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NONINVASIVE BIOMARKERS OF STRESS: SALIVARY ALPHA AMYLASE, MYELOPEROXIDASE AND TOTAL ANTIOXIDANT CAPACITY

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ABSTRACT **Objectives:** The objective of present study is to investigate the effect of stress on salivary parameters in young medical students. **Design and Methods:** Saliva samples of 30 medical students were collected between 10.00 and 11.00 am on the day of their practical examination (stress condition) and 15 days after completion of the examination (relax condition). Salivary alpha amylase (sAA), myeloperoxidase (MPO) activities and total antioxidant capacity (TAC) in saliva were estimated. **Results:** The sAA activity (unit/mg of salivary protein) was significantly increased on the day of examination (32285.56 ± 23805.55) than relaxed (15949.78 ± 9332.09). MPO activity (log transformed) expressed in mU/L, was also significantly increased on the day of examination (56.31 ± 38.25) compared to that on relaxed condition (35.26 ± 26.52). TAC expressed in µM/L, was significantly low in stress (479.96 ± 94.34) than relaxed condition (723.03 ± 228.21). Protein concentration in saliva was not significantly different on the day of examination (3.83 ± 2.22) compare to without stress (3.64 ± 1.66). Heart rate on the day of examination (95.96 ± 9.12) was significantly more than the day of rest (77.8 ± 5.65). Results were expressed as mean ± SD ($p < 0.05$ was taken as significant). After multiple logistic regression analysis, sAA, MPO and TAC were independently indicative of the presence of psychological stress. **Conclusions:** Salivary alpha amylase and myeloperoxidase were significantly increased in stress. Total antioxidant capacity was significantly decreased in stress. These can be used as noninvasive stress markers.

KEYWORDS :

INTRODUCTION:

Salivary biomarkers gained increased popularity over the past decade for diagnosis of many diseases. Saliva is derived from three types of major salivary glands: the parotid, the submandibular and the sublingual glands. Approximately 90% of total salivary volume is secreted by these three major glands and remaining 10% from other minor salivary glands. Saliva offers many distinctive advantages over plasma, serum or other body fluids: saliva sample collection procedure is noninvasive, easy, stress free, good cooperation from patients' side, not a trained technician is required and it eliminates the potential risk of infectious diseases to technicians and patients both due to blood sample collection procedure. (1,2)

Stress means hardship or adversity. It is proposed that psychological and physical stress produces similar physiological effect. (3) Body responses to stress by a complex neuroendocrine integration via two interconnected effector pathways: the Hypothalamic-pituitary-adrenal (HPA) axis and sympathoadrenal medullary (SAM) system. (4) Due to activation of these two pathways in response to stress, salivary composition of different analytes changes which can be used as salivary markers for stress conditions. (5)

Salivary alpha amylase (sAA) helps in digestion of carbohydrate and is a major constituent of salivary proteins. It has been suggested as an index of sympathetic nervous system. Salivary alpha amylase level increases in stress conditions. sAA responses to stress were also found to be associated with heart rate. (5,6)

Oxidative stress is a condition in which body defensive antioxidant mechanisms fail to eliminate elevated concentrations of reactive oxygen species (ROS) which can cause molecular damage to vital structures and functions.(7) Removal of ROS is essential for maintaining health and antioxidant mechanisms specifically removes harmful oxidants.(8) Psychological stress increases oxidative stress by activation of an inflammatory mediator known as NF-kB.(9) Also, psychological stress was found correlated with increased level of C-reactive protein and homocysteine. Homocysteine has been shown to increase reactive oxygen species. (10,11)

Total antioxidant capacity (TAC) is the measure of all antioxidants present (known or unknown antioxidants and their synergistic actions) in the plasma and body fluids. It provides an integrated parameter instead of simple sum of all measurable antioxidants. (12) Psychological stress decreases total antioxidant capacity. (13)

Mature myeloperoxidase (MPO) is a cationic, dimeric, protein with a mass of 146 kDa. It is a heme enzyme released from activated neutrophils, monocytes & macrophages and has role in host defense. MPO generates many cytotoxic reactive oxygen species like hypochlorous acid and nitric oxide. (14,15) MPO activity increases under psychological stress and causes increase in formation of reactive oxygen species in oral cavity and lead to oxidative stress. (16)

The functions of many salivary proteins have been identified. Under stressful conditions salivary protein composition changes eg. IgA, Alpha amylase etc. (17)

Salivary flow rate did not change salivary parameters significantly from rest to stress. No difference of flow rate was found in between male and female in both conditions. So, it is not a confounder in measurement of salivary alpha amylase and other parameters. (18)

MATERIAL AND METHODS:

With approval from the institutional ethics committee, the present study was carried out at the Department of Biochemistry of S. R. T. R Government Medical College, Ambejogai, Maharashtra, India. Subjects were 30 young undergraduate medical students. All subjects included were healthy and free from any medical & dental illness. They were not on any kind of medication at the time of study.

Exclusion criteria included subjects with any history of systemic diseases, any salivary glandular disease, any oral cavity infections (candidiasis, periodontal infections, dental caries etc), history of smoking or any type of tobacco consumption and alcohol intake. Subjects who had received any periodontal or anti-inflammatory treatment in last six months were also excluded. Written informed consent were obtained from all participants.

Sampling Methods:

Saliva samples were collected from all 30 participants (Table 1) on the day of their term ending practical examination (stress condition) and second samples were collected 15 days after examination (relax condition) by passive drooling method. (19) Samples were collected between 10 am to 11 am on both days to avoid diurnal variation of levels of different salivary parameters. (20). Subjects were instructed to do at least 2 hours of fasting before salivary samples collection. Before sample collection subjects were asked to rinse their oral cavity with distilled water five times and drink 30 ml of clean water after rinsing of oral cavity. For the collection of saliva subjects were

instructed to seat in coachman's position with head slightly down and was asked not to swallow saliva. (21)

After collection of samples, different tests were performed for different salivary parameters. sAA activities were done by modified Huggins C et al method. (22) Salivary samples were diluted 1/200 with normal saline and is incubated with buffered starch. After incubation undigested starch reacts with iodine solution and gives blue- violet colour complex. The intensity of this colored solution is measured by colorimeter at 660 nm. One unit of amylase activity is defined as the activity of enzyme that hydrolyses 5 mgs of starch in 15 minutes to a colorless stage at 37^{0}C.

MPO activities were estimated by Krueger AJ et al method (23) in which o-dianisidine in phosphate buffer (pH-6.0) was used. Due to oxidation of o-dianisidine, yellowish orange colored product is formed. Kinetic measurement of product formed was done by spectrophotometer at 460 nm. Molar absorptivity was 11300 L.mol^{-1}.cm^{-1}. One unit of MPO activity was defined as that degrades one micromole of hydrogen peroxide per minute at 25^{0}C.

TAC was determined by Koracevic D et al method (24) using sodium benzoate as a substrate. A standardized solution of Fe-EDTA complex react with hydrogen peroxide by a Fenton-type reaction and form hydroxyl radicals which degrades benzoate and liberates TBARS (Thiobarbituric acid reactive substances). Salivary antioxidant suppress production of TBARS which leads to decrease in color development and absorbance was measured spectrophotometrically at 532 nm against deionized water.

Salivary total protein was estimated by Lowry OH et al method. (25) Pulse rate measurement was done for complete one minute on radial artery during both stress and rest.

Statistical Analysis

Normality of sample distribution was checked by Shapiro- Wilk test before statistical procedure were applied. All salivary parameters were normally distributed except MPO activity. So, log transformed values of MPO activity were taken for analysis. Student paired t test was applied. All analysis was done on SPSS version 21 software. $p< 0.05$ were taken significant.

RESULT:

The sAA activity level expressed in unit per milligram of salivary protein was significantly increased on the day of examination than relaxed. Myeloperoxidase activity (log transformed) expressed in mU/L, was also significantly increased on the day of examination compared to that on relaxed condition. Total antioxidant capacity expressed in µM/L, was significantly low in stress than relaxed condition. Protein concentration in saliva was not significantly different on the day of examination i.e., stress (3.83 ± 2.22) compares to without stress (3.64 ± 1.66). Heart rate on the day of examination (95.96 ± 9.12) was significantly more than the day of rest (77.8 ± 5.65) (mean ± SD). Results are shown in Table 2 and Table 3. $p< 0.05$ was taken as significant.

Table 1: Demography of study population

Male (n)	18
Female (n)	12
Age in years (Mean ± SD)	19.33 ± 0.80
BMI in Kg/m2 (Mean ± SD)	20.92 ± 3.36

Table 2. Student paired t test

Parameters	Stress (mean ± SD)	Without stress (mean ± SD)
sAA (Unit/mg of salivary protein)	32285.56 ± 23805.55*	15949.78 ± 9332.09
MPO (mU/L)	56.31 ± 38.25*	35.26 ± 26.52
TAC (µM/L)	479.96 ± 94.34*	723.03 ± 228.21

*$p < 0.05$

Table 3: Binary logistic regression

Model	Parameters	Nagelkerke R2	Odd's ratio	p- value
1	sAA	0.293	0.919	0.030
2	MPO	0.131	0.979	0.025
3	TAC	0.454	1.009	0.000
4	sAA + TAC	0.587	sAA = 0.927 TAC = 1.009	sAA = 0.026 TAC = 0.025

DISCUSSION:

The aim of this study was to assess whether salivary parameters like alpha amylase, MPO or TAC can be used to detect psychological stress.

Use of saliva as a sample offers many advantages over serum or other body fluids. Collection of saliva is a noninvasive procedure. There is no risk of transmission of infection to patient and technician. It is free of stress related to invasive procedure. Samples can be collected by any one. There is no requirement of a skilled technician or a special equipment to collect the sample. It may provide a cost-effective approach to screen a large population. (2)

Several reports have demonstrated that HPA axis and SAM system respond differently to psychological stressors. sAA reacted and recovered significantly more rapidly than those of cortisol. (26) In our study we found significant increase in sAA level and heart rate on the day of examination (stress condition) which may be due to activation of SAM system or autonomic nervous system. The psychological stress increases the release of sAA which reflects the activity of SAM systems. Therefore, it is supposed that the measurement of sAA may be a useful tool for evaluating the SAM system and stress level of the body. (8)

Psychological stress also increases MPO activity. Increase in MPO activity leads to increase in formation of cytotoxic reactive oxygen species like hypochlorous acid and nitric oxide. This leads to increase in oxidative stress inside oral cavity. (14-16) In our study we found significant increase in salivary MPO activity which reflects raised oxidative stress in oral cavity. High oxidative stress can make the oral cavity more prone to many complications like periodontal disease, dental caries etc. by different mechanisms. (27)

In the present study, significant increase in salivary alpha-amylase & myeloperoxidase activity and significant decrease in TAC was observed on the day of stress than the day without stress. That may be due to increased oxidative stress on the day of examination. The Binary logistic regression analysis supports the above-mentioned hypothesis that sAA, salivary MPO activity and salivary TAC can be seen as an important independent noninvasive biomarker of stress.

The limitation of our study is small sample size. The mechanism involved in changes in sAA, MPO activity and TAC in psychological stress is not cleared from our results. In addition, measurements of endocrine, sympathetic and parasympathetic parameters should be considered in future studies.

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