Biomedical & Pharmacology Journal Vol. **8**(Spl. Edn.), 453-460 (Oct. 2015)

Role of Free Radicals in Oral Carcinogenesis : A Review

T. MANIGANDAN, SARUMATHI and A. AMUDHAN

Department of Oral Medicine and Radiology, Sree Balaji Dental College and Hospital, Bharath University, Chennai, India.
*Corresponding author E-mail: manident@yahoo.com

DOI: http://dx.doi.org/10.13005/bpj/722

(Received: July 25, 2015; accepted: September 10, 2015)

ABSTRACT

Free radical is a molecule or molecular fragment containing an unpaired electron in the valence shell (i.e. radical) and capable of existing independently (i.e. free)[1]. They are unstable, short lived and highly reactive. Oxidative stress is increased and antioxidant defences are compromised in patients with oral cavity cancer. Free radical has a definitive role in cacinogenesis which can also serve as a Potential biomarker for measuring oxidative stress levels in saliva and serum of patients affected with oral cancer and in preventing the possibilities of turning of potentially pre-malignant disorders into malignant disorders. It is necessary to maintain a balance between free radicals and antioxidant defensive mechanism, to prevent the outbreak of Oral Cancer.

Key words: Free radical, oral cancer, Carcinogenesis, biomarker, reactive oxygen species, reactive nitrogen species

INTRODUCTON

Free radical is a molecule or molecular fragment containing an unpaired electron in the valence shell (i.e. radical) and capable of existing independently (i.e. free)[1]. They are unstable, short lived and highly reactive. Generally, it reacts with the nearest stable molecule, "stealing" its electron to gain stability. The attacked molecule looses its electron, it becomes a free radical itself, beginning a chain reaction cascade resulting in disruption of a living cell.

Oxygen is required in many metabolic reactions, particularly for the release of energy. During these processes, molecular oxygen is completely reduced and converted to water. However, if the reduction of oxygen is incomplete, a series of reactive radicals are formed. Reactive oxygen species play an important role in cell signaling and metabolic processes, but also contribute to pathogenic processes in a variety of inflammatory disorders[2,3,4,5,6]. Healthy individuals maintain a balance between the reactive oxygen species and antioxidants.

Free radicals have been implicated in the causation and progression of several diseases such as Cardiovascular disease, Cancer, Inflammatory diseases, Respiratory diseases, Diabetes, Cataract formation, Male infertility, Aging process, Other diseases: Parkinson's disease, Alzheimer's disease, multiple sclerosis, liver cirrhosis, muscular dystrophy, toxemia of pregnancy, etc.

In 1954, Gerschman and colleagues for the first time proposed that damaging effects of oxygen could be attributed to formation of oxygen free radicals[7]. ROS include both free radicals as well as non-radical derivatives of oxygen[8]. The relation between free radicals and disease can be explained by the concept of 'oxidative stress' elaborated by Sies[9]. He defined oxidative stress as an imbalance between oxidants and antioxidants in favour of oxidants, potentially leading to damage[10]. Products of biological damage are referred as biomarkers of oxidative stress[11].

Most Reactive Oxygen Species (ROS) are generated as by-products during mitochondrial electron transport. In addition they are formed as necessary intermediates of metal catalyzed oxidation reactions. Reactive Nitrogen Species (RNS) are formed from interactions of NO with O_2 or O_2^- resulting in formation of dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$).[12]There are two sources of free radicals: endogenous sources and exogenous sources. The endogeneous sources are produced within the body due to physiologic process likemitochondrial respiration- generates superoxide anion radical, enzymatic reactions, respiratory burst and strenuous exercise. Exogeneous source of free radicals include air pollutants, tobacco smoke, drugs, alcoholic beverages, etc.

Classification of free radicals[13]
First classification

1. sigma,
2. pi-delocalized,
3. carbon-centered,
4. oxygen-centered,
5. sulfur-centered,
6. nitrogen-centered,
7. reducing radicals,
8. oxidizing radicals and
9. other reactive molecules in which the reactivity center is nitrogen.

Second classification :

1. Hydroperoxyl (per hydroxyl) radical
2. Superoxide radical
3. Hydrogen peroxide
4. Singlet oxygen and triplet oxygen.

Important physiological roles of reactive oxygen species

1. Free radicals help with cell growth and cell proliferation
2. Free radicals help with cell division
3. Regulate redox balance of the cell
4. Signal transduction
5. Activate protein kinases that regulate gene functions
6. Regulate immune function

Relation between Free Radicals induced Oxidative Stress& Oral Cancer

Oral squamous cell carcinoma (OSCC) is the sixth most common human cancer, with an increasing incidence in younger people, a high morbidity rate, and a 5-year mortality rate of about 50%.[14-19] .Free radicals, such as ROS and RNS, which induce oxidative and nitrative stress, are principal inducers of OSCC. Ma et al.[20] demonstrated that oxidative and nitrative stress contribute to the development of oral carcinogenesis from leukoplakia through DNA damage. RNS in the form of nitrosamines (NO_3 and NO_2) and ROS such as superoxide radicals ($O_2^\wedge$), hydroxyl radicals (OH^), and hydrogen peroxide (H_2O_2), play a key role in human cancer development because they can cause DNA base alterations, strand breaks, damaged tumor suppressor genes, and an enhanced expression of protooncogenes.

ROSinduced mutation could also result from protein damage[21,22]

Salivary nitrosamine production and metabolism are also based on the dietary nitrates (NO3), which are absorbed from the upper gastrointestinal tract and actively concentrated from the plasma into the saliva by the salivary glands through an active transport system similar to that for iodide, thiocyanate, and perchlorate.[23] In the oral cavity the salivary nitrates are turned into nitrites (NO_2), which are of special importance as carcinogenesis promoters because they react with amines and amides to form the carcinogenic nitrosamines.[24,25]

The OSCC-inducing ROS and RNS originate mainly from smoking, alcohol, food, drink, and/or various other volatile sources, which enter freely into the oral cavity through the largest open gate of the body, the mouth. It is of no surprise that evolution armed the oral cavity with an advanced salivary antioxidant system that also contains antinitrosamine inhibitory agents.[26] This salivary antioxidant system is based on enzymatic and nonenzymatic components including peroxidase and superoxide dismutase (SOD) enzymes as well as uric acid (UA)molecules.[27] It also includes another pivotal anticancer salivary enzyme, glutathione S-transferase (GST), which catalyzes glutathione conjugation to the carcinogen electrophilic epoxide intermediates to protect against DNA damage and adduct formation.[28]

Free Radical and Cancer

ROS and RNS are involved in the initiation and promotion of multistep carcinogenesis, both are inhibited by antioxidants.[29,30] However, when the equilibrium is broken either by a reduction in the levels of antioxidants or by enhancement of ROS and RNS levels, DNA is oxidized and cancer evolves. Oral cancer is essentially an event occurring at the gene level, with DNA damage being the final step.

Free Radicals & Carcinogenesis:Initiation, Promotion & Progression

Carcinogenesis is a multistage process definable by at least three steps or stages: Initiation, promotion, and progression, and ROS are found to be involved in all these stages. It participates in the above said stages, by way of causing DNA damage, activating procarcinogens, initiating lipid peroxidation, inactivating enzyme systems and altering the cellular antioxidant defense system.[31]Peroxidation of membrane lipids generates peroxides that decompose to form multiple mutagenic carbonyl products. Lipid hydroperoxides (LHP) and Malondialdehyde (MDA) are well characterized lipid peroxidation end products. They interact with cellular DNA and cause the formation of DNA MDA adducts, causing DNA damage and interference with its repair.[32] Measurement of lipid

peroxidation and antioxidants is therefore valuable in assessing tumor burden at various stages of oral cancer. In addition, it is also important to identify the most influencing parameter, which may take us to a more specific therapeutic intervention.

ROS & 3 Steps to cancer[12]

The effect of oxidative stress at a certain stage of carcinogenesis is directly proportionate to the type and the reactivity of radicals involved. Initiation results when a normal cell sustains a DNA mutation that, when proceeded by a round of DNA synthesis, results in fixation of the mutation, producing an initiated cell. Initiation of cancer by ROS is supported by presence of oxidative DNA modifications in cancer tissues.

The promotion stage is characterized by clonal expansion of initiated cells, by induction of cell proliferation and/or inhibition of apoptosis. Oxidative stress is strongly involved in this stage. ROS can stimulate expansion of mutated cell clones by temporarily modulating the genes which are related to proliferation or cell death and by regulating activity of certain transcription factors such as NFêB, Nrf2, HIF, and p53 which control cell growth and oncogenesis. It can lead to NFêB activation, with subsequent induction of genes encoding for proteins that inhibit apoptosis. It can also act at signal-transduction level to exert pro-survival functions. Oxidative stress can activate ERK/MEK and PI3K/AKT pathways. This could result in inactivation of proapoptotic proteins and upregulation of antiapoptotic genes. A low level of oxidative stress can stimulate cell division in promotion stage and thus promotes tumour growth. This implies that ROS production during this stage is the main mechanism of ROS-related tumour promotion.

Progression. In this stage, generation of large amounts of ROS may contribute to mutate, inhibit antiproteisases, upregulate matrix metalloproteinases (MMPs) and injure local tissues. Increased levels of oxidatively modified DNA bases may contribute to genetic instability and metastatic potential of tumor cells in fully developed cancer. ROS is reported to be crucial for triggering angiogenic response, which is important in cancer metastasis.

This suggests that ROS is involved in all these stages of carcinogenesis. ROS, which are formed through various events and pathways, react with and damage cellular components and contribute to neoplastic transformation. Here is an overview of this (Fig. 1)[12].

Free Radical Damage to DNA and Cancer

DNA is a major target of free radical damage. The types of damages induced are many and include strand breaks (single or double strand breaks), various forms of base damage yielding products such as 8-hydroxyguanosine, thymine glycol or abasic sites, damage to deoxyribose sugar as well as DNA protein cross links. These damages can result in mutations that are heritable change in the DNA that can yield cancer in somatic cells or

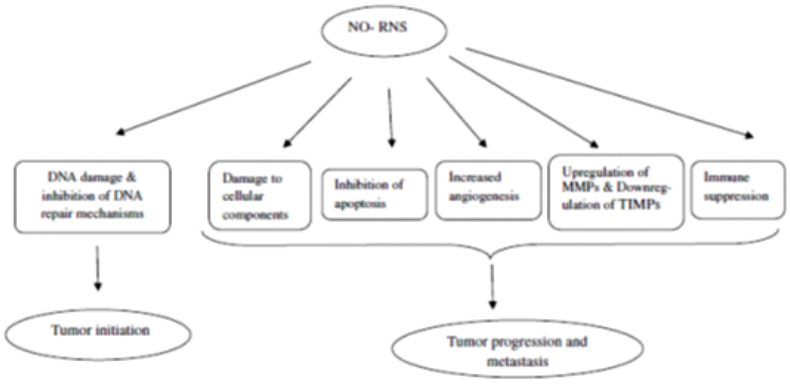

Fig. 1:

foetal malformations in the germcells. The involvement of free radicals with tumor suppressor genes and proto-oncogenes suggest their role in the development of different human cancers (Halliwell and Aruoma, 1993).[33]

Cancer develops through an accumulationof genetic changes. Initiating agents can be tobacco smoking and chewing, UV rays of sunlight, radiation, viruses, chemical pollutants, etc. Promoting agents include hormones (androgens for prostate cancer, estrogens for breast cancerand ovarian cancer). Inflammation induces iNOS (inducible nitric oxide synthase) as well as COX and LOX. These can initiate carcinogenesis.

ROS/RNS can have following DNA damaging effects

(1) Cause structural alterations in DNA, e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification. ROS can produce gross chromosomal alterations and thus could be involved in inactivation or loss of second wild-type allele of a mutated proto-oncogene or tumour-suppressor gene that can occur during tumour promotion and progression, allowing expression of mutated phenotype.

(2) Affects cytoplasmic and nuclear signal transduction pathways.

(3) Modulates activity of proteins and genes that respond to stress and which act to regulate genes that are related to cell proliferation, differentiation and apoptosis.

(4) RNS such as $NO\ddot{y}_2$, ONOO^ , N_2O_3 and HNO_2 are mutagenic agents, with the potential to produce nitration, nitrosation and deamination reactions on DNA bases.

(5) Exposure of cells to H_2O_2, and perhaps other oxidants, suppress DNA repair in addition to inducing damage. Reduced repair will result in elevated DNA lesions and an increased risk of disease.

ROS/RNS mediated DNA damage may participate in carcinogenesis via activation of protooncogenes and inactivation of tumor suppressor genes. In terms of oxidative DNA damage, major interest has focused on modifications of DNA bases. One of the most frequent base modifications is 8-hydroxy-deoxyguanosine (8 oxodG). This base modification formation increases by 35–50% in individuals using tobacco smoke – a well known carcinogenic source of ROS. Accumulation of 8-nitroguanine, which is a potentially mutagenic DNA lesion, and 8-oxodG is found in tissues of patients with oral lichen planus (OLP) oral squmaous cell carcinoma (OSCC) and leukoplakia , whereas no immunoreactivity was observed in normal oral mucosa. Kawanishi et al. from their study concluded that formation of 8-nitroguanine and 8-oxodG may contribute to development of oral cancer from OLP and leukoplakia. They also demonstrated that iNOS dependent DNA damage may lead to p53 accumulation in OLP, leukoplakia and OSCC[61]. All these findings suggest that oxidative and nitrosative DNA damage may be responsible for initiation and promotion of oral carcinogenesis and can be used

as potential biomarkers to evaluate the risk of oral cancer in potentially malignant disorders.

Mitochondrial DNA damage

ROS mediated deletions and mutations in mitochondrial DNA (mtDNA), accumulate with age at a higher rate than in nuclear DNA. mtDNA is more vulnerable to free radical damage because of lack of histone proteins as well as its location is in close proximity to the respiratory chain and thus is frequently exposed to ROS-induced oxidative damage. Moreover, mtDNA repair is less complete than chromosomal DNA repair making it an important contributor to carcinogenesis.

In linking oxidative stress with promotion, it must not be forgotten that biomolecules other than DNA may be oxidatively modified which may have significant effects in carcinogenesis.

Oxidative damage to proteins

Studies have proved that proteins are major initial cell targets of ROS. Oxidative damage of proteins involves loss of histidine residues, oxidative scission, introduction of carbonyl groups, and formation of protein-cantered alkyl, Rÿ, alkoxyl, ROÿ, and alkylperoxyl, ROOÿ, radicals. Protein oxidation is connected with formation of inter- and intra-protein cross linkages. It may result in fragmentation, cross-linking, and aggregation of proteins. Amino acid residue side chains are very susceptible to attack by ROS and RNS. Radical-protein interaction can damage functions of some important proteins such as DNA repair enzymes, which can lead to increased frequency of mutations. Proteins oxidation leads to earlier formation of protein carbonyls in biological systems, and increased levels of protein carbonyls has been proposed as ‘‘a sign of disease-associated dysfunction”.

Advanced oxidative protein products (AOPP), are generated by different oxidation patterns that lead to production of either NO or, H2O2 which sets a cascade of reactions with potential to damage cellular micro-molecules. Nayar et al. in their study found high serum levels of AOPP in patients with speckled leukoplakia and OSCC as compared to healthy individuals[34]. It is required to find whether AOPP and other protein oxidation products can be used as reliable oral cancer biomarkers.

Oxidative damage to lipids

Cell membranes are very sensitive to ROS damage. Methylene group between two double bonds of polyunsaturated fatty acid (PUFA) in cell membranes make them more sensitive to oxidation. ROS induced lipid peroxidation of cell membranes has been implicated in malignant transformation. It results in formation of reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxy- 2-nonenal (4-HNE), which demonstrate high reactivity with proteins and DNA. Aldehyde end-products of lipid peroxidation can bind to DNA and are potentially mutagenic. Lipid peroxides can decompose to a range of mutagenic carbonyl products. MDA has been found to be mutagenic in bacterial and mammalian cells and carcinogenic in rats[42]. Levels of these lipid peroxides can serve as markers of cellular damage due to free radicals. Increased levels of lipid peroxidation products such as lipid hydroperoxides, 4-HNE and MDA have been reported in oral cancer and precancer patients. This could be due to increased formation of free radicals which suggest that there may be relationship between free radical activity and malignancy.

Oxidative modification of sugars [Advanced glycation endproducts (AGEs)] and ROS mediated damage to extracellular components can also occur. Whether these have any relation with oral cancer needs to be evaluated. Along with ROS, RNS is also known to play role in carcinogenesis process.

Mechanism of action of RNS in cancer

NO^{-}, an abundant reactive radical, acts as an important oxidative biological signalling molecule in various physiological processes. It becomes genotoxic and mutagenic when generated at higher concentrations for prolonged periods of time. Reaction of NOÿ with oxygen or other free radicals generates RNS, which causes multiple biological effects. NO may mediate DNA damage through formation of carcinogenic nitrosamines, generation of RNS and inhibition of DNA damage repair mechanisms. It can thus be considered a tumour initiating agent. It may also have an impact on other stages of cancer

development by inhibiting apoptosis, promoting angiogenesis, by modulating host defence mechanisms, and by interacting with MMPs . NO has also been implicated in oral carcinogenesis. The high incidence of oral cancer and precancer has been linked with tobacco chewing and smoking habits. NO radicals released from tobacco-related compounds were shown to cause nitrosative stress and DNA strand breaks in immortalized hamster cheek pouch cells. Damage to genes sustained by elevated ROS/RNS could be one of the mechanism by which cancer arises in long-term tobacco abuse. NO products and NOS enzymes have been found to be raised in blood and tissues of oral cancer patients. Raised levels of NO products are also noted in serum of oral precancer patients and in healthy individuals with tobacco habit. Thus NO may serve as a biomarker for estimation of oral cancer risk in patients with potentially malignant disorders or in individuals with tobacco habit.

Oxidative Stress In Oral Cancer And Precancer

Tobacco (smoking and smokeless) use and excessive consumption of alcohol are amongst the major risk factors for oral cancer. ROS has been implicated in oral cancer development in tobacco chewers and smokers. Oxidative stress is increased and antioxidant defences are compromised in patients with oral cavity cancer . In vitro studies have demonstrated that free radicals are produced when human oral epidermal carcinoma cells are incubated with smokeless tobacco extract. There is continuous endogenous damage to cellular DNA by free radicals generated by the use of tobacco, and accumulation of such damage plays significant role in oral carcinogenesis. Tobacco chewing and smoking causes oxidant/ antioxidant imbalance which elevates oxidative stress. This is accompanied by increased lipid peroxidation, oxidative DNA damage, damage to macro and micro-molecules of cells and disturbances of antioxidant defence which can induce malignant process. The heat (generated during smoking) as well as pH (change during chewing) of body fluids due to tobacco consumption affects formation and stabilization of free radicals. Furthermore, free radicals produced during auto-oxidation of areca nut-polyphenols in saliva of tobacco users are crucial in initiation and promotion of oral cancer. This establishes the role of ROS in oral cancer in tobacco users. Areca (betel) nut chewing is the most important environmental factor for oral cancer in South and Southeastern Asians. Areca nut is identified as a human group I carcinogen. Areca ingredients induce formation of ROS and DNA adducts. Alkaline conditions observed in betel nut chewing are favourable for formation of free radicals . Hsuan-Hsuan Lu in their study confirmed a significant increase in ROS for

Fig. 2:

areca nut extract-treated OSCC cells[35].suggesting role of areca nut in oral carcinogenesis through generation of ROS as one of the mechanisms.

Alcohol increases oral cancer risk in humans regardless of the form it takes. Petti and Scully in their extensive study found a strong association between alcohol-drinking profiles and oral cancer mortality[36] . Free radicals are produced in excessive amounts in alcoholics. Ethanol is oxidised by Cytochrome P450 2E1 (CYP2E1) to acetaldehyde which is further oxidised to acetate. Chronic ethanol ingestion can induce single nucleotide polymorphism of CYP2E1. Resulting increased CYP2E1 activity leads to increased generation of ROS which leads to lipid peroxidation and its products such as 4-hydroxynonenal (4HNE), which binds to DNA to form mutagenic adducts . Vitamin C deficiency and marked tissue depletion of vitamin E found in alcoholics is due to their increased utilization for scavenging free radicals produced in chronic alcoholics. All this suggest that in alcoholics there is oxidant–antioxidant imbalance resulting from increased oxidative stress and weakened antioxidant system which may result in initiation of oral cancer.Fig. 2[6] shows role of ROS in oral carcinogenesis.

CONCLUSION

It is clear that free radical has a definitive role in cacinogenesis. Free radicals can also serve as a Potential biomarker for measuring oxidative stress levels in saliva and serum of patients affected with oral cancer and in preventing the possibilities of turning of potentially pre-malignant disorders into malignant disorders. It is necessary to maintain a balance between free radicals and antioxidant defensive mechanism, to prevent the outbreak of Oral Cancer.

REFERENCES

1. Mukherji SM, Singh SP. Free radicals. In: Mukherji SM, Singh SP, editors. Reaction mechanism in organic chemistry. Madras: Macmillan India Press; . 285 (1984).
2. Bloomer RJ. Decreased blood antioxidant capacity and increased lipid peroxidation in young cigarette smokers compared to nonsmokers: impact of dietary intake. *J Nutr* **6**: 39–42 (2007).
3. Rao V. Bioavailability and in vivo antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer. *Nutr Cancer* **31**:199-203 (1998).
4. Prasad KN, Kumar A. High doses of multiple antioxidant vitamins: Essential ingredients in improving the efficacy of standard cancer therapy. *J Am Coll Nutr* **18**: 13-25 (1999).
5. Singh, Gaby. Premalignant lesions: Role of antioxidant vitamins and beta carotene in the risk reduction and prevention of malignant transformation. *Am J Clin Nutr* **53**: 386S-90S (1991).
6. Kaugars, Silverman, Lovas. A clinical trial of antioxidant supplements in the treatment of oral leukoplakia. *Oral Surg Oral Med Oral Path* **78**: 462-8 (1994).
7. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. Oxygen poisoning and X-ray radiation: a mechanism in common. *Science* **119**: 623-6 (1954).
8. Shalu Rai, Rohit Malik, Deepankar Misra, Akshay Sharma. Future prospective and current status of antioxidants in premalignant and malignant lesions of oral cavity. *International Journal of Nutrition, Pharmacology, Neurological Diseases* **4**(4): 198-202 (2014).
9. Sies H. Biochemistry of oxidative stress. *Angew Chem Int Ed Ingl* **25**(12): 1058–71 (1986).
10. Sies H. Oxidative stress: from basic research to clinical application. *Am J of Med* **91**: 31S–8S (1991).
11. Sies H. Oxidative stress: introductory remarks. In: Sies H, editor. Oxidative stress. London: Academic Press; 1-8 (1985).
12. Sheetal Korde Choudhar et al. Oxidative and antioxidative mechanisms in oral cancer and precancer: A review. *Oral Oncology,* **50**:10–18 (2014).
13. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd ed. Oxford: Clarendon Press (1989).
14. Kantola S, Parikka M, Jokinen K, et al.

Prognostic factors in tongue cancer—relative importance of demographic, clinical and histopathological factors. *Br J Cancer.* **83**: 614–619 (2000).
15. Alho OP, Kantola S, Pirkola U, Laara E, Jokinen K, Pukkala E. Cancer of the mobile tongue in Finland—increasing incidence, but improved survival. *Acta Oncol.* **38**: 1021–1024 (1999).
16. Myers JN, Elkins T, Roberts D, Byers RM. Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. *Otolaryngol Head Neck Surg*. **122**:44–51 (2000).
17. Ribeiro KC, Kowalski LP, Latorre MR. Impact of comorbidity, symptoms, and patients' characteristics on the prognosis of oral carcinomas. *Arch Otolaryngol Head Neck Surg.* **126**: 1079–1085 (2000).
18. Sparano A, Weinstein G, Chalian A, Yodul M, Weber R. Multivariate predictors of occult neck metastasis in early oral tongue cancer. *Otolaryngol Head Neck Surg.* **131**: 472–476 (2004).
19. Hinerman RW, Mendenhall WM, Morris CG, Amdur RJ, Werning JW, Villaret DB. Postoperative irradiation for squamous cell carcinoma of the oral cavity: 35-year experience. *Head Neck*. **26**: 984–994 (2004).
20. Ma N, Tagawa T, Hiraku Y, Murata M, Ding X, Kawanishi S. 8-Nitroguanine formation in oral leukoplakia, a premalignant lesion. *Nitric Oxide.* **14**:137 143 (2006).
21. Patel RP, McAndrew J, Sellak H, et al. Biological aspects of reactive nitrogen species. *Biochim Biophys Acta*. **1411**: 385–400 (1999).
22. Liu RH, Hotchkiss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutat Res.* **339**: 73–89 (1995).
23. Tenovou J. The biochemistry of nitrates, nitrites, nitrosamines and other potential carcinogens in human saliva. *J Oral Pathol.* **15**: 303–307 (1986).
24. Xia DS, Deng DJ, Wang SL. Destruction of parotid glands affects nitrate and nitrite metabolism. *J Dent Res*. **82**: 101–105 (2003.
25. Deng DJ. Progress of gastric cancer etiology: N-nitrosamides 1999s. *World J Gastroenterol*. **6**: 613–618 (2000).
26. Stich HF, Rosin MP, Bryson L. The inhibitory effect of whole and deproteinized saliva on mutagenicity and clastogenicity resulting from a model nitrosation reaction. *Mutat Res.* **97**: 283–292 (1982).
27. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Reznick AZ. Characterization of the differentiated antioxidant profile of human saliva. *Free Radic Biol Med*. **32**: 268–277 (2002).
28. Sturgis EM, Wei Q. Genetic susceptibility—molecular epidemiology of head and neck cancer. *Curr Opin Oncol.* **14**: 310–317 (2002).
29. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic Biol Med*. **8**: 583–599 (1990).
30. Oberley LW, Oberley TD. Role of antioxidant enzymes in cell immortalization and transformation.*Mol Cell Biochem.* **84**:147–153 (1988).
31. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic Biol Med* **8**: 583 99 (1990).
32. Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. *Toxicol Pathol* **38**:96 109 (2010).
33. Halliwell B and Aruoma OI. (eds) DNA and Free Radicals, Boca Raton Press (1993).
34. Nayyar AS, Mubeen Khan M, Vijayalakshmi KR, Suman B, Gayitri HC, Anitha M. Serum total protein, albumin and advanced oxidation protein products(AOPP) – implications in oral squamous cell carcinoma. *Malaysian. J Pathol* **34**:47–52 (2012).
35. Lu HH, Kao SY, Liu TY, Liu ST, Huang WP, Chang KW, et al. Areca nut extract induced oxidative stress and upregulated hypoxia inducing factor leading to autophagy in oral cancer cells. *Autophagy* **6**(6): 725–37 (2010.
36. Petti S, Scully C. Oral cancer: the association between nation-based alcohol drinking profiles and oralcancer mortality. *Oral Oncol* **41**(8): 828–34 (2005).