaction mixture added 0.05ml of saliva and vortexed for 10 s. A decrease in the OD/30 seconds for about 3 minutes was monitored at 340nm by using (Beckman DU-530) UV-Vis Spectrophotometer. A blank solution was used without saliva. The enzyme activity was expressed in U/ml.

2.4 Statistical Analysis

All analysis was carried out at least three separate experiments (triplicates). The level of significance was expressed using one way and two way ANOVA. All the analysis was carried out using Sigmastat 3.5 software.

3. Results analysis

Saliva is a complex secretion whose components exert a well documented role in health and disease and its diagnostic use is spreading (Bald and Glowacki, 2005). Moreover, saliva contains various antioxidants, including uric acid, which contributes more than half of the total radical trapping capacity (Moore *et al.*, 1994) and the remaining half by the glutathione and its precursors (Zappacosta *et al.*, 2002). The ubiquitous tripeptide glutathione (GSH) and other amino thiols are also involved in the production of volatile sulphur compounds responsible for the bad breath in periodontopathic patients (Bald and Glowacki, 2005) and generally salivary GSH levels decrease in periodontal diseases (Öztürk *et al.*, 2008). Glutathione associated metabolism is a major mechanism for cellular protection against agents, which generate oxidative stress and lipid peroxidation. Genetic and biochemical evidence has demonstrated that glutathione and glutathione-dependent enzymes play a central role in the cellular defense against toxic environmental agents (Ludmila Gavriiliuc *et al.*, 2007). A major function of GSH is to serve as a reductant in redox processes and the reduction of oxidized glutathione (GSSG) is consequently of fundamental importance for the metabolic function of glutathione. This reduction was mediated by a class of enzymes called glutathione reductases (EC 1.6.4.2). Therefore, it is of special significance to investigate the enzymatic reduction of GSSG to GSH by GR. Ludmila Gavriiliuc *et al.* (2007) have reported that GR participates in the first level of defense in the saliva against mediators of oxidative stress and an increase in the salivary GR activity (114%) was observed in the patients affected with fluorosis.

Previous reports on the characterization of GR distributed in various species like *Trachemys scripta elegans* (Turtle), *Euglena gracilis* etc., showed an optimum pH range between 4.0 and 10 (William Willmore and Kenneth Storey, 2007). The glutathione reductase from sheep liver had a specific activity under the optimal pH 8.0 (Halis Sakoiroglu *et al.*, 2005). The investigation of optimum pH of salivary glutathione reductase from normal individuals/ non-smokers revealed two optimum peaks at pH 4.2 and 6.8. The activity of GR at 4.2 was found to be less than its activity at 6.8 because this pH may be the optimum pH for salivary juice. A study carried out by Johan Aps and Luc Martens (2005) revealed that the proportion of

bicarbonate as a salivary buffering component during resting conditions is approximately 50% and during these conditions, the parotid gland will produce almost no saliva, while both other major glands will be responsible for the production of saliva. The latter results in a high viscous and protein rich secretion, which will stabilize the oral fluid's pH at around 7.0. It was observed that the enzyme activity increases after a pH of 6.2 and then it records a maximum activity at pH 6.8 (Fig.1). However, the activity decreases sharply beyond pH 6.8 and completely vanished after pH 7.4. Generally, GR catalyze a bisubstrate reaction (GSSG and NADPH), where, NADPH form strong hydrogen bond with the active site amino acids like Thr 369, Arg 291, Asp 331 and the another substrate GSSG form hydrogen bond with the amino acids such as Ser 30, Arg 37, Tyr 114 and Arg 347. So, even a small change in the pH may cause the protonation or deprotonation of amino acid side chains and leads to a change in the three dimensional structure of the biological native enzyme and thereby a decrease/ complete loss in its activity (Donnie Berkholz, 2006). The results revealed that pH 6.8 as an optimum pH for salivary GR.

The studies were extended to explore the optimum temperature of salivary GR for normal individuals/ non-smokers. The thermal energy plays a pivotal role in maintaining the specific heat capacity of any compound. The normal homeostasis of any higher class mammalian system can be maintained at a temperature of 36.9°C. The thermal energy released during the protein folding that followed the first law of thermodynamics and thereby, a classical intra non-covalent interaction was also maintained. This made an enzyme as a stabilized one in the system. When there is an elevation of temperature due to an environmental insult, naturally the thermal energy of the system raised and this excess energy may be utilized for disrupting the non-covalent interactions that maintained the three dimensional structure of an enzyme. This may have a drastic effect in reducing an enzyme action. It was observed that the enzyme activity was sharply increasing from 17°C to 37°C. A decline in the activity was observed furthermore and the activity was completely disappeared beyond 67°C. The results proved that 37°C was optimum temperature for salivary GR (Fig.2).

The optimum pH and temperature found for salivary GR was then adopted to explore the binding efficiency (K_m) between GR and GSSG using Michaelis – Menten steady state kinetics. The results revealed that the K_m value was 0.058mM (Fig.3).

The incidence of SCC in cigarette smokers is four to seven times higher than in non- smokers, and if alcohol is also consumed this incidence may be even higher. Moreover, compared with non-smokers, the higher cigarette smoke-related risk for SCC is manifested by a reduction in the mean age of development of the disease by 15 years (Nagler and Abraham Reznick, 2004). Smoking further increases the risk of oral cancer, along with cancer of the larynx, and oesophagus. Smoking affects the activity of several antioxidant enzymes present in the saliva. The glutathione reductase activity in both normal males and females were found to be 0.108U/ml and 0.097U/ml respectively, under optimal pH, temperature and K_m . In male acute smokers the GR activity was found to be 0.0378 U/ml (65% reduction compared to normal

activity) and for females it was observed as 0.0552U/ml (43.1% reduction compared to normal activity). Similarly, the activity in the male chronic smokers was found to be 0.0128U/ml (88% reduction compared to normal activity) and 0.0457U/ml activity has been recorded for females (52.9% reduction) (Fig.4). Statistical analysis using two way ANOVA for the salivary GR activity obtained among non smokers was proved to be not significant at 5% level ($p > 0.05$) whereas it was proved to be significant ($p < 0.05$) between non smokers and smokers (acute and chronic) (Table 1). In addition, a significant difference at 5% level ($p < 0.05$) in the activity of salivary GR has been observed between the acute smokers and chronic smokers. A significant correlation ($r^2 = 0.988$) has been observed between the GR activity of smokers and non smokers indicating a risk for the development of oral cancer. As a result, salivary GR may be utilized as a biomarker in diagnosing the oral, throat and neck cancers. The method is simple, cheap, requires non expertise and moreover, cost effective (data not shown).

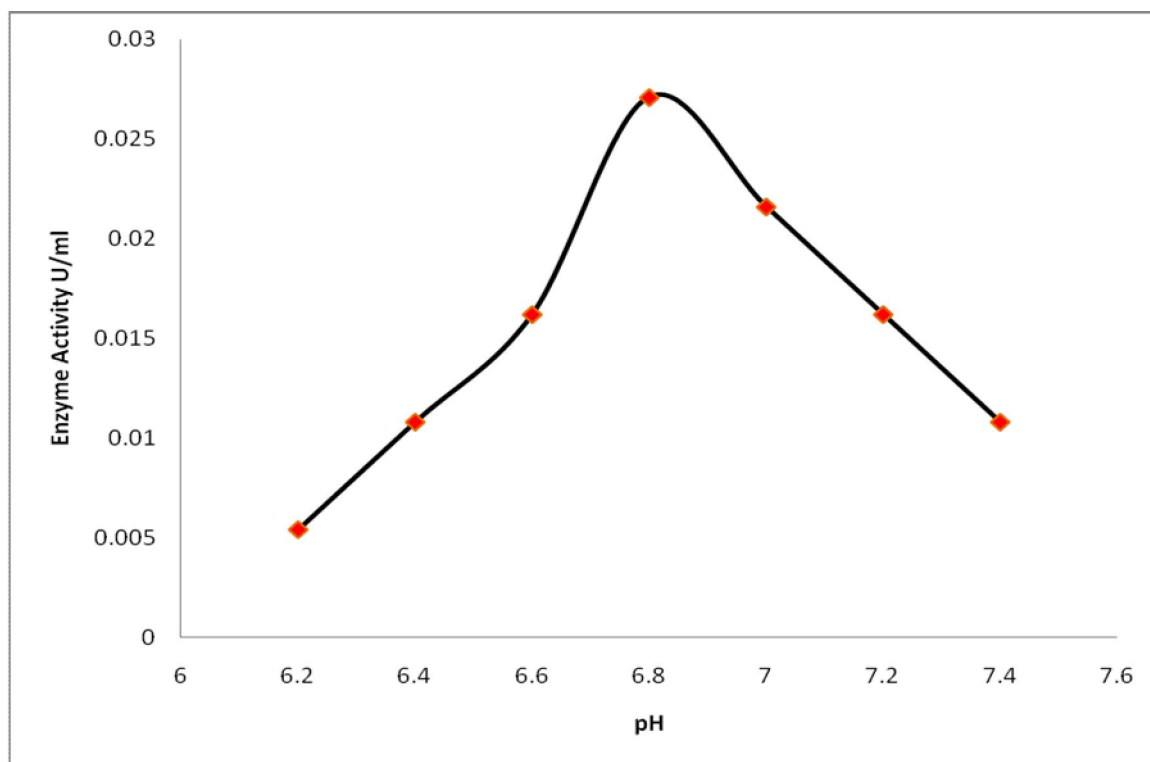


Figure 1. Effect of pH on salivary glutathione reductase catalyzed reaction

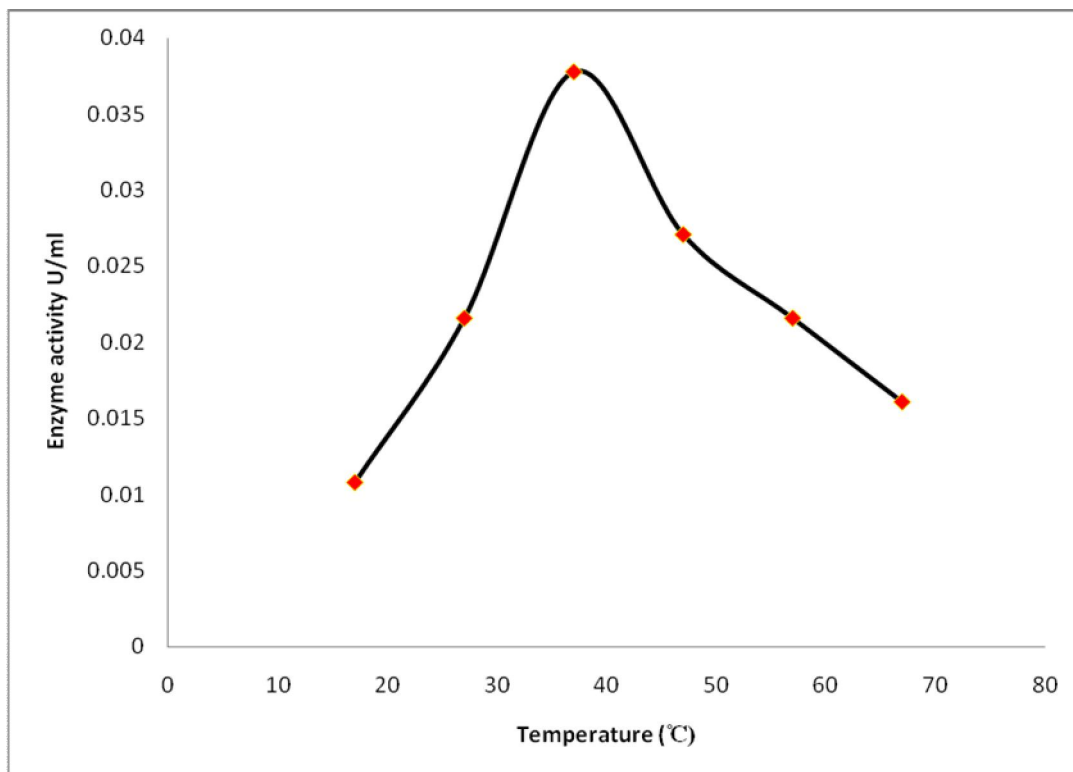


Figure 2. Effect of temperature on salivary glutathione reductase catalyzed reaction

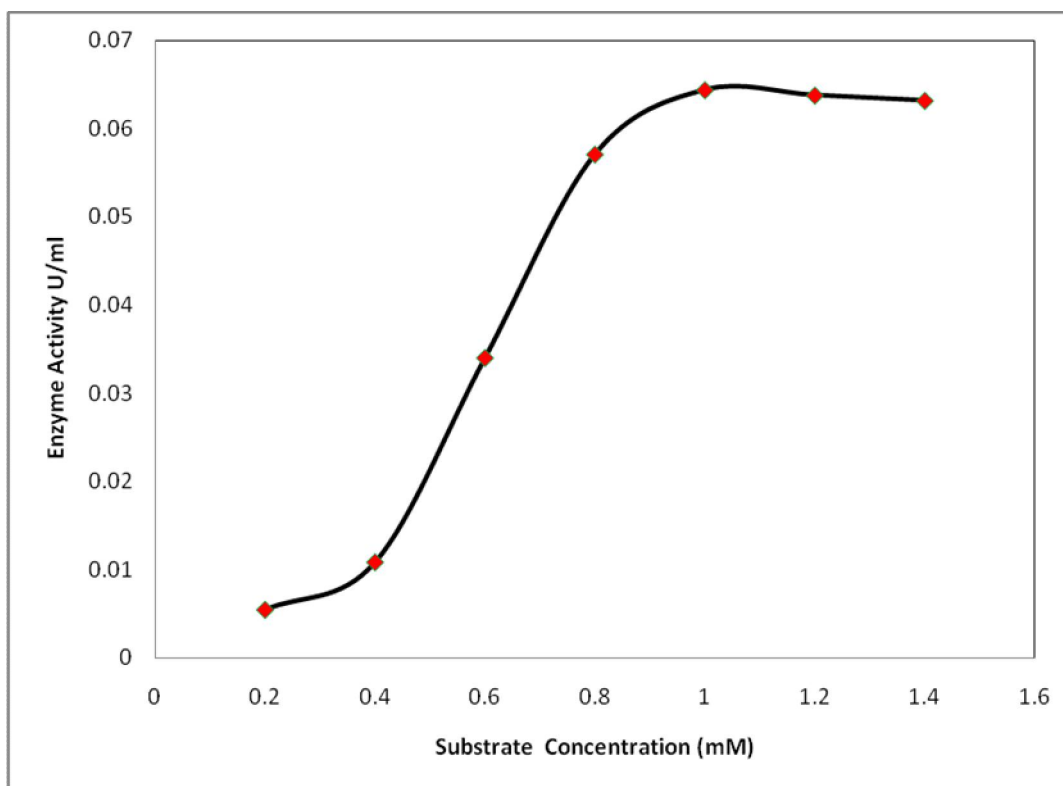


Figure 3. Effect of GSSG concentration on salivary glutathione reductase catalyzed reaction

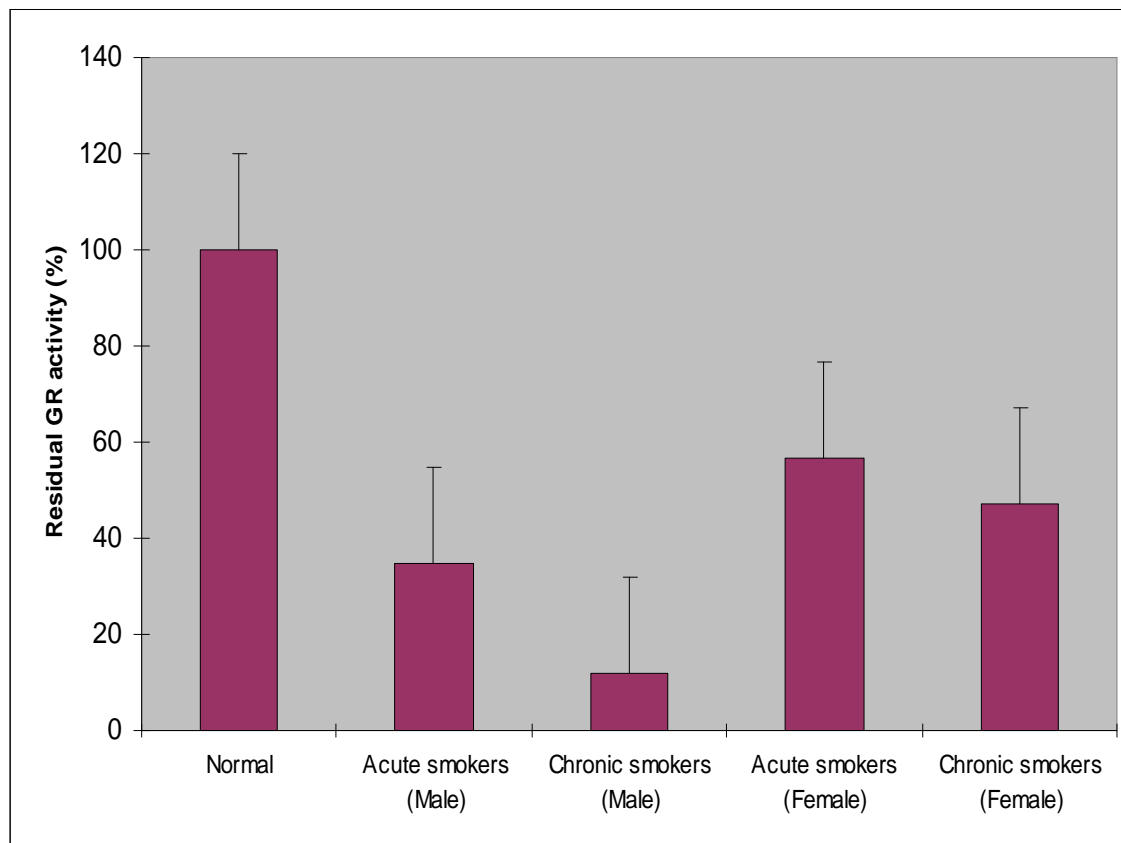


Figure.4 Comparison of salivary GR activity between Normal, acute and chronic smokers

Table 1. Two way ANOVA for GR activity of non smokers and smokers

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	0.00299	.00299	1.65652	0.42051
Factor B	1	2.4025E-3	2.4025E-3	0.13301	0.77736
Model	2	0.00323	0.00323	0.89477	0.59873
Error	1	0.00181	0.00181	-	-
Corrected Total	3	0.00504	-	-	-

Factor A = Salivary GR activity among non smokers ($p > 0.05$)

Factor B = Salivary GR activity between the non smokers and smokers ($p < 0.05$)

4. Conclusion

Saliva is a biological fluid that offers several opportunities in diagnosis, toxicology and in forensic science. Furthermore, many salivary proteins offer great potential in clinical and epidemiological research, in oral as well as in general health studies. In conclusion, the studies revealed that the pH optimum of salivary glutathione reductase of non smokers was found to be 6.8, temperature optimum as 37°C and the K_m as 0.058mM (GSSG). A significant reduction in the salivary GR of both acute and chronic smokers has

been observed than the non smokers. Especially, the GR activity was decreased rapidly in chronic smokers than the non smokers proving the possibility of utilizing the enzyme as an oral cancer detecting biomarker.

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