STUDIES ON KINETIC PARAMETERS OF SALIVARY GLUTATHIONE REDUCTASE IN NORMAL INDIVIDUALS AND ITS IMPLICATIONS ON SMOKERS

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

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The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students. It was submitted for the viva voce examination submitted on 24.04.2008.

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CONTENTS

CONTENTS

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Saliva is the first biological secretion confronted by external material produced during mastication or by uptake of fluids. The salivary glands produces various enzymes that can scavenge free radicals. Post prandial salivary samples collected from the normal individuals and smokers of various age groups were assessed for the antioxidant property. Various *in vitro* antioxidant assays like FRAP, CUPRAC and Cerium sulphate (IV) proved that the salivary samples of non smokers possess a significant antioxidant power than the smokers. The pH optima of salivary glutathione reductase was found to be 6.8, the temperature optima was at 37 □ C and the K_m was found to be 0.058mM. The glutathione reductase activity was decreased rapidly in chronic smokers and a slight decrease in the acute smokers compared to the non smokers, proving that glutathione reductase activity can be utilized as a potent biomarker for detecting oral cancer.

Keywords : Saliva, Antioxidant, free radical scavenging, Glutathione reductase, oral cancer.

1.INTRODUCTION

Atmospheric oxygen is a diatomic molecule with the formula O_2, in which the two oxygen atoms are doubly bonded to each other (Triplet oxygen). This form of oxygen has two unpaired electrons that can act as a radical. Whereas most radicals are highly reactive molecules, triplet oxygen is fortunately unreactive. Triplet oxygen released by plants as a product of photosynthesis is necessary for aerobic respiration in mammalian cells. In mammalian cells, aerobic respiration is coupled to oxidative phosphorylation, which produces ATP that is required both for cell-type-specific functions and for the maintenance of viability. Because of its radical nature, oxygen can undergo unintended electron transfer reactions with cellular constituents, with damaging consequences targeted towards important biological macromolecules (Scott Budinger and Navdeep Chandel, 2007)

1.1 Reactive oxygen species

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell and Gutteridge, 1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller *et al.,* 1990). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet -}$) (Miller *et al.,* 1990) (**Fig. 1.1)** Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalyzed processes (Marian Valko *et al.,* 2006).

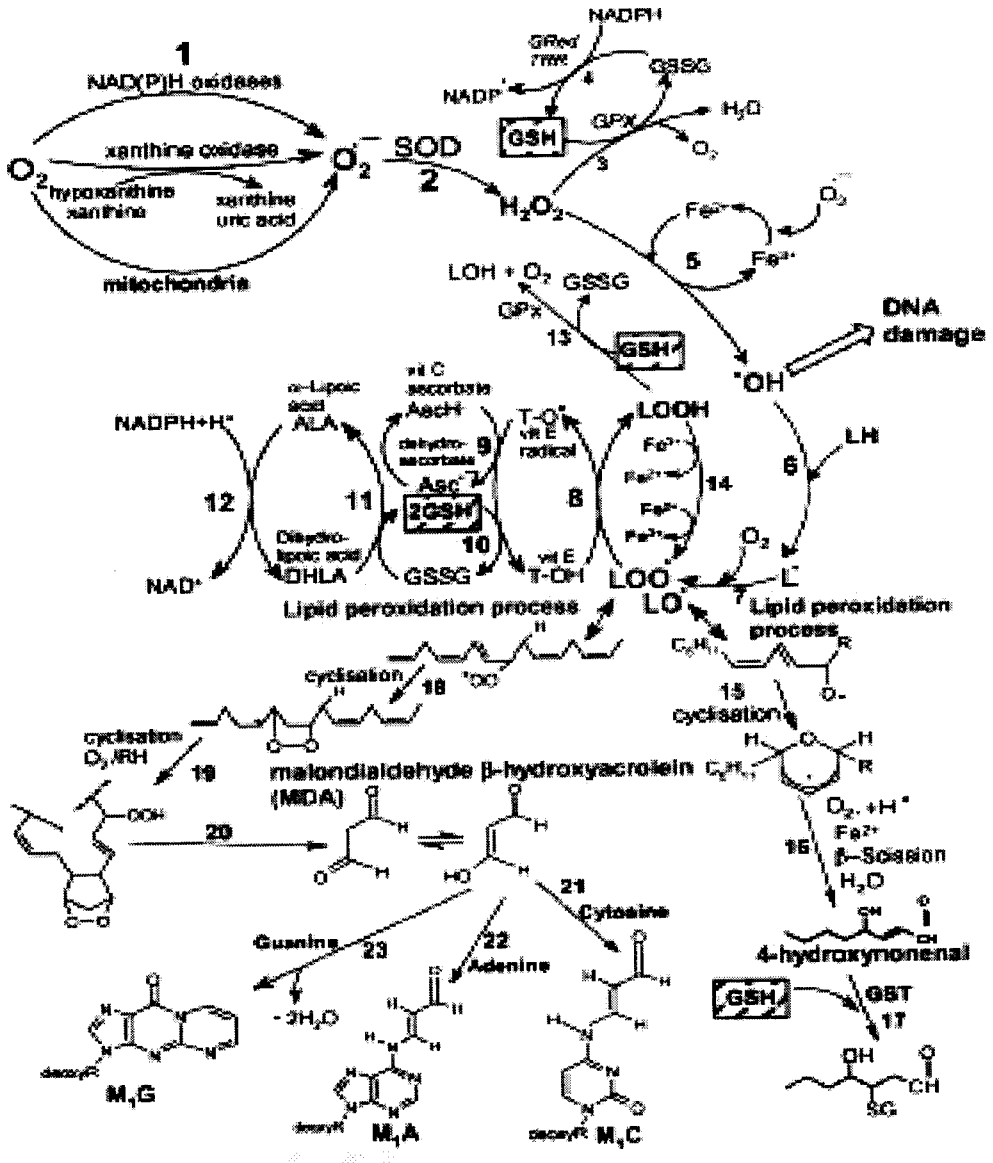

Fig. 1.1 Pathways of ROS formation (Courtesy: Marian Volko et al., 2006)

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Marian Valko *et al.,* 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999). The most extensively studied DNA lesion is the formation of 8-OH-Guanine. Permanent modification of genetic

material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing.

1.2 Reactive nitrogen species

NO• is a small molecule that contains one unpaired electron on the antibonding $2\pi^*y$ orbital and is, therefore, a radical. NO• is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline with the formation of NO• *via* a five electron oxidative reaction (Ghafourifar and Cadenas, 2005). Nitric oxide (NO•) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation (Bergendi *et al.,* 1999). NO• has a half-life of only a few seconds in an aqueous environment. NO• has greater stability in an environment with a lower oxygen concentration (half life >15 s).

Overproduction of reactive nitrogen species is called nitrosative stress (Klatt and Lamas, 2000). This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Carr *et al.,* 2000)

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$$

1.3 Antioxidants

Antioxidants are molecules that inhibit or prevent the oxidation of a substrate, that evolved to protect biological systems against damage induced by RONS (Halliwell and Gutteridge, 1989). A sophisticated, co- operative array of antioxidant defense mechanisms, the antioxidant network, is found in biological systems. The network works in concert in order to avoid oxidative stress through different mechanisms: RONS scavenging, reduction of peroxides and metal chelating activity (Mauro Serafini, 2006). There are two types of antioxidants viz., enzymatic and non-enzymatic. The major non-enzymatic antioxidants are vitamins C, E, carotenoids, and phenolic compounds, especially of flavonoids that includes isoflavones, flavonols, anthocyanins, glycosides etc., These antioxidants can be derived mainly from the plant sources in the form of vegetables, fruits, nuts and cereals. Similarly, enzymatic antioxidants like catalase,peroxidases,Superoxidedismutase(SOD),Glutathioneperoxidase(GPx), and Glutathione Reductase (GR) also plays a pivitol role in the removal of free radicals. It has been observed that these enzymes are widely distributed in various organisms.

In higher class mammalian systems including *Homo sapiens* possesses different modes of biological secretions such as salivary, gastric and intestinal juices that act as the primary defense barriers, which removes threat posing microorganisms and its products from the sytem. Most of these fluids possess several free radical scavenging molecules (antioxidants) that scavenge the radicals that cause several different disorders, especially of cancer.

1.4 Cancer

Cell cycle plays a significant role in maintaining the normal cell content in a tissue and its architecture. Several signaling molecules involved in a cascade process to control the cell cycle and thereby normal mitosis. If any impairment occurs in the signal transduction pathway, it leads to an uncontrolled cell proliferation and abnormal cell growth ("Lumps formation") in a particular location that is termed as cancer. Tumors can be Benign or Malignant

Benign tumors are not cancer, which are rarely life-threatening. Generally, benign tumors can be removed, and they usually do not recur. Cells from benign tumors do not invade the tissues around them i.e., they are localised. Cells from benign tumors do not spread to other parts of the body.

Malignant tumors are generally more serious than benign tumors. They may be life-threatening and can be often removed, but sometimes they grow may recur. Cells from malignant tumors can invade and damage nearby tissues, organs and can spread to other parts of the body. Generally, the tumor cells produce various types of proteases that can hydrolyze the cell matrix proteins like collagen, elastin, fibronectin, proteoglycan etc., As a result the cells can move away from the original cancer (primary tumor) site and may enter into the bloodstream or lymphatic system through which they may invade other organs, forming new tumors and damaging these organs. The spread of cancer is called metastasis.

2. LITERATURE REVIEW

2.1 Saliva

Saliva is a complex secretion whose components exert a well documented role in health and disease (Mandel, 1987). In addition to its lubricant properties (Bruno Zappacosta *et al.,* 1999), saliva contains many biochemical systems known to be involved in soft-tissue repair including lysozyme, lactoferrin and salivary peroxidase (Carlsson, 1987). Saliva contains various antioxidants, including uric acid, which contributes almost 70% of the total radical-trapping antioxidant capacity (Moore *et al.,* 1994). The first contact with cigarette smoke products occurs in the mouth. Saliva is the first biological secretion confronted by external material secreted during mastication. it possess defense mechanism due to the presence of defense proteins like immunoglobuins or lysozymes, peroxidase, cystatins, histatins etc.

When foods and beverages are injected, these are first mixed with saliva and then swallowed into the stomach being mixed with gastric juice. Part of H_2O_2 in the foods and beverages may be decomposed in the oral cavity by salivary peroxidase. Previous reports are there for the formation of H_2O_2, and OH-radical in the gastric juice. If H_2O_2 is present in saliva and gastric juice, H_2O_2 react with Fe(II) and HNO_2 the mixture of saliva and gastric juice producing OH radicals and reactive nitrogen species, respectively. On the other hand, it is known that saliva contains SCN^- (0.3–2 mM) and NO_2^- (0.05–0.3 mM) which is produced by the oralbacterium-dependent reduction of salivary NO_3^-, and that gastric juice contains ascorbic acid (AA) (around 0.1 mM)and iron ions (about 0.04 mM). When saliva and gastric juice are mixed, reactions among the above components are possible. In fact, it has been reported that the mixture of saliva and gastric juice can produce NO. If H_2O_2 is present in saliva and gastric juice, H_2O_2 can react with Fe(II) and in the HNO_2 mixture of saliva and gastric juice producing OH radicals and reactive nitrogen species respectively. It has been reported the formation of OH radical in the stomach.

Saliva serves many roles, some of which are important to all species, and others to only a few

- *Lubrication:* The mucus in saliva is extremely effective in binding masticated food into a slippery bolus that (usually) slides easily through the esophagus without inflicting damage to the mucosa. Saliva also coats the oral cavity and esophagus, and food basically never directly touches the epithelial cells of those tissues.

- *Oral hygiene:* The oral cavity is almost constantly flushed with saliva, which floats away food debris and keeps the mouth relatively clean. Flow of saliva diminishes considerably during sleep, allow populations of bacteria to build up in the mouth resulting in *dragon breath* in the morning. Saliva also contains lysozyme, an enzyme that lyses many bacteria and prevents overgrowth of oral microbial populations.
- *Initiates starch digestion:* In most species, the serous acinar cells secrete an alpha-amylase which can begin to digest dietary starch into maltose. Amylase is not present, or present only in very small quantities, in the saliva of carnivores or cattle.
- *Provides alkaline buffering and fluid:* This is of great importance in ruminants, which have non-secretory for stomachs.
- *Evaporative cooling:* clearly of importance in dogs, which have very poorly developed sweat glands - look at a dog panting after a long run and this function will be clear.

Diseases of the salivary glands and ducts are common in animals and man, and excessive salivation is a symptom of almost any lesion in the oral cavity. The dripping of saliva seen in rapid animals is not actually a result of excessive salivation, but due to pharyngeal paralysis, which prevents saliva from being swallowed.

2.2 Salivary glands

Saliva is secreted from the salivary glands. The basic secretory units of salivary glands are clusters of cells called an acini. These cells secrete a fluid that contains water, electrolytes, mucus and enzymes, all of which flow out of the acinus into collecting ducts.

Within the ducts, the composition of the secretion is altered. Much of the sodium is actively reabsorbed, potassium is secreted, and large quantities of bicarbonate ion are secreted. Bicarbonate secretion is of tremendous importance to ruminants because this along with phosphate, provides a critical buffer that neutralizes the massive quantities of acid produced in the forestomachs. Small collecting ducts within salivary glands lead into larger ducts, eventually forming a single large duct that empties into the oral cavity (Chicharro *et al.*, 1998).

There are three major pairs of salivary glands that differ in the type of secretion they produce

- *parotid glands* produce a serous, watery secretion.
- *submaxillary (mandibular) glands* produce a mixed serous and mucous secretion.
- *sublingual glands* secrete a saliva that is predominantly mucous in character.

The basis for different glands secreting saliva of differing composition can be seen by examining salivary glands histologically. Two basic types of acinar epithelial cells exist:

- *serous cells*, which secrete a watery fluid, essentially devoid of mucus
- *mucous cells*, which produce a very mucus-rich secretion

Acini in the parotid glands are almost exclusively of the serous type, while those in the sublingual glands are predominantly mucus cells. In the submaxillary glands, it is common to observe acini composed of both serous and mucus epithelial cells.

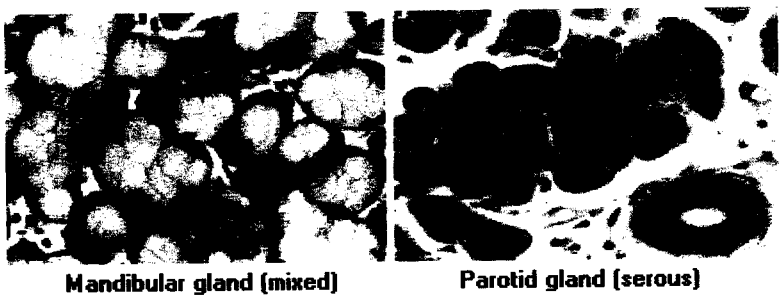

Figure 2.2.1 Sections of canine salivary gland

The histologic sections of canine salivary gland **(Fig 2.2.1)**, reveals both the serous and mucus cells. The cells that stained pink are serous cells, while the white, foamy cells are mucus-secreting cells. Secretion of saliva is under control of the autonomic nervous system, which controls both the volume and type of saliva secreted. Parasympathetic stimulation from the brain results in greatly enhanced secretion, as well as increased blood flow to the salivary glands. Potent stimuli for increased salivation include the presence of food or irritating substances in the mouth, and thoughts of or the smell of food. Knowing that salivation is controlled by the brain will also help explain why many psychic stimuli also induce excessive salivation.

2.3 Secretion of saliva

Healthy adult subjects normally produce 500–1500 ml of saliva per day, at a rate of approximately 0.5 ml/min (Silvia Chiappin *et al.,* 2007) but several physiological and pathological conditions can modify saliva production quantitatively and qualitatively, e.g., smell and taste stimulation, chewing, psychological and hormonal status, drugs, age, hereditary influences, oral hygiene (Aps and Martens, 2005) and physical exercise. Each type of salivary gland secretes a characteristic type of saliva. Differences in the concentration of salts/ions (Kalk *et al.,* 2002) and total proteins (Hu, *et al.,* 2004) among glands can be observed. Salivary proteins are expressed differently among individual glands, like submandibular and sublingual glands. For example cystatin C is secreted by the submandibular gland and MUC5B mucin and calgranulin are secreted by the sublingual gland. Moreover, salivary composition varies, depending upon whether salivary secretion is basal or stimulated (Silvia Chiappin *et al.,* 2007).

Salivary output and composition depend on the activity of the autonomic nervous system: the serous part of the glands is under the control of the sympathetic system and the mucous part of both parasympathetic and sympathetic systems. The α–β adrenergic and cholinergic stimuli (neural or pharmacological) can modify the quantity; viscosity and ionic and protein concentrations can vary. Parasympathetic stimulation results in a high flow of saliva containing low levels of organic and inorganic compounds. Sympathetic stimulation produces a low volume of protein-rich and K^+-rich saliva (Aps and Martens, 2005; Silvia Chiappin *et al.,* 2007). The presence of food in the mouth can affect salivary composition as a stimulus for selective protein release; after a meal an increase of total proteins and of α-amylase in saliva has been shown (Messenger *et al.,* 2003).

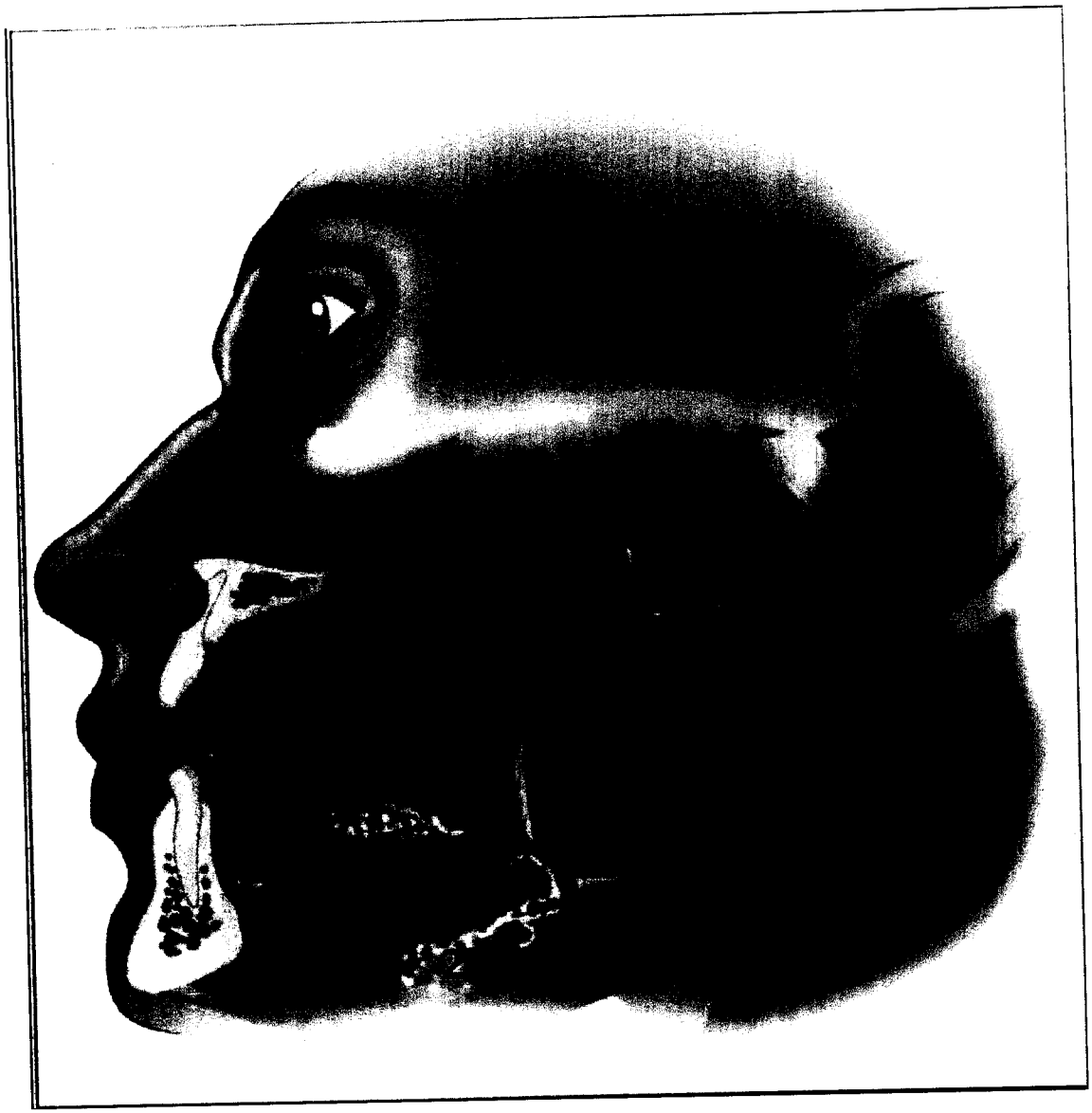

Figure 2.3.1 The three pairs of large salivary glands; the glandula parotis (1), the glandula submandibularis (2) and the glandula sublingualis (3). (Figure from Van Nieuw Amerongen, 2004.)

2.4 Salivary composition

Whole saliva contains mainly water, strong and weak ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, HCO_3^-, HPO_3^{2-}) which can generate buffer capacity. The primary secretion from salivary glands is plasma ultrafiltrate (isotonic compared to plasma) but in salivary ducts there is energy-dependent reabsorption of Na^+ and Cl^- resulting in a hypotonic fluid secretion, with a lower ion concentration compared to plasma (Silvia Chiappin *et al.,* 2007). In salivary gland ducts mineralocorticoid receptors are present, so salivary glands are mineralocorticoid-responsive, for this reason salivary K^+ concentration is higher than the plasma concentration (25 Vs.4 mmol/l) and Na^+ concentration is lower in saliva compared with that in plasma (2 Vs. 145 mmol/l) (Aps and Martens, 2005).

Small amounts of organic non-protein compounds can be detected in saliva. Uric acid is one of the most important antioxidant compounds in saliva (Guan e*t al.,* 2003), bilirubin and creatinine are also detectable (Lioyd *et al.,* 1996). Saliva moreover contains glucose, amino acids, lipids like cholesterol and mono/diglycerides of fatty acids (Agha-Hosseini *et al.,* 2006; Silvia Chiappin *et al.,* 2007). In saliva amines, such as putrescine, cadaverine and indole, are also detectable (Cooke *et al.,* 2003). Fatty acids, too, are measurable in saliva and in particular α-linoleic acid and arachidonic acid can be measured in human saliva: their concentration seems to correlate with a dietary fatty acid intake (Actis *et al.,* 2005).

2.5 Salivary proteins

The salivary levels of total protein increase was mainly through β-sympathetic activity in the salivary glands, since saliva secretion is mainly evoked by the action of adrenergic mediators (Silvia Chiappin *et al.*, 2007). Human saliva proteins can have a wide range of functional properties. They can be related to the immune response and oral defence, like lysozyme, lactoferrin, lactoperoxydase, immunoglobulins, agglutinins, chitinases and mucins which in saliva participate in the protection of the oral tissues; other proteins possess bacteria-killing properties, like histatins and defensins. The salivary immunoglobulins are mainly s-IgA (N85%) and are produced directly by the B lymphocytes present near the salivary glands. The remaining 5–15% of salivary immunoglobulins are mainly IgG and IgM, derived from crevicular fluid or from plasma leakage (Van Nieuw Amerongen, 2004). Other proteins are also present: for example, enzymes and enzyme inhibitors, hormones such as growth factors, and cytokines such as interleukin-8 (Simpson *et al.*, 2005; Silvia Chiappin *et al.*, 2007)

2.6 Salivary hormones

Some hormones commonly measured in plasma, such as steroids, non-steroids, peptide and protein hormones, can be detected in the oral fluid. Catecholamines can be recognized in saliva ranging from 250 to 800 pg/ml (Silvia Chiappin *et al.*, 2007), but their source is still uncertain. They seem to originate by diffusion from serum, but there is also an amount of salivary catecholamines derived by direct release from sympathetic nervous terminations, so their concentration is poorly correlated with that of plasma. There is little information about thyroxin and triiodothyronine levels in saliva: in preliminary studies they were detected in saliva and their salivary levels (1.10±0.07 nmol/l for T_4 in healthy subjects) seem to correlate with plasma

levels (Putz *et al.,* 1985). Steroid detection is perhaps the most interesting application in salivary hormonal studies. Steroids have often been studied because salivary-free steroid hormones can give good information on serum-free levels (Silvia Chiappin *et al.,* 2007). The most commonly assayed biomarkers in saliva are cortisol, testosterone, dehydroepiandrosterone (DHEA) (Patel *et al.,* 2004), 17-hydroxyprogesterone, progesterone and aldosterone. In saliva also other protein hormones have been detected, such as prolactin (Huang, 2004), insulin-like growth factor I (IGF-I) and melatonin. Melatonin levels in saliva reached 500 pmol/l at night time and were found to correlate significantly with plasmatic concentrations.

2.7 Salivary enzymes

Saliva contains both host-derived and microbial derived factors, including several enzymes that degrade proteins, proteoglycans, lipids and carbohydrates (Nieminen, et al., 1993; Nurdan Ozmeric, 2004). Enzymes in saliva can originate from cells in salivary glands, microorganisms, epithelial cells, PMN and can be derived from GCF. Reports are there for the presence of several salivary proteins like elastase, α-amylase, arginase, myeloperoxidase, lysozyme, β-glucoronidase, chitinase, glutathione peroxidase etc., that

can be act as good markers for several periodontal diseases and also for oral cancer (Nurdan Ozmeric, 2004). Previous reports are also there for the reduced activity of enzymes like oral peroxidases and SOD in smokers.

2.8 Cell cycle

Dysregulation of the cell cycle machinery is a fundamental hallmark of cancer progression (Todd *et al.*,2002). The cellular programs of proliferation, differentiation, senescence, and apoptosis are intimately linked to the cell cycle regulatory machinery. Many of the molecular alterations that cause abnormal biologic behavior of cancer cells are based on aberrations of cell cycle regulation. For example, escape from dependence on mitogens or induction of resistance to anti-mitogens, tolerance to DNA damage, apoptosis resistance, and progression of cells with activated oncogenes and inactivated tumor suppressor genes through multiple checkpoints resulting in increased genomic instability and all are affected by cell cycle regulatory proteins (Todd *et al.*,2002).

2.9 Cigarette smoking

All cigarettes can damage the human body. Any type of smoking is dangerous. Cigarettes are the only legal product whose advertised and intended use smoking is harmful to the body and causes cancer. Although some people try to make their smoking habit safer by smoking fewer cigarettes, most smokers find that hard to do. Research has found that even smoking as few as 1 to 4 cigarettes a day can lead to serious health outcomes, including an increased risk of heart disease and a higher risk of dying at a younger age. Some think that switching from high-tar and high-nicotine cigarettes to those with low tar and nicotine makes smoking safer, but this is not true. When individuals switch to brands with lower tar and nicotine, they often end up smoking more cigarettes, or more of each cigarette, to get the same nicotine dose as before. The smoke has carbonmonoxide, ammonia , dimethylnitrosamine , formaldehyde, hydrogen cyanide and acrolein(Ross G. Cooper, 2006).Cigarette Smoking increases the risk of developing numerous cancers including the oral/mouth, throat, lungs, pharynx, esophagus, pancreas, larynx, uterine cervix, urinary bladder and

kidneys. Cigarettes usually contains nicotine present ,Inhaled nicotine reaches the brain within 15 seconds and concentrations in brain tissue remain high for about 2 hours. Tobacco smoke contains large numbers of gas and tar phase radicals and other oxidants (Ross G. Cooper, 2006). It has been estimated that a single puff of a cigarette contains as much as 10^{15} gas phase radicals and 10^{14} tar phase radicals potentially capable of modifying endogenous macromolecules including lipids.

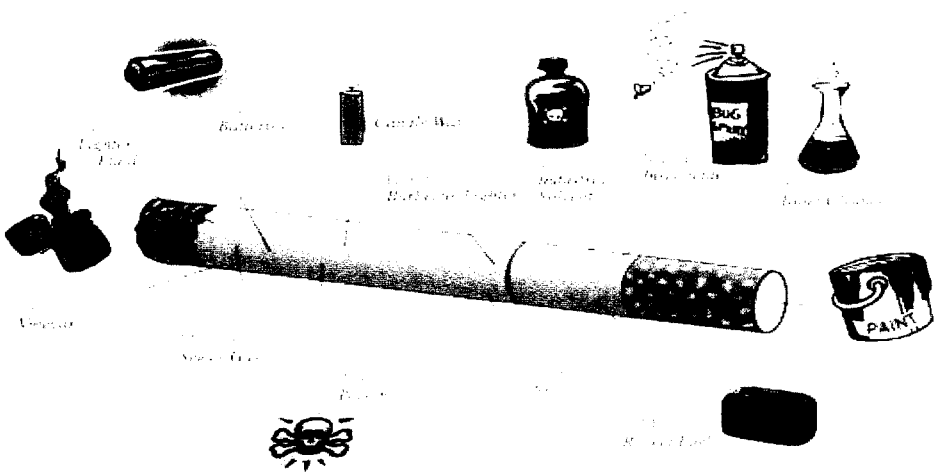

Figure 2.9.1Cigarette composition (www.ihec.org/.../images/whatisincig.jpg).

Nicotine is an organic compound that is found naturally in the tobacco plant. It is composed of carbon, nitrogen, hydrogen and oxygen and belongs to a group of compounds called alkaloids. Plants usually produce these types of chemical poisons so that animals are deterred from eating them. Nicotine is transformed by oxidation in the liver to conitine which is then excreted in the urine where it can be detected by a laboratory test. In the brain, it activates the dopamine reward system and increases certain neurotranmitters and hormones including norepinephrine, epinephrine, endorphins, and acth/cortisol . Other activators of the dopamine reward system include opiates,

amphetamines, and cocaine . Nicotine is 10 times more potent than heroin in its addictive properties. Nicotine acts a stimulant as improving attention, learning, reaction time, problem solving ability, lifts the mood, decreases tension, and lessens depressive feelings and Skeletal muscles promotes relaxation of muscle tone ,where as in digestive tract possessing nausea and appetite suppression and respiratory system results local irritation, impaired ciliary motion which impacts the auto-cleaning mechanisms of the lungs as cardiovascular effects increases heart rate and blood pressure, platelet adherence and vasoconstriction resulting in abnormally active clotting of the blood and accounts for the increased risk for stroke and heart disease. Average dose of nicotine delivered by smoking one cigarette is about 0.5 mg. Fatal dose of nicotine is 60 mg and causes respiratory muscle paralysis.

Cigarette smoke has both gaseous and particulate phases. Inhaled smoke delivers chemicals to the bloodstream of the smoker and deposits chemicals and particles in the smoker's mouth, bronchial airway passages and in the lungs. Mainstream smoke comes from the mouthpiece during puffing as the smoker exhales. Sidestream smoke is generated between puffs from the burning cone and mouthpiece. Environmental tobacco smoke is pollution from the mainstream and sidestream smoke and is harmful to the smoker and those who inhaled it.

Smoking cigars causes cancers of the lung, oral cavity (lip, tongue, mouth, throat), larynx (voice box), esophagus (swallowing tube), and probably cancers of the bladder and pancreas. Cigar smokers have a greater risk of dying from cancer of the mouth, larynx, or esophagus than non-smokers. Cigar smokers who inhale deeply and smoke several cigars a day are also at increased risk for heart disease and chronic lung disease. Pipe smokers have an increased risk of dying from cancers of the lung, throat, esophagus, larynx, pancreas, and colon and rectum.

SAMPLE REPRESENTATION - FOR REFERENCE ONLY
WARNINGS FOR SMOKED TOBACCO PRODUCTS 2006

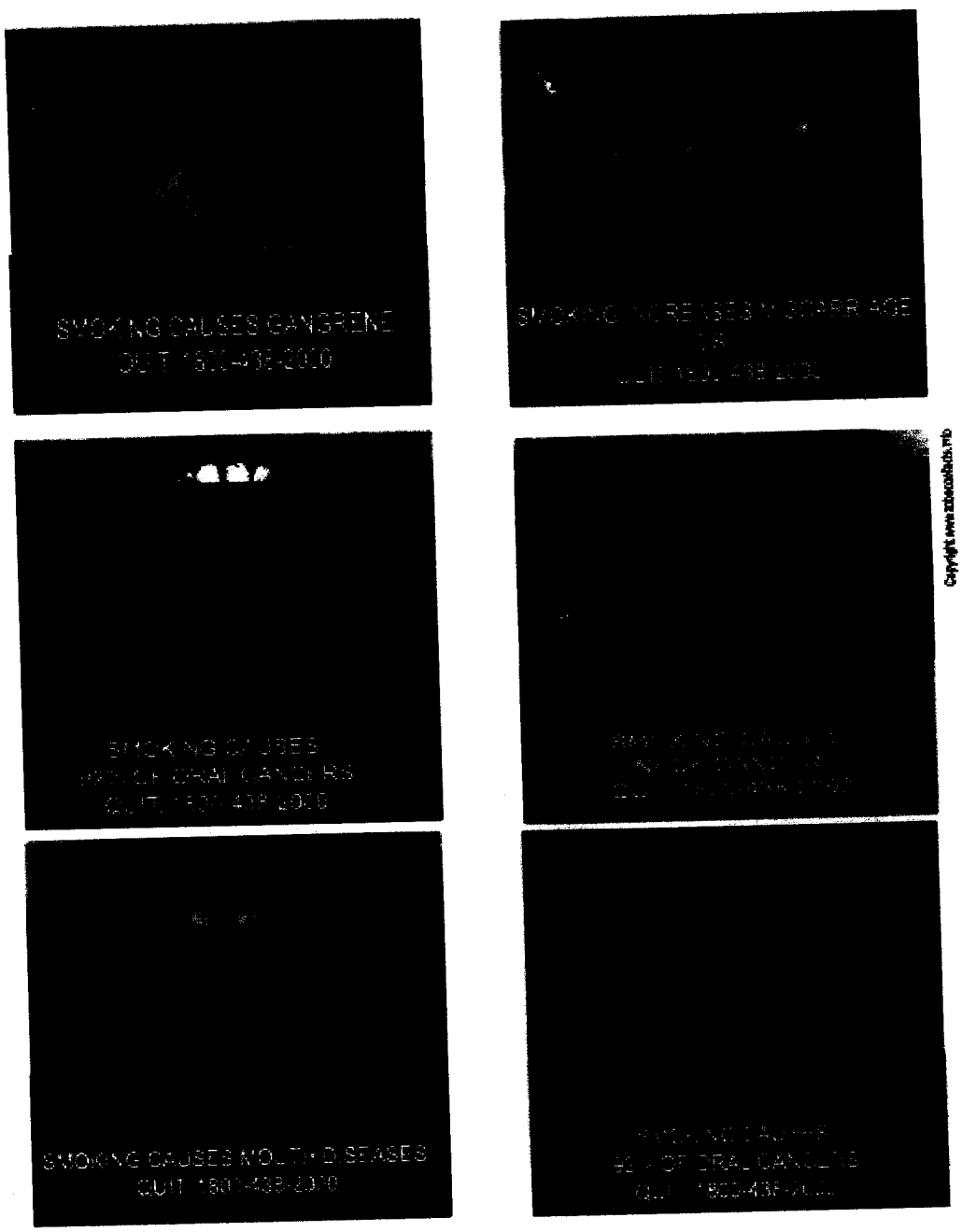

Figure 2.9.2 Different types of cancer due to smoking

(www.dogflu.co/images/smoking_deaths_heart_dis)

Figure 2.9.3Various diseases caused due to smoking.

(www.cfiwest.org/.../images/funeral15.jpg)

Cancer of the oesophagus	Heart attack	Diabetes
Stomach cancer	Coronary heart disease	Asthma
Kidney cancer	Cardiovascular disease	Stomach ulcers
Cancer of the bladder	Congestive heart failure	Cataracts
Cancer of the pancreas	Stroke	Gum disease
Liver cancer	Atherosclerosis	Crohn's disease
Cancer of penis	Peripheral artery disease	Premature aging of the skin
Cancer of the anus	Ischaemic heart disease	Loss of smell and taste
Cervical cancer	Angina	Osteoporosis (women)

Table 2.9.1 List of diseases, illnesses and other effects due to smoking

Bidis or "beedies" are flavored cigarettes imported mainly from India. They are hand-rolled in an unprocessed tobacco leaf and tied with colorful strings on the ends. Their popularity has grown in recent years in part because they come in many candy-like flavors such as strawberry, vanilla, and grape, they usually cost less than regular cigarettes, and they often give the smoker an immediate buzz. Even though bidis contain less tobacco than regular cigarettes, they deliver higher levels of nicotine (the addictive chemical in tobacco) and other harmful substances such as tar and carbon monoxide. Because they are thinner than regular cigarettes, they require about 3 times as many puffs per cigarette. They are also unfiltered. Bidis appear to have all of the same health risks of regular cigarettes, if not more. Bidi smokers have much higher risks of heart attacks, chronic bronchitis, and some cancers than non-smokers.

2.10 Oral cancer

Smoking is by far the major risk factor for developing oral cancer. The use of alcohol and smoking further increases the risk of oral cancer, along with cancer of the larynx, and esophagus. According the the American Cancer Society statistics, there were 28,000 new cases and 7,400 deaths from Oral cavity and pharynx cancer in the United States in 2002. Patients with head and neck cancer that smoke are more likely to develop spread of the cancer to their lymph nodes and once in the nodes the cancer is more likely to spread into the soft tissues. Although smoking is a risk factor development of oral cancer, the human papilloma virus is an even greater risk in developing cancer of the base of tongue and tonsil. The viral DNA has been found in over 70% of tumors. The virus is known to be spread by sexual contact.

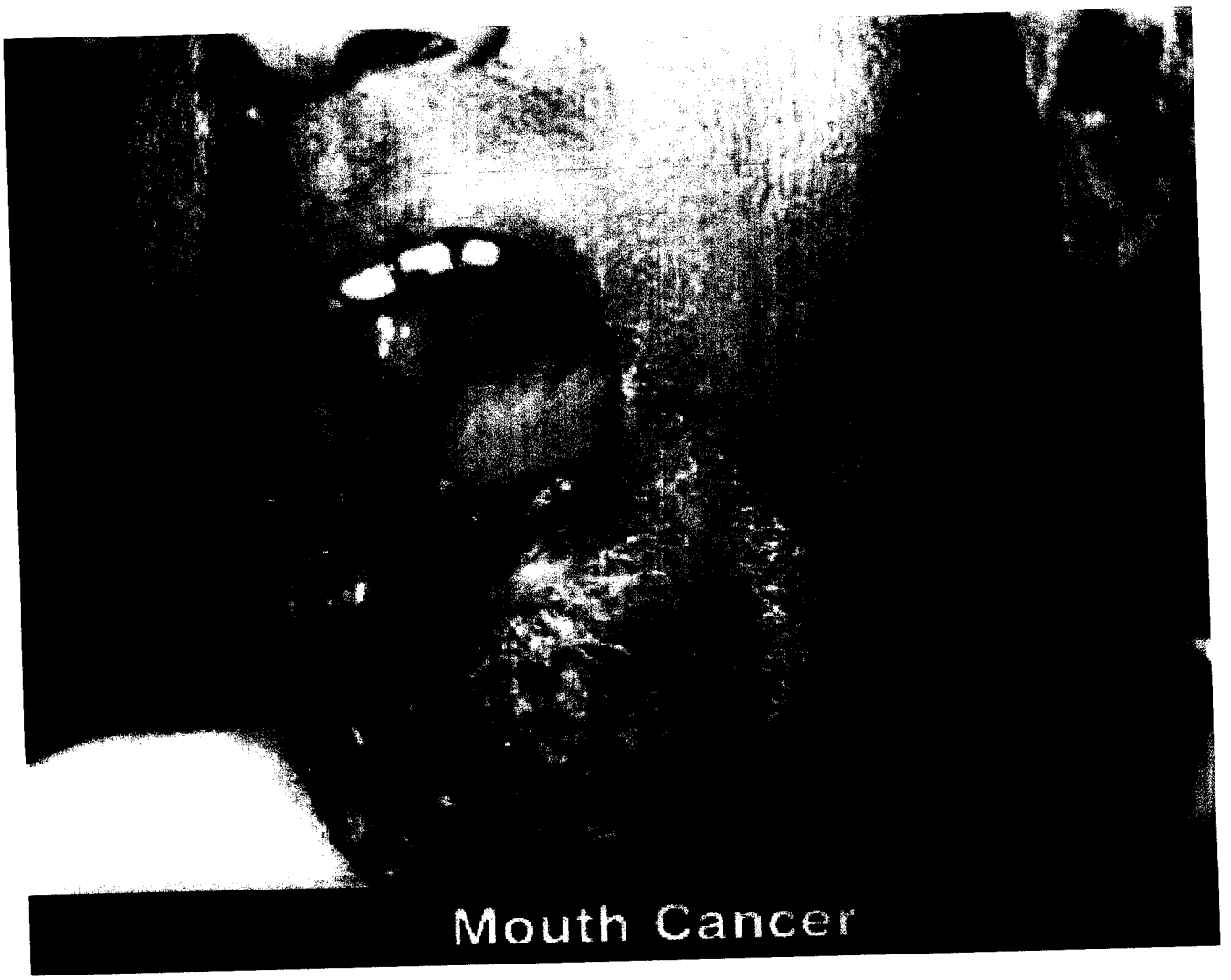

Figure 2.10.1 Mouth cancer (www.cfiwest.org/.../images/mouth cancer.jpg)

2.11 Larynx cancer

Smoking is by far the major risk factor for developing laryngeal cancer. The use of alcohol and smoking further increases the risk of cancer of the larynx, oral cavity, and esophagus. Exposure to the carcinogens in tobacco can cause permanent genetic damage in the cells and tumors can develop years later. If they are hoarsed for more than four weeks, then definitely need to have the voice box (larynx) examined. Cancers usually grow slowly, but can sometimes grow fast. If someone smokes more than half a pack per day or more he will have a far greater chance of developing cancer.

Figure 2.11.1 Larynx cancer (www.cfiwest.org/.../images/larynx cancer.jpg)

2.12 Lung cancer

Smoking causes 87% of all lung cancer cases. Smokers have approximately one chance in 10 of developing lung cancer over his/her lifetime. Kentucky has the highest incidence of adult smoking and the highest incidence of lung cancer (in 1997 to 1998 82 cases per 100,000 people per year). Lung cancer rates are 52% higher in Kentucky than the national average.

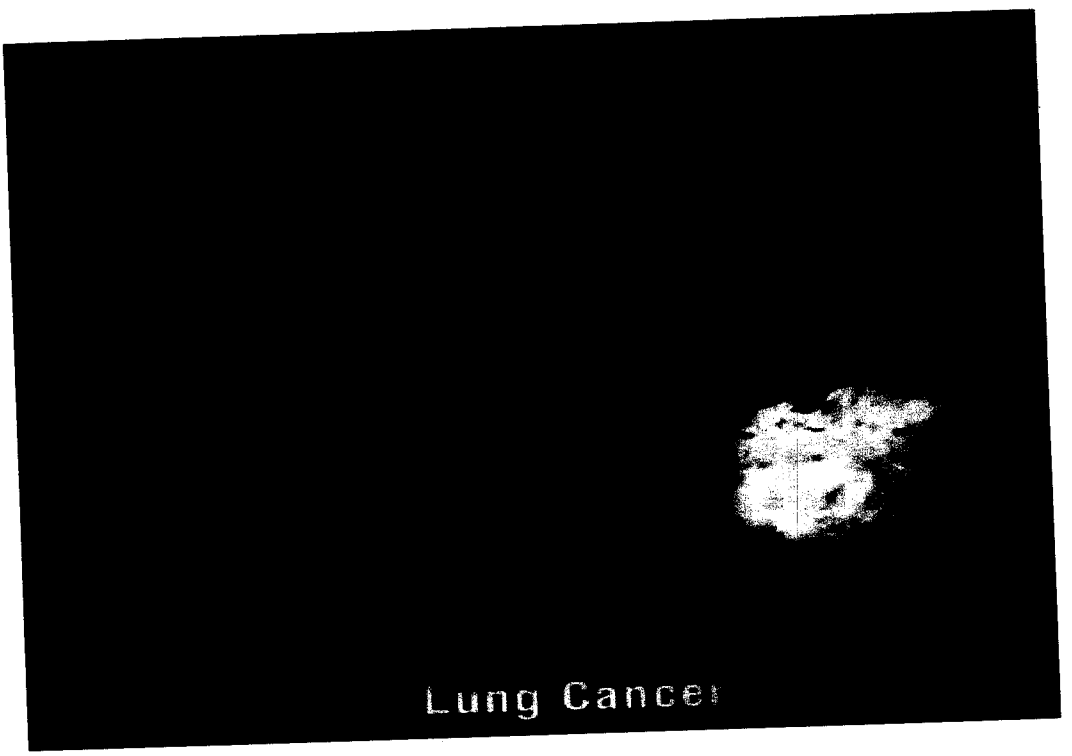

Figure 2.12.1 Lung cancer (www.cfiwest.org/.../images/lung cancer.jpg)

Surgery and radiation therapy or combination of chemotherapy and radiation therapy are the treatments of choice.

2.13 Alcohol

Although between 80 and 95 percent of alcoholics smoke cigarettes, a rate that is three times higher than the general population, and 70 percent of alcoholics are heavy smokers compared with just 10 percent of the population. smoking stimulates and alcohol relaxes, smokers use alcohol to prevent over-stimulation from smoking and alcoholics use cigarettes to prevent sedation. alcoholic smokers use tobacco mostly for the nicotine in it." smoking kills more alcoholics than alcohol does. According to the American Cancer Society, smoking is the most preventable cause of death in American society. Nearly one in five deaths in the U.S. results from the use of tobacco; more than 400,000 die from smoking in the U.S. each year.

2.14 Glutathione reductase

Glutathione reductase is an enzyme (EC 1.8.1.7), which reduces the oxidized glutathione (GSSG) to reduced glutathione (GSH) and is secreted by the salivary glands. It is an important and the least studied antioxidant enzyme that maintains the sulfhydryl pool in the salivary juice (Mauro Serafini, 2006). GR plays many important roles because of the importance of reducing equivalents. Glutathione reductase is critical in preventing high levels of oxidative stress because its activity can counteract oxidation. Glutathione reductase also is important in synthesis of DNA precursors as well as proton transport across membranes. These are substances that protect cells from damaging effects of oxygen radicals & highly reactive species. Antioxidant defenses of the body are also composed of enzymatic components like catalase, peroxidases, SOD, GPx, GR. how, the composition of the network markedly differs in terms of concentration and components, in different environments. Protection at cellular level is mainly guaranteed by these enzymes and glutathione (GSH) (Mauro Serafini, 2006). The effectiveness of the non-enzymatic antioxidant network can

capacity (TAC),defined as the moles of oxidants neutralized by one litre of the tested sample(Bruno Zappacosta *et al.*,2002). TAC considers the cumulative action of all the antioxidants present in the matrix, thus providing an integrated parameter rather than the simple sum of measurable antioxidants, giving an insight into the balance between antioxidants molecules. Experimental evidence has show that plasma TAC of patients affected by different chronic diseases such as diabetes, AIDS, ulcerative colitis, Crohn's disease, meningitis,CVD, colorectal, lung and breast cancer is lower compared to healthy controls suggesting the deep impairment of the antioxidant network during the development of these pathologies.

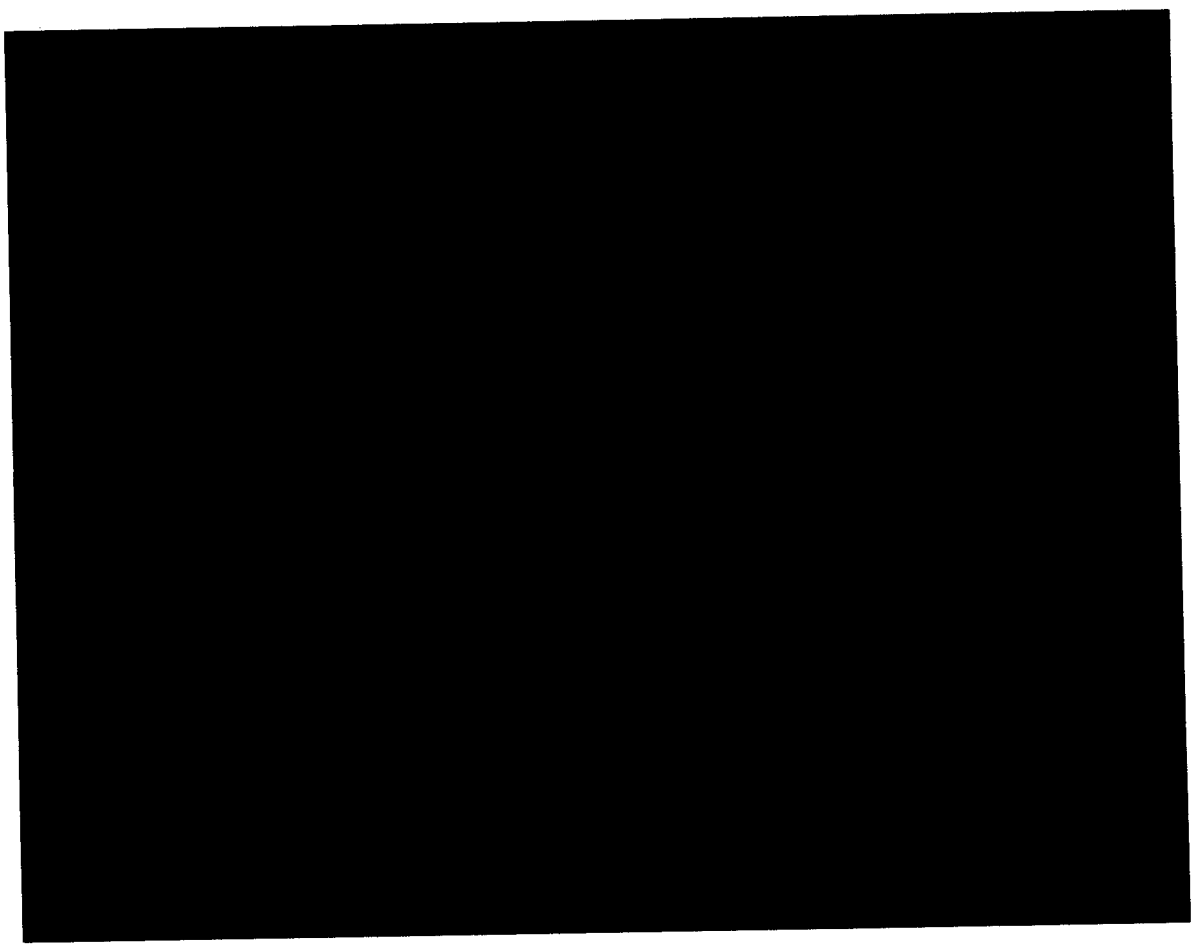

Figure 2.14.1 Structure of Glutathione reductase. (Donnie Berkholz,2006)

Glutathione reductase operates in a pingpong fashion, meaning the NADPH binds and transfers a hydride to FAD, then leaves, before the diglutathione ever binds. In other words, the two substrates are mutually exclusive (Donnie Berkholz,2006).

Tyr197 forms a protective shield over FAD to keep it from being oxidized when NADPH is not bound, then swings out of the way when NADPH binds and forms a strong hydrogen bond with Thr369. Arg291 and Asp331 form another ion pair near FAD. In three separate cases, ion pairs exist near the flavin with the positive residues of the pair pointed at the flavin. Other residues known important in binding of the NADPH phosphate include Arg218, Arg224, His219 and some integral waters. The nicotinamide ring is where the hydride is transferred to FAD, and there are a number of residues suspected to be critical there. Residues involved in binding the nicotinamide include Leu337, Glu201, Lys66, Val370 and Thr369. Glu201 andLys66 also form a salt bridge.

In glutathione binding, residues directly hydrogen bonding to glutathione include side chains of Ser30, Arg37, Tyr114, Arg347, His467' and Glu473' as well as main chain atoms of Met406' and Thr469'. Tyr114 moves about 1A on GSSG binding and fits well between the glycine moieties of the two glutathiones. It's well positioned to be a proton donor for a leaving glutathione, but unlikely because of its normally high pKa. It could be interesting to examine whether any nearby conserved residues could modify its pKa. Through-water hydrogen bonds occur with the side chains of Lys67, Tyr106, Asn117, Ser470', Glu472' and Thr476' and the main chain of Gly55, Pro468' and Thr476'.

The first step of the reaction is the NADPH-dependent reduction of FAD. Tyr197 may also play a role here, acting as a "spring" to force the nicotinamide ring closer to the FAD to ease hydride transfer. In addition, the Lys66-Glu201 salt bridge is near where the hydride transfer occurs. Next, the flavin reduces the disulfide Cys58-Cys63, likely through and intermediary adduct with the sulfur of Cys63. Few important residues have been proposed in this step of catalysis. Finally, the reduced, former disulfide reduces GSSG and is reoxidized to the disulfide. Cys63, His467' and Glu472' have been proposed to act similarly to the catalytic triad in serine proteases – glutamate activates the histidine, which in turn creates a highly nucleophilic cysteine (Donnie Berkholz,2006). The importance of His467' as a proton acceptor and donor has also been shown via mutagenesis.

Inspite of several scientific documentation there have been no reports on

1. The total antioxidant activity in the salivary juice of non smokers and smokers
2. Characterization and Activity of salivary glutathione reductase (GR) in non smokers and smokers.

So, it is considered worthwhile to investigate and explore the scientific evidence for the above mentioned parameters in both smoking and non smoking samples.

3.OBJECTIVES

1. To screen the antioxidant power of salivary juice in the normal individuals & smokers.

2. To characterize the salivary glutathione reductase activity like pH, temperature and substrate concentration in the normal individuals.

3. To investigate the salivary glutathione reductase activity in normal individuals and smokers.

4.MATERIALS AND METHODS

4.1 Modified ferric ion reducing/ antioxidant power assay (FRAP assay)

(Resat apak *et al.*, 2007)

PRINCIPLE

The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity. The antioxidants present in the sample reduced the oxidant probe and the respective product interacted with some colouring agents to form a coloured complex.

In this method, the antioxidants reduced the Fe^{3+} to Fe^{2+}. This ion then conjugated with the ferricyanide ion to form a Prussian blue coloured product, which was spectrophotometrically measured at 700nm. The presence of SDS prevents the formation of turbidity in the solution. The increase in the absorbance is directly proportional to concentration of total antioxidants present in sample.

$$Fe^{3+} + \text{antioxidant} \rightleftarrows Fe^{2+} + \text{oxidizedantioxidant},$$
$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^-$$

or

$$Fe(CN)_6^{3-} + \text{antioxidant} \rightleftarrows Fe(CN)_6^{4-} + \text{oxidizedantioxidant}$$
$$Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe[Fe(CN)_6]^-$$

REAGENTS

See Appendix 1

PROCEDURE

1. Pippetted out 2ml (concentration varying from 0.02 to 0.1ml) of the sample into series of test tubes.
2. Added 2ml of phosphate buffer (pH = 6.6) and 2ml of 1% potassium ferri cyanide into all tubes.
3. To the 'blank' tube pipetted out 4ml of phosphate buffer and 2ml of 1% potassium ferri cyanide.
4. All the tubes were boiled at 50□ C for 20 minutes.
5. The reaction was arrested by adding 2ml of 10% TCA in all test tubes.
6. The tubes are centrifuged at 650g for 10 mintues and 4ml of supernatant was pippetted out.
7. To this added 0.8ml of 0.1% $Fecl_3$.
8. The blue color formed was colorimetrically read at 700nm and an increase in OD reading showed an increased antioxidant activity.

4.2 Modified CUPRAC (cupric ion reducing antioxidant capacity) assay

(Resat apak *et al.*, 2006)

PRINCIPLE

Antioxidants especially the hydroxyl groups of phenolic compounds are converted to their respective quinones [oxidized form] in the presence of cupric chloride (Cu^{2+}) which in turn is reduced to Cu^{+}. The formed Cu^{+} then reacted with the chromogen, neocuproine (2,9-dimethyl-1,10-phenanthroline) to form a yellow coloured complex which is spectrophotometrically measured at 450nm.

REAGENTS

See Appendix 2

PROCEDURE

1. Pippetted out 2ml (concentration varying from 0.02 to 0.1ml) of the sample into series of test tubes.
2. Added 1ml of cupric chloride, 1ml of ammonium acetate, 1ml of neocuproine and finally, 1ml of absolute ethanol into all test tubes.
3. To the 'blank' tube pippeted out 1ml of distilled water and 1ml of cupric chloride, 1ml of ammonium acetate, 1ml of neocuproine and finally, 1ml of absolute ethanol.
4. All the tubes were incubated at room temperature for 30 minutes.
5. The increase in the absorbance at 450 nm was used to measure the cupric ion reducing power of the salivary sample.

4.3 Cerium(IV) sulphate assay

(Resat apak *et al.*, 2007)

PRINCIPLE

Cerium(IV) sulphate assay is based on the oxidation of antioxidant compounds with Ce(IV) sulphate and the absorbance of unreacted Ce(IV) ion is measured at 320 nm. The decrease in the absorbance is directly proportional to the concentration of total antioxidants present in sample.

REAGENTS

See Appendix 3

PROCEDURE

1. One milliliter of 2.0×10−3M Ce(IV) solution was added to 1mL of salivary sample of varying concentration(200-1000µl/ml), and the mixture was diluted to 10mL with distilled water.
2. After shaking for a few minutes, the solution was let to stand for 30 min at room temperature.
3. The absorbance of the reaction mixture was measured at 320 nm against a blank composed of distilled water.
4. The decrease in the absorbance at 320 nm was used to measure the unreacted Ce(IV) ion in the salivary sample which indicates the increase in antioxidant power of the sample.

4.4 Glutathione reductase (GR) assay

(Carlberg and Mannevvik, 1975 and modified by Mohandas *et al.*, 1984)

PRINCIPLE

$$GSSG + \beta\text{- NADPH} \xrightarrow{GR} GSH + \beta\text{- NADP}$$

Glutathione is a tripeptide comprised of glu-cys-gly. It plays a major role as a redox buffer in scavenging the free radicals. The unusal structure of glutathione is such that the peptide bond is formed between the γ- carboxyl group of glutamate (side chain carboxyl), not the α- carboxyl group.

GLUTATHIONE

In this assay the oxidized glutathione (GSSG) was reduced to reduced glutathione (GSH) in the presence of glutathione reductase (GR). NADPH is the reducing agent where, the disulphide linkage (−S−S−) of GSSG will be reduced to thiol group (−SH) i.e., as GSH and NADP will be formed. A decrease in the UV absorption of NADPH at 340nm as a function of time is used to calculate the glutathione reductase activity.

REAGENTS

See Appendix 4

PROCEDURE

1. To 1.65ml of phosphate buffer, added 0.05ml of 1mM GSSG, 0.1ml of 1mM β- NADPH and 0.1ml of 0.5mM EDTA
2. To this reaction mixture added 0.05ml of the enzyme extract and vortexed for 10 s.
3. A decrease in the OD/30 seconds for about 3 minutes was monitored at 340nm by using (Beckman DU-530) UV–Vis Spectrophotometer.
4. A blank solution was used without the enzyme extract.

CALCULATION

$$\text{Units/ml enzyme} = \frac{(\text{OD of test} - \text{OD of blank}) \times 1.68 \times df}{6.22 \times 0.05}$$

1.68 = Total assay volume

6.22 = mM extinction co efficient of NADPH at 340nm

0.05 = Volume of enzyme extract

5. RESULTS AND DISCUSSION

5.1 Antioxidant assays

As originally defined by Sies (1986), oxidative stress is an imbalance between oxidants and antioxidants on a cellular or individual level. Oxidative damage is one result of such an imbalance and includes oxidative modification of cellular macromolecules, induction of cell death by apoptosis or necrosis, as well as structural tissue damage. Chemically speaking, oxidants are compounds capable of oxidizing target molecules. This can take place in three ways: abstraction of hydrogen, abstraction of electrons or addition of oxygen. All cells living under aerobic conditions are continuously exposed to a large numbers of oxidants derived from various endogenous and exogenous sources (Jens Lykkesfeldt, 2007). The endogenous sources of oxidants are several and include the respiratory chain in the mitochondria, immune reactions, enzymes such as xanthine oxidase and nitric oxide synthase and transition metal mediated oxidation. Various exogenous sources of ROS also contribute directly or indirectly to the total oxidant load. These include effects of ionizing and non-ionizing radiation, air pollution and natural toxic gases such as ozone, and chemicals and toxins including oxidizing disinfectants. A poor diet containing inadequate amounts of nutrients may also indirectly result in oxidative stress by impairing cellular defense mechanisms (Jens Lykkesfeldt, 2007).

Tobacco smokers constitute a convenient population to study effects of increased exogenous oxidative stress *in vivo* since they voluntarily inhale large amounts of toxic fumes as a result of their habit. Tobacco smoke contains large numbers of oxidants that damage several macromolecules like DNA, proteins and lipids (Jens Lykkesfeldt, 2007).

Exposure to passive smoke is clearly a health risk. This includes an enhanced exposure to carbon monoxide of a non smoking visitor in a recreational environment and in the workplace (Jo *et al.,* 2004). There is some

indication of an elevated breast cancer risk associated with passive smoking exposure of 5 h or more per day at work (overall risk = 1.6). Occupational exposure to tobacco smoke is damaging to children (Mpabulungi and Muula, 2004; Ross G. Cooper, 2006).

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas, 1997). Defense mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defenses, and (iv) antioxidant defences. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), αtocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Marian Valko *et al.,* 2006).

Saliva secretion, mainly from the parotid gland, is stimulated as food is chewed, and is the primary step in the digestive process. Saliva is a complex liquid comprising about 99% water, the remainder being mainly proteins, glycoproteins, electrolytes and small organic molecules. Saliva has multiple roles in relation to the gastrointestinal tract: bolus formation, enzymatic digestion, and buffering. It is also involved in the immunological defense system, which is based on the protein enzymatic defense process and the secreted immunoglobulins. Another significant function of saliva has been discovered, in that saliva acts as an antioxidant system comprising various molecules that it contains of which the main ones are uric acid, and the peroxidase enzymes (Gorelik, *et al.,* 2007). Salivary peroxidase is secreted by the salivary glands, and myeloperoxidases by polymorphonuclear neutrophils ,

both being heme-peroxidases that require reducing substrates for their catalytic cycle. To date the involvement of saliva in the oxidation process involved in food digestion has been little studied. It has been shown that human parotid saliva has antioxidant effects on lipid peroxidation of liposomal phospholipids and fish meat, and that it can reduce fatty acid hydroperoxides.

The redox state of any cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits (Marian Valko *et al.,* 2006). Antioxidants in the salivary sample may disrupt the Fe^{3+} to Fe^{2+} transformation by competing with $O_2^{\cdot-}$ and thereby causing a decrease in the formation of hydroxyl radicals which was measured as the reducing power of the sample. An increased absorbance as a function of increased volume was noticed and indicated saliva's high reducing potentiality. Statistical analysis at 5% level was proved to be significant ($p < 0.05$) for the salivary sample obtained from non smokers. The standard error was found to have a trifling deviation (0.0058) proving a high reducing power. A high correlation between the volume and the absorbance was observed according to Karl – Pearson Correlation of analysis (0.997). It was also observed that the smoker's samples possess a drastic decrease in the antioxidant activity and a high correlation (Karl – Pearson Correlation of analysis, 0.997) between the antioxidant capacity and smoking was observed.

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	1.40002E-5	1.40002E-5	0.02191	0.88408
Factor B	*1*	***0.0023***	***0.0023***	***3.59419***	***0.07511***
Model	2	0.0023	0.00116	1.80805	0.19412
Error	17	0.01086	6.9071E-4	-	-
Corrected Total	19	0.9318	-	-	-

Table 5.1.1 Two way ANOVA (Reducing power Assay)

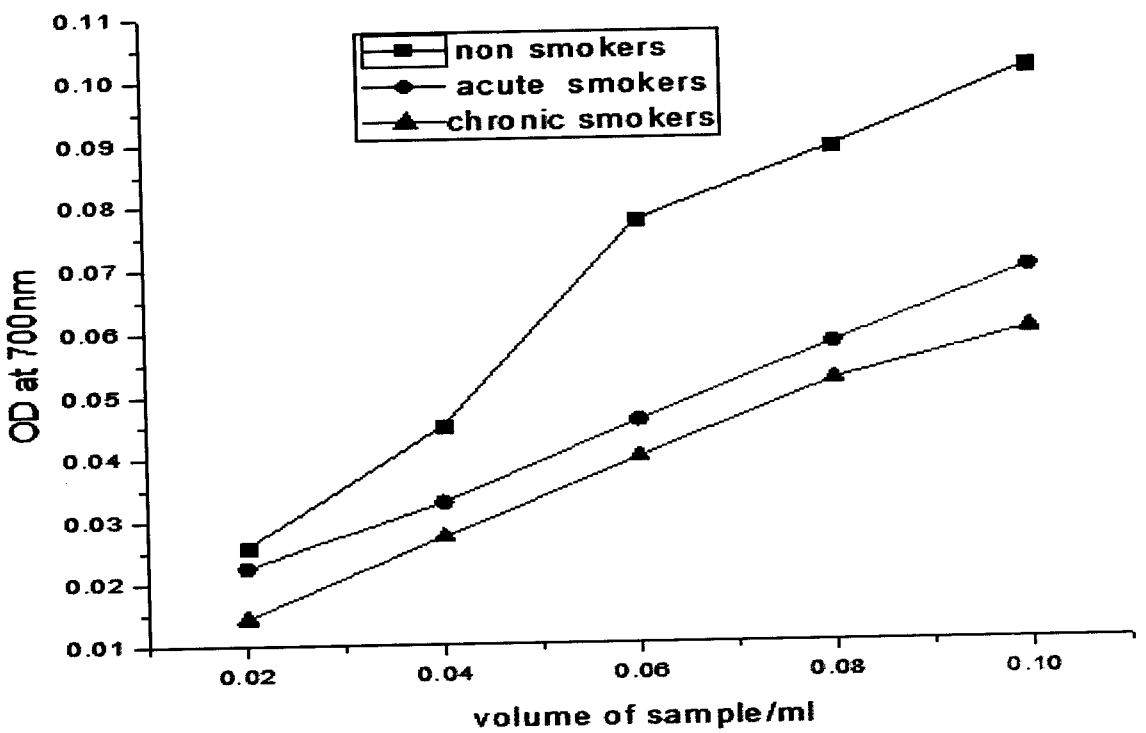

Figure 5.1.1 Effect of antioxidant activities of non-smokers, acute and chronic smokers(FRAP assay)

The production of hydroxyl radical occurs *in vitro* occurs even due to the presence of Cu^{2+} (Marian Valko *et al.,* 2006). In this aspect recently,(Res at Apak *et al.,* 2006) proposed a method to evaluate the cupric ion reducing power present in the samples. An increased absorbance as a function of increased volume was noticed and indicated saliva's high reducing potentiality. Statistical analysis at 5% level was proved to be significant ($p < 0.05$) for the salivary sample obtained from non smokers. The standard error was found to have a trifling deviation (0.0079) proving a high reducing power. A high correlation between the volume and the absorbance was observed according to Karl – Pearson Correlation of analysis (0.996). It was also observed that the smoker's samples possess a drastic decrease in the antioxidant activity and a high correlation (Karl – Pearson Correlation of analysis, 0.996) between the antioxidant capacity and smoking was observed.

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	7.74469E-4	7.74469E-4	1.17021	0.29447
Factor B	*1*	***0.01222***	***0.01222***	***18.46776***	***4.8768E-4***
Model	2	0.013	0.0065	9.81898	0.00146
Error	17	0.01125	6.61822E-4	-	-
Corrected Total	19	0.02425	-	-	-

Table 5.1.2 Two way ANOVA (Modified CUPRAC Assay)

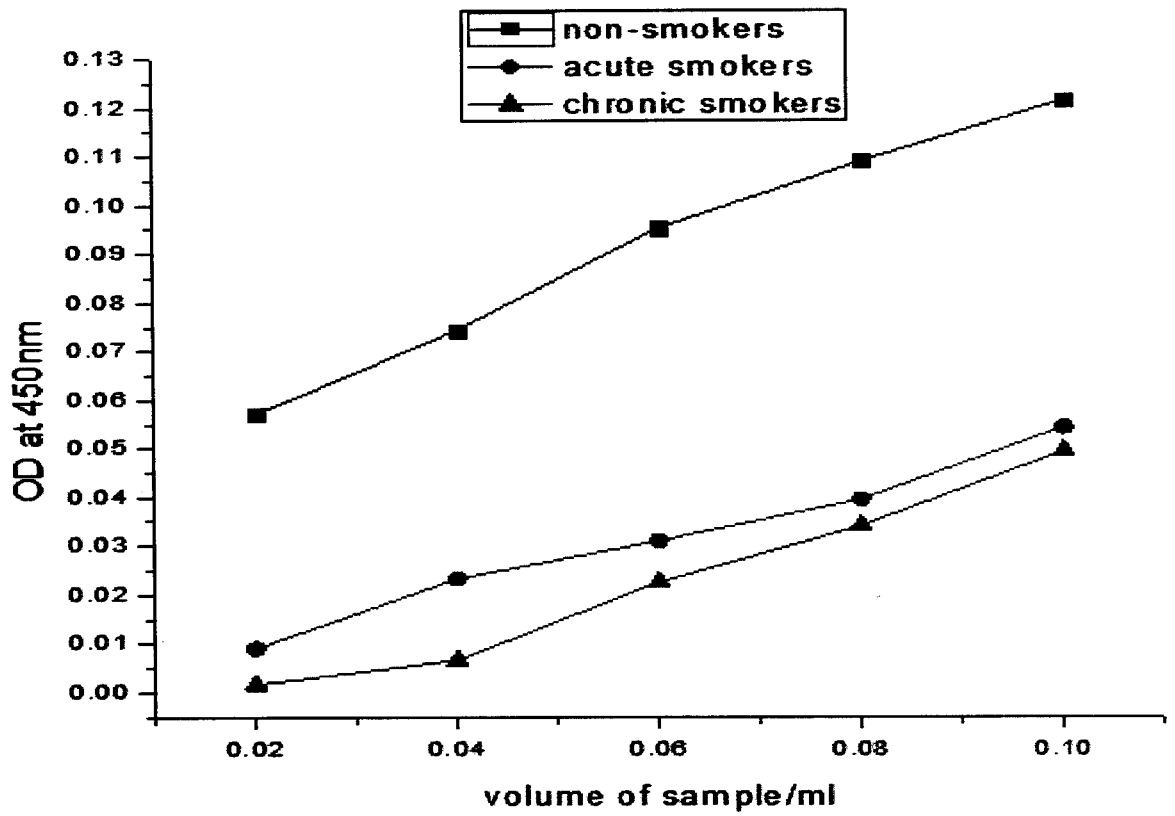

Figure 5.1.2. Effect of antioxidant activities of non-smokers, acute and chronic smokers (CUPRAC assay)

The production of hydroxyl radical occurs *in vitro* occurs even due to the presence of Mn^{+}(Marian Valko *et al.*, 2006). In this aspect recently (Res at Apak *et al.*,2006) proposed a method to evaluate the cerium ion reducing power present in the samples. An decreased absorbance as a function of increased volume was noticed and indicated saliva's high reducing potentiality. Statistical analysis at 5% level was proved to be significant ($p < 0.05$) for the salivary sample obtained from non smokers. The standard error was found to have a trifling deviation (0.079) proving a high reducing power. A high correlation between the volume and the absorbance was observed according to Karl – Pearson Correlation of analysis (0.998). It was also observed that the smoker's samples possess a drastic decrease in the antioxidant activity and a high

correlation (Karl – Pearson Correlation of analysis, 0.998) between the antioxidant capacity and smoking was observed.

	DF	Sum of Squares	Mean Square	F value	P value
Factor A	1	0.03727	0.03727	1.16998	0.29451
Factor B	*1*	***0.22542***	***0.22542***	***7.07582***	***0.0164***
Model	2	0.26269	0.13134	4.1229	0.03469
Error	17	0.54157	0.03186	-	-
Corrected Total	19	0.80426	-	-	-

Table 5.1.3 Two way ANOVA (Cerium (IV) sulphate Assay).

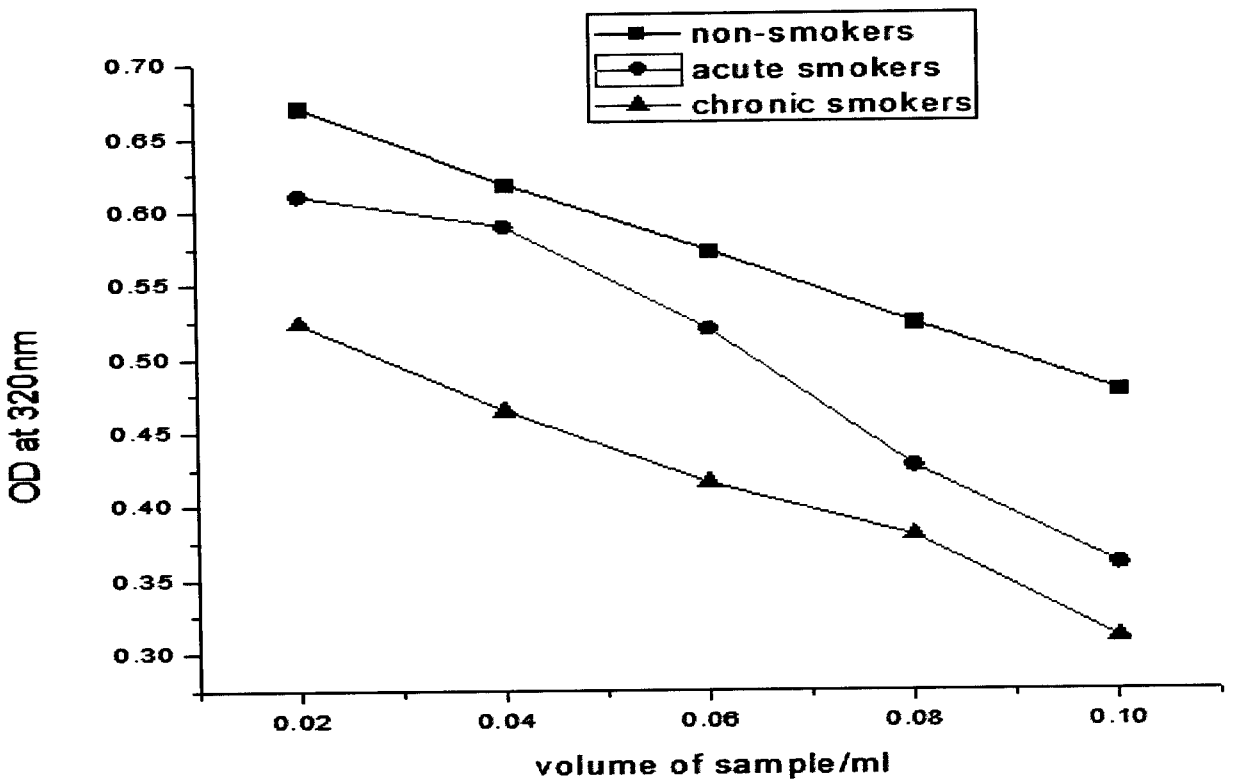

Figure 5.1.3 Effect of antioxidant activities of non-smokers, acute and chronic smokers(Cerium (IV) sulphate assay)

5.2 Glutathione reductase (GR) activity

The ubiquitous tripeptide glutathione (GSH), which is the most abundant low molecular weight thiol in almost all cells, is involved in a wide range of enzymatic reactions. A major function of GSH is to serve as a reductant in oxidation-reduction processes; a function resulting in the formation of glutathione disulfide (GSSG). It is well established in reduced form , and the reduction of 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 1.8.1.7). Therefore, it is of special significance to investigate the enzymatic reduction of GSSG (Ricard D. Mavis And Earle Stellwagen, 1968).

Shigeru Shigeoka et al.,(1987) reported that the glutathione reductase in *Euglena gracilis* retained full activity up to 67°C between pH 5.8 and 8.4. It was also reported that the activity was lost completely at 78°C. The optimum pH reported was 8.2 and optimum temperature was 52°C. Similarly, *Trachemys scripta elegans* (Turtle) glutathione reductase, possessed high activity under a broad pH range between pH 4 and 10.The optimum pH was 6.5 and the enzyme maintains activity under the pH drop that occurs under anoxic conditions. The high affinity of turtle GR suggests that turtles have high redox buffering capacity of tissues to protect against oxidative stress encountered during anoxia/reoxygenation. (William and Kenneth, 2007). The glutathione reductase from sheep liver had a specific activity under the optimal pH 8 at 60°C (Halis pakuroulu ,2005).

The investigation of optimum pH of salivary glutathione reductase revealed two peaks at 4.2 and 6.8. Further refined pH studies proved that the pH optimum was 6.8. It was also observed that the enzyme activity increases after 6.2 and then it shows maximum activity at pH 6.8 (Figure.4). However, the activity decreases sharply after pH 6.8 and completely vanished after pH 7.4. The substrate (both NADPH and GSSG) binding site amino acids present in the glutathione reductase was found to be mainly arginine, histidine, serine and glutamate. So, even a slight change in the $[H^+]$ level may protonate/ deprotonate the side chains of the above mentioned amino acids. This may lead to change in the three dimensional structure of the biological native enzyme and thereby a decrease in its activity.

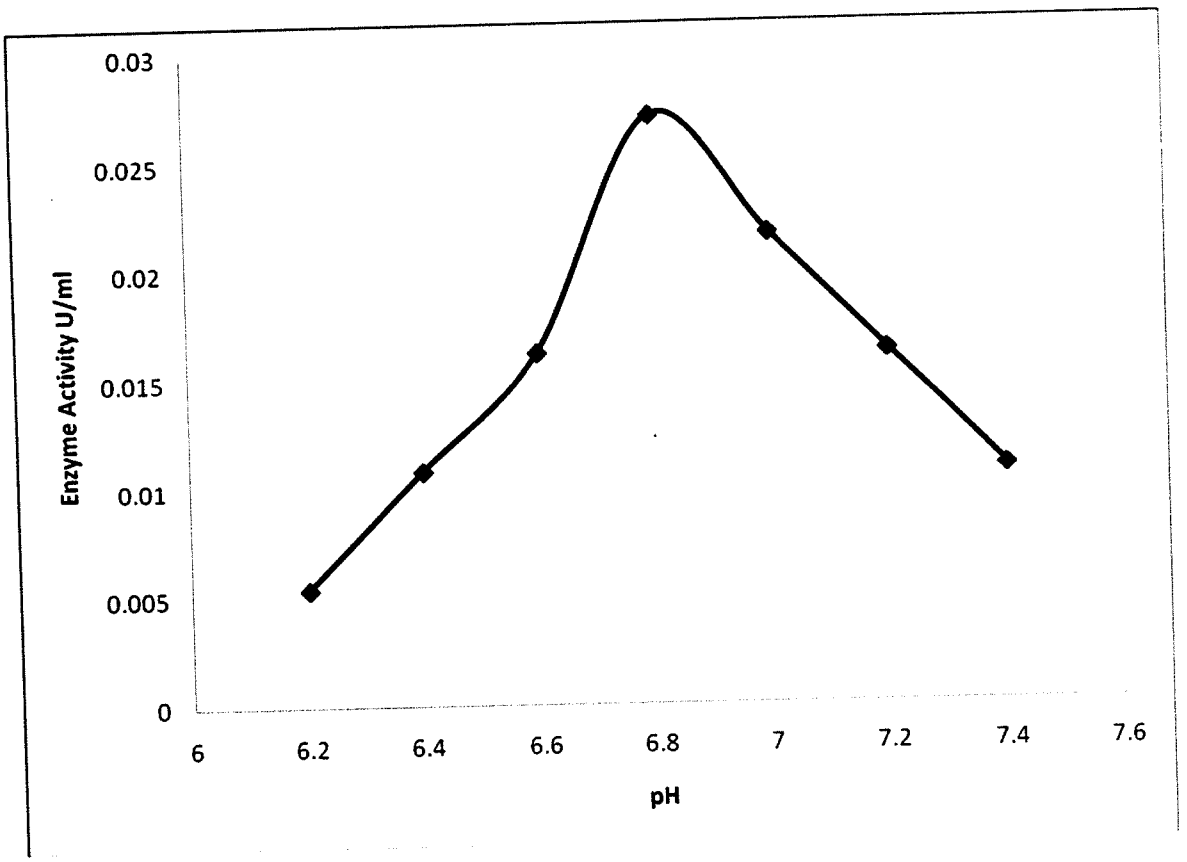

Figure 5.2.1. Effect of pH on salivary Glutathione reductase catalysed reaction

Thermal energy plays a vital role in maintaining the specific heat capacity of any compound. The normal homeostasis temperature in any higher class mammalian system was found to be 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 interactions 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 gets raised and this excess energy may be utilized for breaking the non-covalent interactions that maintained the three dimensional structure of an

enzyme. This drastically reduced an enzyme action. The studies on temperature optimum of salivary glutathione reductase proved to be 37°C. It was observed that the enzyme activitiy 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.

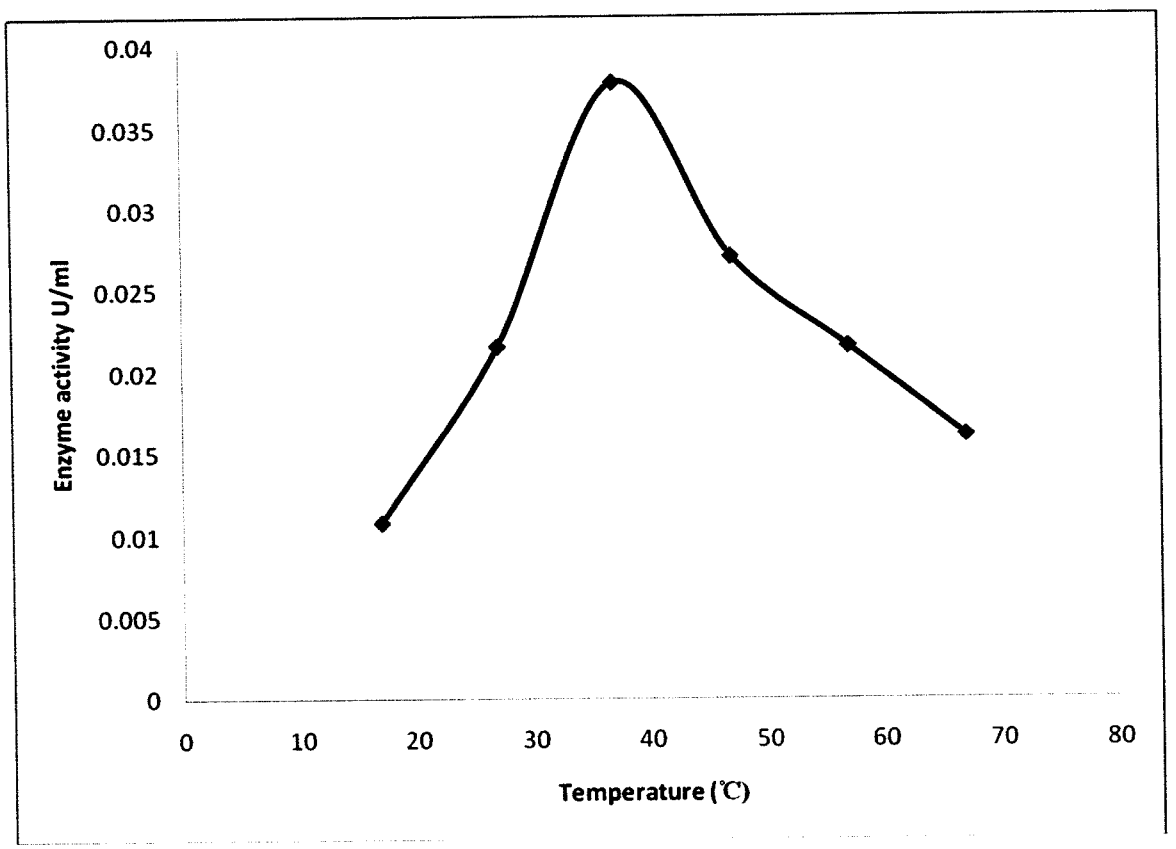

Figure 5.2.2 Effect of temperature on salivary Glutathione reductase catalysed reaction

Furthermore, the activity of enzyme with its own substrates was investigated at optimum pH and 37°C revealed that the K_m value was 0.058mM.

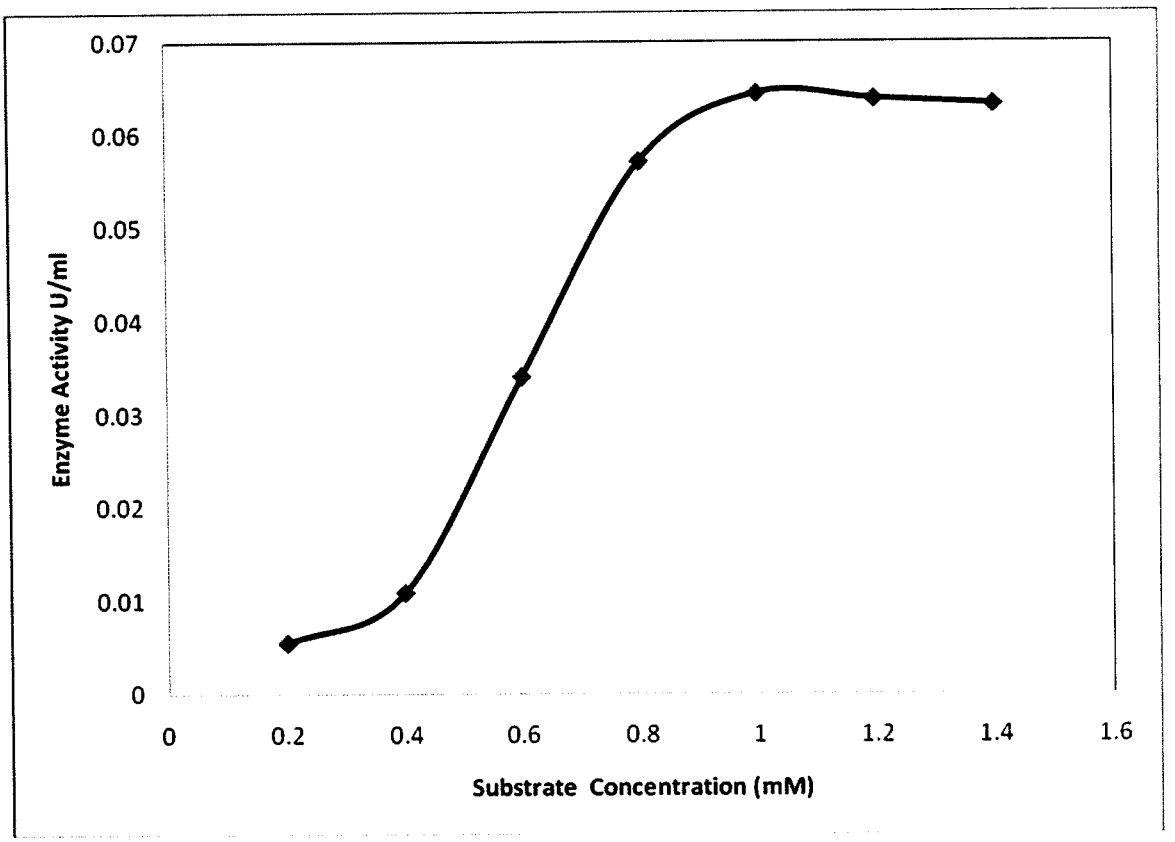

Figure 5.2.3. Effect of substrate concentration on salivary Glutathione reductase catalysed reaction

Smoking is by far the major risk factor for developing oral cancer. The use of alcohol and 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 activity of GR was measured in both smokers (20 yrs to 65 yrs of both genders) and non smokers

females was found to be 0.108U/ml and 0.097U/ml respectively, whereas in acute smokers the activity was found to be 0.0378 U/ml for both genders. Similarly the activity of the chronic smokers was found to be 0.0108U/ml. Statistical analysis using Students t-Test for the salivary samples obtained from non smokers was proved to be not significant at 5% level ($p < 0.05$) whereas it was proved to be significant between non smokers and smokers(acute and chronic). Furthermore it was proved to be significantly different between acute smokers and chronic smokers. A significant correlation (0.988) has been observed between the GR activity and the smokers showing a tremendous decrease indicating a risk for oral cancer.

	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	-	-	-

Table 5.2.1 Two way ANOVA (Glutathione Reductase Assay)

S.No	REAGENTS	COST (in Rupees)
1	GLUTATHIONE (OXIDIZED)	0.01
2	NADPH	3.98
3	EDTA	0.01
4	BUFFER	0.01
5	SAMPLE COLLECTION TUBE	1.00
6	GLOVES	1.00
7	MISCELLANOUS	3.00
	TOTAL	9.01

Table 5.2.2 Cost analysis per test

6. 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 saliva possess a significant antioxidant activity in non smokers compared to that of smokers which has been revealed by *in vitro* antioxidant assays. The pH optima of salivary glutathione reductase was found to be 6.8, the temperature optima was at 37°C and the K_m was found to be 0.058mM. The glutathione reductase activity was decreased rapidly in chronic smokers and a slight decrease was found in the acute smokers, proving the possibility that the enzyme can be utilized as a oral cancer detecting biomarker. This optimized protocol was found to be highly cost effective.

APPENDIX

APPENDIX 1

MODIFIED FERRIC ION REDUCING/ ANTIOXIDANT POWER ASSAY

(FRAP ASSAY)

a) Sample preparation

Mixed saliva (about 5 ml) was collected 2–3 h after breakfast. The pH of saliva was about 7.0. The saliva was centrifuged for 15 min at 16,000 rpm to remove cellular components. The supernatant obtained was used for experiments.

b) 0.2 M phosphate buffer

Mixed 26.5ml of Na_2HPO_4 with 73.5ml of NaH_2PO_4 [0.2 M Na_2HPO_4 = 35.6g/l , 0.2 NaH_2PO_4 = 31.2g/l].

c) 1% potassium ferric cyanide

Dissolved 1g of potassium ferricyanide in 100ml of distilled water.

d) 10% Tricarboxylic acid

Dissolved 10g of tricarboxylic acid and made upto 100ml with distilled water.

e) 0.1% Ferric chloride

Dissolved 0.1g of ferric chloride in 100ml of distilled water.

APPENDIX 2

MODIFIED CUPRAC (CURIC ION REDUCING ANTIOXIDANT CAPACITY) ASSAY

a) Sample preparation

Mixed saliva (about 5 ml) was collected 2–3 h after breakfast. The pH of saliva was about 7.0. The saliva was centrifuged for 15 min at 16,000 rpm to remove cellular components. The supernatant obtained was used for experiments.

b) 0.0075M Neocuproine (2,9-dimethyl-1,10-phenanthroline)

Dissolved 0.031g of neocuproine in 20ml of distilled water.

c) 0.01M Cupric chloride

Dissolved 0.034g of cupric chloride in 20ml of distilled water.

d) 1M Ammonmium acetate

Dissolved 1.54g of ammonium acetate in 20ml of distilled water.

e) Absolute ethanol

APPENDIX 3

CERIUM (IV) SULPHATE ASSAY

a) Sample preparation

Mixed saliva (about 5 ml) was collected 2–3 h after breakfast. The pH of saliva was about 7.0. The saliva was centrifuged for 15 min at 16,000 rpm to remove cellular components. The supernatant obtained was used for experiments.

b) 2.0×10−3M Ce(IV) solution

A cerium(IV) sulfate solution containing 2×10−3MCe(IV) was prepared by dissolving 0.0809 g $Ce(SO_4)_2 \cdot 4H_2O$ in 25ml distilled water, adding 17ml concentrated H_2SO_4 and thoroughly mixing by the aid of a magnetic stirrer until total dissolution at room temperature. This solution was totally transferred to a 100 ml flask, and diluted to the mark with distilled water.

APPENDIX 4

GLUTATHIONE REDUCTASE (GR) ASSAY

a) Sample preparation

Mixed saliva (about 5 ml) was collected 2–3 h after breakfast. The pH of saliva was about 7.0. The saliva was centrifuged for 15 min at 16,000 rpm to remove cellular components. The supernatant obtained was used for experiments.

b) 0.1M phosphate buffer (pH = 7.4)

Sodium dihydrogen phosphate = 0.16g in 10ml of distilled water **(A)**

Disodium hydrogen phosphate = 0.45g in 25ml of distilled water **(B)**

Mixed 5ml of (A) with 20ml of (B) and made upto 50ml with distilled water.

c) 1mM Oxidized glutathione (GSSG)

Dissolved 15mg of oxidized glutathione in 25ml of distilled water

d) 1mM β- NADPH

Dissolved 17mg of β- NADPH in 20ml of distilled water

e) 0.5mM EDTA

Dissolved 19mg of EDTA in 100ml of distilled water

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