Original Article

# Estimation of Malondialdehyde Levels and Determination of Total Antioxidant Capacity in Serum and Saliva of Patient Affected with Sickle Cell Anemia

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## Abstract

**Introduction:** Hemoglobinopathy is an inherited blood disorder in which an individual has an abnormal form of hemoglobin (variant) or decreased production of hemoglobin. The molecular pathogenesis of sickle cell disease explains the phenomenon well when there is adeficiency of oxygen carriage by the erythrocytes. The horrendous patterns of sickling/unsickling bring about the accumulation of lipid oxidation items and reactive oxygen speciesthat gather inside sickle-shaped red blood cells (RBCs). **Aims:** To estimate the malondialdehyde levels and determine the total antioxidant capacity in serum and saliva of sickle cell anemic patients. **Methods and Material:** Fiftysubjects aged 5–40 years were selected for the study. The subjects were divided into two groups. Blood and saliva samples were collected from both groups and were subjected to the phosphomolybdenum method. Absorbance was read spectrophotometrically at 695 nm. SPSS software was used, and statistical analysis was performed using Student's unpaired ttests. **Results:** Oxidative stress results f an imbalance between reactive oxygen species formation and enzymatic and non-enzymatic antioxidants. **Conclusion:** Salivary levels of total antioxidant capacity, malondialdehyde, and electrolytes make saliva a potential prognostic and diagnostic medium as there is an alteration of oxidative stress in sickle cell anemia (SCA).

**Keywords:** Antioxidant, malondialdehyde, saliva, serum, sickle cell anemia

**Key Message:** This is a cross-sectional research study to determine the correlation of sickle cell anemia to serum and saliva and its relevance in its diagnosis. An increase in rapid and less invasive diagnostic tests has made saliva an important diagnostic and prognostic tool.

## Introduction

India has the largest concentration of tribal populations globally. According to the Census of India 2011, the tribal population of India is 8.6% of the total population which is about 67.8 million people.[1] The states of Madhya Pradesh, Maharashtra, Orissa, Jharkhand, Chhattisgarh, Andhra Pradesh, West Bengal, and Karnataka account for around 83% of the total scheduled tribe population in the country residing in rural areas.[1] Hemoglobinopathies are common in tribal populations, and the great incidence of sickle cell anemia has led to this research.[2,3]

The molecular pathogenesis of sickle cell disease states that when there is a deficiency of oxygen carriage by the erythrocytes, polymerization of the deoxyhemoglobin S (HbS) molecules occurs, which causes the formation of fibers and aggregation of the RBC, resulting in its sickle shape. Multiple cycles of sickling and unsickling cause rupture of the erythrocytes, releasing hemoglobin fibers and molecules in the circulation.[4-6] When the oxygen-carrying capacity of the cell lowers, ischemia and infarction result, which creates a vaso-occlusive crisis.[7,8] Sickle cell anemia is characterized

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by chronic hemolysis, inflammation, vaso-occlusion, and ischemia–reperfusion injury, which are the hallmarks leading to strokes and infarctions. Ischemia–reperfusion injury is characterized by intermittent cessation of blood flow and the production of reactive oxygen species (ROS), which contribute to the oxidative stress implicated in sickle cell anemia.[9]

This has led to the evolution of this research study for the aims set: (a) To estimate the malondialdehyde levels and determine the total antioxidant capacity in serum and saliva of patients affected with sickle cell anemia. (b) To compare the two results between the sickle cell anemic patients and healthy patients.

## Materials and Methods

Fifty subjects aged 5–40 years were selected for this cross-sectional study. The subjects were divided into two groups. The patients were informed about the procedure, and consent was obtained for the procedure. The ethical clearance certificate for conducting the research was obtained from the institution's ethical committee vide no. TIDSHRC/IEC/2018/D011. The study was performed for a period of two years. Selection criteria included patients previously diagnosed with SCA; fully conscious and cooperative patients were selected. Exclusions were indicated for patients with severe jaundice and hemolytic anemia; patients indulged in chronic smoking and alcoholics; children who utilized mouthwashes or orthodontic braces as well as presenting lesions in their oral mucosa, patients with findings of any physical or mental abnormality and patients below the age of five years and above the age of 40 years.

## Methodology

A total of 50 subjects aged 5–40 years were selected and divided into two equal groups, (a) 25 suffering from sickle cell anemia and (b) 25 healthy controls. Sample size was determined using the formula = $z^2pq/d^2$, where n = sample size, z = 1.96 at 95% confidence level, p = 0.96, q= (1-p), and d = 0.05 [desired marginal error]

$n= [(1.96)^2 \times 0.96 \times 0.04]/(0.05)^2 = 50$

Blood and saliva samples were collected aseptically from both groups and were subjected to the phosphomolybdenum method. Absorbance was read spectrophotometrically at 695 nm. The concentration of total antioxidants was obtained by plotting the absorbance of the test against the standard graph. Five ml of unstimulated saliva was obtained from all the patients by spit method in a sterile test tube following standard precollection protocol. Saliva was centrifuged at 12,000 rpm for 24 min at 4°C to obtain a supernatant. The malondialdehyde (MDA) content of the saliva was evaluated by using thiobarbituric acid (TBA). The MDA obtained from the lipid peroxidation reaction reacts with TBA to yield a yellow fluorescent product. The absorbance of the 2-ml colored layer was measured using a spectrophotometer at 695 nm. Five ml of blood sample was collected from the median cubital vein of the patient. A tourniquet was tied 2–3 cm above the hand, isopropyl alcohol was applied, and blood was withdrawn from the syringe. Blood collection tubes were drawn in a specific order to avoid cross-contamination of additives between tubes. The recommended order of draw was, first—blood culture bottle or tube (yellow or yellow–black top), second—coagulation tube (light blue top), third—non-additive tube (red top), and last draw—additive tubes in this order of serum separator tube (SST) (red–gray or gold top), sodium heparin (dark green top), plasma separator tube (PST) (dark green top with gold rim) containing lithium heparin anticoagulant and a gel separator, ethylenediamine tetraacetic acid (EDTA) (lavender top), and oxalate/fluoride (light gray top) as additive. The blood sample was labeled and centrifuged in a centrifugation machine.

Statistical analysis was done using SPSS (version 20.0; SPSS Inc. Chicago, IL, USA) software. Comparisons of proportions between serum and salivary TAC and malondialdehyde in sickle cell anemic patients and healthy patients were conducted withStudent'sunpaired "ttest" and close correlation was studied in both groups using Pearson's rvalue test. $P \leq 0.05$ was considered statistically significant.

## Results

This quantitative assay was based on the conversion of molybdenum (Mo VI) by reducing agents like antioxidants to molybdenum (Mo V), which further reacts with phosphate under acidic pH resulting in the formation of a green-colored complex, the intensity of which can be read spectrophotometrically at 695 nm. This procedure was followed in the phosphomolybdenum assay.

When the TAC of serum was compared between the patient with SCA and healthy controls, using the Student's unpaired ttest, the result was highly significant ($P$ = 0.002) [Table 1 and Figure 1]. When the serum malondialdehyde was compared between the patient with SCA and healthy controls, using the Student's unpaired ttest, the result was highly significant ($P$ = 0.000) [Table 2 and Figure 2]. When the TAC of saliva was compared between the patient with SCA and healthy controls, using the Student's unpaired"ttest," the result was highly significant ($P$ = 0.000) [Table 3 and Figure 3]. When the salivary malondialdehyde was compared between the patient with SCA and healthy controls, using the Student's unpaired"ttest," the result was highly significant ($P$ = 0.000) [Table 4 and Figure 4]. On evaluating

**Table 1: Comparison of mean serum TAC between the groups**

| Groups | Mean | SD | Mean difference | *t* | *P* |
|---|---|---|---|---|---|
| Case | 0.33 | 0.07 | 0.09 | 3.358 | 0.002 |
| Control | 0.42 | 0.1 | | | |

*Statistically significant. INFERENCE: The values of TAC evaluated, in the study groups, in serum are given in Table 1. Mean in serum of sickle cell anemia was found to be 0.33, while that of healthy children it is 0.42. The difference of mean serum TAC between the groups is statistically significant ($P$<0.05)

the correlation, a positive correlation was present between serum and salivary TAC [Table 5] and serum and salivary malondialdehyde [Table 6] in cases of SCA, but it was not statistically significant ($P > 0.05$). A positive correlation was present between serum and salivary TAC [Table 7] and serum and salivary malondialdehyde [Table 8] in cases of control, but it was statistically significant ($P > 0.05$).

The MDA was increased in patients with SCA, and a significant correlation between the MDA of serum and saliva indicates that changes in serum may be reflected equally in saliva. Therefore, the assessment of MDA in the saliva of SCA patients could serve as a noninvasive alternative to that in serum. In the present study, increased oxidative stress may account for raised MDA level, which serves as a biomarker.

**Table 2: Comparison of mean serum malondialdehyde between the groups**

| Groups | Mean | SD | Mean difference | *t* | *P* |
|---|---|---|---|---|---|
| Case | 9.32 | 1.3 | 4.63 | 14.281 | 0.000 |
| Control | 4.69 | 0.8 | | | |

*Statistically significant. INFERENCE: The values of malondialdehyde evaluated, in the study groups, in serum are given in Table 2. Mean in serum of sickle cell anemia was found to be 9.32, while that of healthy children it is 4.69. The difference of mean serum malondialdehyde between the groups is statistically significant ($P<0.05$)

**Table 3: Comparison of mean salivary TAC between the groups**

| Groups | Mean | SD | Mean difference | *t* | *P* |
|---|---|---|---|---|---|
| Case | 0.30 | 0.05 | 0.15 | 6.516 | 0.000 |
| Control | 0.45 | 0.1 | | | |

*Statisticallysignificant. The difference of mean salivary TAC between the groups is statistically significant ($P<0.05$). INFERENCE: The values of TAC evaluated, in the study groups, in saliva aregiven in Table 3. Meanin serum ofsickle cell anemia was found to be 0.30, while that of healthy children it is 0.45. The difference of mean serum TAC between the groups is statistically significant ($P<0.05$)

**Table 4: Comparison of mean salivary malondialdehyde between the groups**

| Groups | Mean | SD | Mean difference | *t* | *P* |
|---|---|---|---|---|---|
| Case | 0.79 | 0.2 | 0.46 | 10321 | 0.000 |
| Control | 0.33 | 0.1 | | | |

*Statistically significant. INFERENCE: The values of malondialdehyde evaluated, in the study groups, in saliva aregiven in Table 4. Meanin saliva ofsickle cell anemia was found to be 0.79, while that of healthy children it is 0.33. The difference of mean salivary malondialdehyde between the groups is statistically significant ($P<0.05$)

**Table 5: Correlation between serum and salivary TAC in cases**

| Groups | Mean | SD | Pearson's *r* | *P* |
|---|---|---|---|---|
| Serum TAC | 0.33 | 0.07 | 0.195 | 0.350 |
| Salivary TAC | 0.30 | 0.05 | | |

*Not statistically significant. INFERENCE: Comparative correlation of TAC between serum and saliva of patients of sickle cell anemia. Positive correlation present with no statistical significance

## Discussion

Reactive oxygen species (ROS) consist of free radicals (FR) such asO2•, OH•, and other oxygen derivatives such as hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), and singlet oxygen ($1O_2$) that are formed *in vivo* as a part of inflammatory processes or due to environmental factors exposure. Free radicals are reactive oxygen species that circulate freely in the body and can cause tissue damage

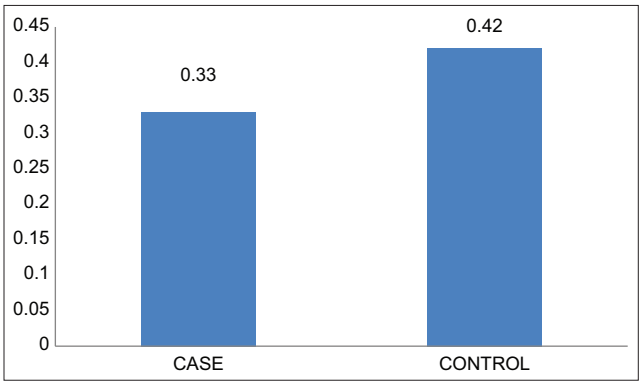


**Figure 1:** Comparison of mean serum TAC between the groups

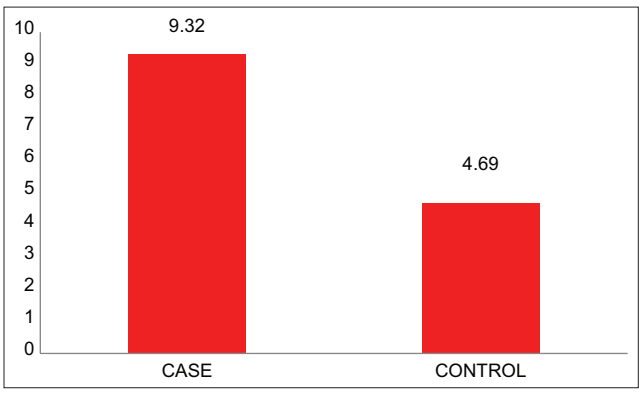


**Figure 2:** Comparison of mean serum malondialdehyde between the groups

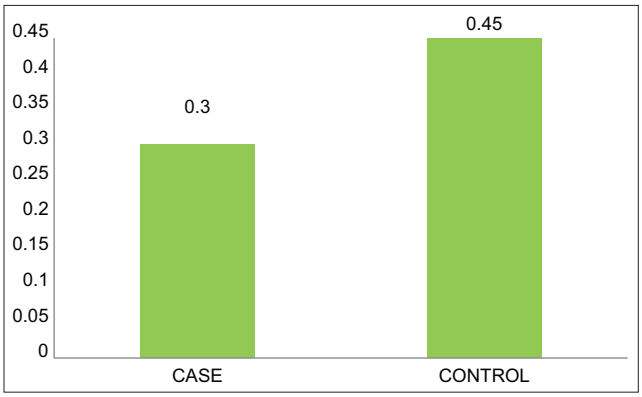


**Figure 3:** Comparison of mean salivary TAC between the groups

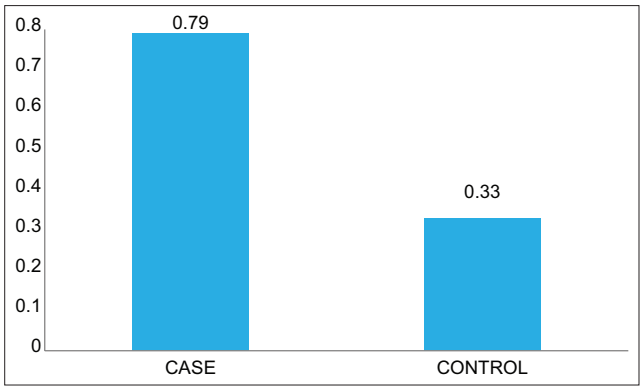


**Figure 4:** Comparison of mean salivary malondialdehyde between the groups

**Table 6: Correlation between serum and salivary malondialdehyde in cases**

| Groups | Mean | SD | Pearson's *r* | *P* |
|---|---|---|---|---|
| Serum malondialdehyde | 9.32 | 0.13 | 0.318 | 0.121 |
| Salivary malondialdehyde | 0.79 | 0.2 | | |

INFERENCE: Comparative correlation of malondialdehyde between serum and saliva of patients of sickle cell anemia. Positive correlation present with no statistical significance

**Table 7: Correlation between serum and salivary TAC in controls**

| Groups | Mean | SD | Pearson's *r* | *P* |
|---|---|---|---|---|
| Serum TAC | 0.42 | 0.1 | 0.973 | 0.000 |
| Salivary TAC | 0.45 | 0.1 | | |

INFERENCE: Comparative correlation of TAC between serum and saliva of patients of control group. Positive correlation present with statistical significance

**Table 8: Correlation between serum and salivary malondialdehyde in controls**

| Groups | Mean | SD | Pearson's *r* | *P* |
|---|---|---|---|---|
| Serum malondialdehyde | 4.69 | 0.8 | −0.450 | 0.024 |
| Salivary malondialdehyde | 0.33 | 0.1 | | |

INFERENCE: Comparative correlation of malondialdehyde between serum and saliva of patients of sickle cell anemia. Positive correlation present with statistical significance

through mechanisms that include DNA damage, lipid peroxidation (through cyclooxygenases and lipoxygenases), protein damage, oxidation of important enzymes like α 1-antitrypsin, and stimulation of pro-inflammatory cytokines release by monocytes and macrophages. Various researches arebeing directed toward preventing or controlling diseases through FR manipulations.[10,11] Congenital hemoglobin mutations can alter the delicate balance of free-radical generation and antioxidant defense by favoring the FR, giving rise to oxidative stresses in the tissues. Currently, there is an increasing interest in using and measuring antioxidants.[12,13]

Due to recent advancements in salivary research, its role as a diagnostic and prognostic medium has exponentially advanced in the last ten years.[14,15] Most organic compounds in saliva are produced locally in the salivary glands, but some molecules pass into saliva from blood.[16] The biomolecules enter saliva by either passive diffusion of lipophilic molecules or active transport of proteins via ligand -receptor binding. Hence, in the present study, we have compared the salivary and serum components. Therefore, the present study evaluated both saliva and serum in patients with SCA and healthy controls to utilize the potential of saliva to be used as an investigative tool.[16,17]

No single assay can be considered a TAC assay, even though it can be performed in an aqueous and lipophilic environment.[16,17] The phosphomolybdenum method, a quantitative assay, was used to determine TAC in the present study. It has been reported that TAC is higher in unstimulated saliva than in stimulated saliva. Therefore, in the present study, the determination of antioxidant capacity was done using unstimulated saliva.[17] In a study evaluating TAC levels in serum and saliva, it has been reported that both saliva and serum could serve as markers for assessing the severity of the disease process. Still, compared to serum, the salivary analysis is noninvasive and easier to perform.[18] The results showed reduced TAC levels in SCA patients compared to healthy individuals. It further emphasized the role of oxidative stress in the pathophysiology of SCA, and any intervention aimed at increasing the antioxidant capacity of these patients may be beneficial.[18,19] The antioxidant capacity has been stated to be related to the intake of dietary antioxidants and may alter as a function of age. In the present study, the TAC increased in older patients with SCA.[20] Even though the study involved SCA patients from low socioeconomic status, statistical tests revealed a positive correlation between the TAC values and advancing age. This may be related to a dietary shift to food containing larger volumes of antioxidants in the form of micronutrients with increase in age. Similar findings were also seen in healthy patients.[21]

Malondialdehyde (MDA) is the most commonly studied end product of lipid peroxidation in response to oxidative stress. The end products of lipid peroxidation are more stable than FRs and can react with molecules, such as DNA and proteins, far from their production site. Increased production of ROS in SCA has been stated to have a role in oxidative stress and induction of lipid peroxidation, the by-product of which is MDA.[22] ROS cannot be measured directly due to its extreme instability and short half-life. Thus, determining MDA levels may be useful in quantifying the level of oxidative stress *in vivo* and in vitro. In the present study, estimation of MDA was done by the thiobarbituric acid (TBA) method that yields "thiobarbituric acid reactive substances (TBARS)," which can be easily measured by spectrophotometry.[21,22] In this study, a weak positive correlation was found between the MDA levels in serum and saliva in patients with SCA and healthy controls. The MDA levels were found to be increased in the serum of patients with SCA compared to healthy controls. The increase in MDA levels in the present study could be attributed to enhanced ROS formation

in SCA.[23] Since RBCs contain hemoglobin, which is one of the most powerful catalysts for the initiation of peroxidative reactions, they are susceptible to peroxidative damage. Thus, MDA in excess quantity can promote erythrophagocytosis.[24]

In the present study, the MDA decreased in older patients with SCA. This study also revealed a relation in which there was no age correlation between the patients with sickle cell anemia and healthy patients. At present, a fast-growing group of sickle cell anemic individuals who will face the prospect of growing old in a healthier state than before encounter all the non-sickle-related aging problems in their distinctive way.[24]

## Conclusion

The increasing evidence of the role of reactive oxygen species in the pathophysiology of SCA suggests new therapies for the disease. It gives new insights into the mechanism of action of some currently accepted therapies. As a diagnostic tool, saliva offers advantages over serum because it can be collected noninvasively by individuals with modest training, and it offers a cost-effective approach for screening of large population.[17] There is also minimal risk of contracting infections during saliva collection, and saliva can be used in clinically challenging situations, such as obtaining samples from children or handicapped or anxious patients, in whom blood sampling could be difficult. As we talk about the treatment of sickle cell anemia, supplementation of antioxidants can serve as a treatment option as they help neutralize the effects of ROS produced in sickle cell anemia.[18]

### Limitations

The use of saliva requires manpower and skills, along with new assays and devices, to be produced at commercially feasible rates for the common population of the country on the rural and urban scales. It requires the coordination of the government and stakeholders to produce the yield of the diagnostic kits at a cheap scale also, which can be agreed upon by the insurance companies to cover.

### Future prospects

Saliva serves as a reliable diagnostic and prognostic tool. Being a noninvasive method, it helps in diagnosing the entire population in a short period. Saliva possesses a wide range of antioxidant mechanisms, including low molecular antioxidants such as glutathione, ascorbic, and uric acid, as well as melatonin; it may form the first line of defense against free radical-mediated oxidative stress in SCA.[24]

### Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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