Original Article

Comparative evaluation of salivary, serum and urinary 8-OHdG in gutka-associated oral submucous fibrosis

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Abstract

Background: Gutka chewing is the most common deleterious oral habit prevalent in the geographical distribution of the Indian subcontinent. Gutka leads to the production of numerous free radicals, which causes oxidative stress in regional oral tissues. Oxidative stress brings about the oxidation of guanine bases of DNA that generates 8-OHdG as its main byproduct. The presence of 8-OHdG can be evaluated not only in tissue but also in saliva, blood and urine. The availability of 8-OHdG in these samples is quite documented. In addition, a comparative assay of 8-ohdg DNA damage marker in multiple samples is yet to be done.

Material and Methodology: A sample size of 60 was divided into two groups, i.e., gutka consumers without any lesion and gutka consumers with OSMF. Ten samples each of saliva, serum and urine were collected from these two groups and healthy controls. Samples were centrifuged at 1000 RPM at 2–8°C for 15–20 minutes. A volume of 1.5 ml resultant supernatant was pipetted out in labelled Eppendorf tubes and stored at -80°C. The ELISA test was performed to measure the concentration of 8-OHdG protein in different samples at 450 nm after adding stop solution in 96-well microplate.

Results: 8-OHdG concentration was found to be highest in saliva followed by urine and serum. 8-OHdG concentration in serum was significantly less than that in saliva and urine (P-value <0.05). Intergroup difference in concentration of 8-OHdG of urine, saliva and serum was significant (P-value <0.05). *Post hoc* analysis revealed that concentration of 8-OHdG in saliva and urine was non-significantly different (P-value >0.05).

Conclusion: Saliva appears to be the most appropriate sample type as compared to serum and urine for the evaluation of 8-OHdG in OSMF subjects.

Keywords: DNA damage, gutka-induced oxidative stress, gutka-induced oxidative stress in OSMF, salivary serum urinary 8-OHdG

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INTRODUCTION

Oral submucous fibrosis (OSMF) is an oral potentially malignant disorder, which poses regional and global oral health problems, especially in East and Southeast Asia.[1] The malignant transformation rate of OSMF to oral squamous cell carcinoma (OSCC) accounts for 7%–13%.[2] Gutka chewing in different forms is the most common causative factor of malignant transmission in OSMF.

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Gutka chewing is one of the most common deleterious habits seen in the Indian subcontinent population. Gutka is made by adding various carcinogenic ingredients such as betel nut, tobacco, limestone, catechu and crusted glass.[3] These components of gutka help in the production of various free radicals like H_2O_2, ROO^-, O_2, ROOH, RO, OH_2, O_2^- and·OH. Among all these free radicals., OH is the most reactive type of free radical molecule. ·OH is formed by radiolysis of water and by the reaction of H_2O_2 with ferrous (Fe^{2+}) ions; the latter process is termed as Fenton reaction. The reactive oxygen species, hydroxyl (·OH) radical is one of the potential inducers of DNA damage.[4]

A free radical (FR) is any molecular species that contains at least one unpaired electron. The unpaired electron increases the chemical reactivity of an atom or molecule that generates a high instability.[5] Due to the increase in free radicals, oxidative stress arises. The oxidative stress has been defined as a disturbance in the balance between the production of free radicals and the antioxidant defence system capacity to counter-act their action. The oxidative stress occurs from excess generation or from a deterioration of the antioxidant protective ability.[6] This process leads to the oxidation of biomolecules with consequent loss of its biological functions, whose manifestation is the potential oxidative damage to cells and tissues. Accumulation of free radicals can result in several adverse effects such as lipid peroxidation, protein oxidation and DNA damage.[7]

DNA is chemically unstable and vulnerable to oxidation, due to its susceptibility to endogenous and exogenous damage. The exogenous genotoxic agents are mainly produced by gutka consumption.[8] The oxidative stress leads to DNA damage by the direct modification of nucleotide bases or oxidation of nucleosides, which could cause DNA strand breaks; this type of damage could have teratogenic or carcinogenic consequences.[9]

In recent years, 8-hydroxy-2'-deoxyguanosine (8-OHdG or 8-oxodG) has appeared as a marker of oxidative stress in tissues and body fluids. The 8-OHdG is the most common stable product of oxidative DNA damage caused by free radicals. Among all purine and pyridine bases, guanine is most susceptible to oxidation. Hydroxyl radical addition to the eighth position of the molecule leads to the formation of guanine-modified product 8-OHdG.[10] Oxidative-modified DNA in the form of 8-OHdG can be quantified to indicate the extent damage to genetic material is the most frequent and most mutagenic lesion in nuclear DNA and is important in mutagenesis and carcinogenesis processes.[11]

8-OHdG is used as a standard biomarker of oxidative-induced DNA damage mainly because of its reliable detectability. Elevated levels of 8-OHdG from cancer patients compared with healthy subjects have been observed in lung cancer, basal cell carcinoma, colorectal cancer, bladder cancer and renal cell carcinoma.[12] With respect to periodontitis, published data on oxidative damage to DNA have been reported by many authors around the world who investigated 8-OHdG levels in the saliva of periodontitis patients. These studies demonstrated that levels of 8-OHdG in samples from different groups of patients were significantly higher than those from healthy controls and indicated that 8-OHdG levels may be a useful marker for disease activity and may indirectly reflect disease severity parameters.

MATERIALS AND METHODS

This comparative study was planned and conducted in the Department of Oral Maxillofacial Pathology and Oral Microbiology, Government College of Dentistry, Indore, after ethical clearance. The study was approved by the Ethical Committee dated 27-08-2023, No 145/IEC/SS/2022.

Study design

The study was designed using two groups, OSMF and Control, with gutka habit without any lesion. A sample size of 60 was divided between these groups. Totally, 30 samples of saliva, serum and urine, and 10 from each collected from clinically diagnosed male OSMF patients of the age group of 20–60 years having gutka-chewing habit. OSMF-treated, debilitated and female patients were excluded. Similar sampling was done for control patients. Samples were centrifuged at 1000 RPM at 2–8°C for 15–20 minutes, and a volume of 1.5 ml resultant supernatant was pipetted out in labelled Eppendorf tubes and stored at -80°C.

Sample collection

After obtaining informed and written consent, clinical examination was done and patient history recorded. Approximately 4ml of unstimulated saliva was collected using drooling methods in morning around 9 am in quiet, resting conditions. 10 ml of random urine sample in sterile labelled container from each of the subjects. These saliva and urine samples were then transferred to labelled sterile centrifuge tubes and centrifuged at 1000 RPM at 2-8°C for 20 minutes. A volume of 1.5 ml supernatant pipetted out in Eppendorf tubes and stored. 3 ml of blood sample was aspirated with 20 gauge needle from antecubital vein after sobbing the area with swab and transformed in clot activator vial. It was left for 10–15 minutes before centrifuged at 1000

RPM for 15 minutes. The resulting supernatant was pipetted out in 1.5-ml Eppendorf tubes and stored at a -80°C.

ELISA

The ELISA test was performed for the quantification of 8-OHdG using Biotinylated Detection antibody ELISA kit of Elabscience Technology Laboratory. Test was run using Thermo Fisher microplate reader with serial number 357-910509. The 96-well microplate was read at a wavelength of 450 nm on normal reading speed. Optical density (OD value) of each well was immediately determined using the microplate reader set at 450 nm within 15 minutes after adding the stop solution.

Statistical analysis

Descriptive statistics were analysed using SPSS 21.0 version software. Data were analysed for probability distribution using the Kolmogorov–Smirnov test, and *P* value >0.05 indicated that the data were not normally distributed. Inter-group comparison of continuous variable was done using one-way ANOVA followed by *post hoc* analysis. Intra-group comparison of continuous variable was done using repeated-measures ANOVA followed by *post hoc* analysis. Correlation between the variables was assessed using Pearson's correlation coefficient. *P* value <0.05 was considered statistically significant.

RESULT

The mean age of male OSMF patients having gutka-chewing habit was 36.4 years, ranging from 20–60 years. The mean age of control with gutka habit patients was 26.4 years.

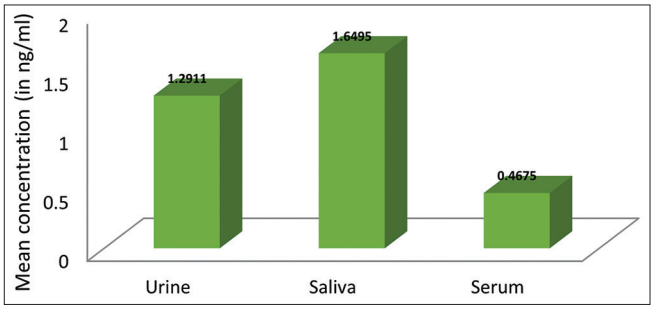
The mean 8-OHdG concentration among the controls in urine, saliva and serum was 1.2911 ± 0.48726 ng/ml, 1.6495 ± 0.29322 ng/ml and 0.4675 ± 0.26786 ng/ml and slightly raised in patients of OSMF 1.3231 ± 0.48496 ng/ml, 1.6920 ± 0.60871 ng/ml and 0.6428 ± 0.54765 ng/ml. These levels showed a non-significant difference (*P* > 0.005) in mean comparison by using the one-way ANOVA.

Within control (A) group subjects, the 8-OHdG concentration was found to be highest in saliva followed by urine, followed by serum. The difference in the 8-OHdG concentration in urine, saliva and serum was highly significant (*P* value <0.001). *Post hoc* analysis revealed that the 8-OHdG concentration in saliva and urine was non-significantly different (*P* value >0.05). The 8-OHdG concentration in serum was significantly less than that in saliva and urine (*P* value <0.05) [Table 1, 1a and Graph 1].

Within OSMF (B) group subjects, the 8-OHdG concentration was found to be highest in saliva followed by urine, followed by serum. The difference in the 8-OHdG concentration in urine, saliva and serum was statistically significant (*P* value <0.05). *Post hoc* analysis revealed that the 8-OHdG concentration in saliva and urine was non-significantly different (*P* value >0.05). The 8-OHdG concentration in serum was significantly less than that in saliva and urine (*P* value <0.05) [Table 2, 2a and Graph 2].

DISCUSSION

Gutka is commercially prepared form of tobacco, which comprises of multiple components. Some of these are betel nut, slaked lime, catechu and paraffin wax.[13] Consumption of gutka induces release of the free radicals like superoxide (O2-), hydroxyl (.OH) and peroxyl (ROO).



Graph 1: Concentration of 8-hydroxy deoxyguanosine (8-OHdG) in urine, saliva and serum of group A subjects

Table 1: Comparison of concentration of 8-hydroxy deoxyguanosine (8-OHdG) in urine, saliva and serum of group A subjects

	Mean	Standard deviation	*F*	*P*
Urine	1.2911	0.48726	24.7	0.001
Saliva	1.6495	0.29322		
Serum	0.4675	0.26786		

Table 1a: *Post hoc* analysis of Group A

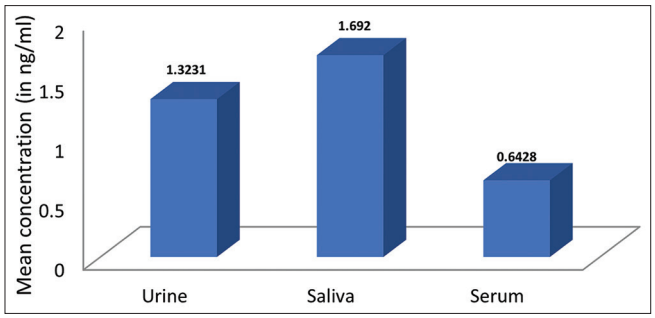
Pairwise	Difference in mean	*P*
Urine vs saliva	-0.358	0.075
Urine vs serum	0.824	0.002
Saliva vs serum	1.182	0.001

Table 2: Comparison of concentration of 8-hydroxy deoxyguanosine (8-OHdG) in urine, saliva and serum of group B subjects

	Mean	Standard deviation	*F*	*P*
Urine	1.3231	0.48496	7.912	0.003
Saliva	1.6920	0.60871		
Serum	0.6428	0.54765		

Table 2a: *Post hoc* analysis Group B

Pairwise	Difference in mean	*P*
Urine vs saliva	-0.369	0.218
Urine vs serum	0.680	0.037
Saliva vs serum	1.049	0.002



Graph 2: Concentration of 8-hydroxy deoxyguanosine (8-OHdG) in urine, saliva and serum of group B subjects

The presence of slaked lime ($Ca(OH)_2$) creates an alkaline pH in the oral cavity favouring more ROS generation. The production of free radicals is further enhanced by Fe^{2}+, Fe^{3}+ and Cu_2+ ions in gutka. These excessive releases of free radicals in gutka consumption bring about redox imbalance favouring a state of oxidative stress in tissues.

Oxidative stress leads to several harmful molecular events in cells that target cellular membranes, lipids, proteins and DNA. Excess of free radicals can cause lipid peroxidation of cell membrane damage to cell membranes. The synthesis of incorrect protein products and changes in protein homeostasis are caused by defective or inadequate protein translation due to excess free radicals, which promotes diseases. DNA damage is yet another target of oxidative stress. This brings about genetic mutations and epigenetic changes at the promoter gene. That brings transcriptional changes and protein modification. These molecular events play a key role in the malignant transformation of OSMF.[14,15] This oxidative stress-induced DNA damage can be measured by 8-OHdG byproduct of DNA damage. The main byproduct of DNA damage is 8-OH-Gua (8-hydroxyguanine) and FapyGua (2,6-diamino-4-hydroxy-5-formamidopyrimidine). 8-OHdG only one directly oxidized base product is more studied.[16]

8-OHdG is excreted from cells to extracellular fluid by ATP-dependent active cellular transport method and reaches blood by passive absorption. 8-OHdG presents either as free 8-OHdG or DNA incorporated 8-OHdG. Free form of 8-OHdG get excreted in saliva through salivary gland by $Na^+/K^+/2Cl^-$ channel and in urine through kidney glomerular filtration due its small molecular size.[17,18]

The variation in distribution of 8-OHdG protein in saliva, urine and serum indicates that the causative factors responsible for oxidative stress-induced 8-OHdG protein production from DNA oxidation is not equally distributed among all participants of groups. The availability of 8-OHdG protein in all body samples is not equally distributed. The total 8-OHdG protein availability gets affected due to variation in method of collection of samples from individuals and centrifugation at different RPM affects its availability too.[19] The sensitivity of ELISA assay kits for 8-OHdG protein in different samples is not equal.[20]

Comparative evaluation of the samples of urinary, saliva and serum 8-OHdG in OSMF and control participants revealed maximum concentration in saliva followed by urine and serum. 8-OHdG concentration was quite significant as compared to the urinary and serum. In conclusion, the present study elicits the fact that the mean salivary 8-OHdG levels showed significant differences not only between the controls but also between patients with OSMF showing the highest mean 8-OHdG levels. Thus, salivary 8-OHdG can be used as a novel biomarker of DNA damage to assess disease progression from OSMF to OSCC.

CONCLUSION

Comparative evaluation of 8-OHdG in saliva, serum and urine samples was done and statistically analysed. The highest concentrations of 8-OHdG were noted in saliva followed by urine and serum among gutka users with OSMF and without oral lesion control. Salivary 8-OHdG appears to be the most appropriate sample type for the evaluation of gutka-induced oxidative stress DNA damage through the assessment of 8-OHdG.

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Conflicts of interest
There are no conflicts of interest.

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