Salivary Ferritin as a Predictive Marker of Iron Deficiency Anemia in Children

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Objectives: *The objective of the study was to determine the salivary Ferritin levels in children with iron deficiency anemia and its reliability as a predictive marker of the disease and whether these levels could give a new hypothetical insight on the oral epithelial changes seen in patients with iron deficiency anemia.* ***Study Design:*** *The study comprised of 60 children of which the study group comprised of 30 individuals; aged 8–14 years with iron deficiency anemia. Venous blood was collected and hematological examination was performed to determine the hemoglobin and serum ferritin levels to confirm the diagnosis. Saliva was then collected from 30 children with iron deficiency anemia and an equal number of controls. The ferritin levels in saliva were then analyzed using solid phase ELISA.* ***Result:*** *The mean value of salivary ferritin in iron deficient cases was 153.24±46.58 µg/dl and the mean ferritin levels in control subject were 93.87±30.15 µg/dl. Thus the salivary ferritin was found to be significantly higher in iron deficient subjects compared to the controls.* ***Conclusion:*** *The result of our research affirms the fact that the expression of ferritin in saliva of iron deficiency anemia may be due to the enzymatic functions in the saliva and the endocytosis of ferritin which can possibly elevate the salivary ferritin. The diminished level of cytochrome oxidase, together with its relatively high Ferritin content, depicts the association of oral epithelial changes and ferritin occurring in iron deficiency anemia.*

Keywords: *Ferritin, anemia, children, saliva, epithelium, ELISA*
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INTRODUCTION

Dietary deficiency of iron and the resulting anemia appears to be the most prevalent disorder in human being.[1] The continued persistence of anemia in many parts of the world is a challenge that needs to receive the highest priority for attention and action. Despite the magnitude of this problem, the constantly expanding body of research findings relating to pathogenesis, diagnostic modalities and efficacious interventions, are riddled with inconsistencies and disagreements among practitioners.[2] Considerable emphasis is placed to develop strategies about the prevention and management of iron deficiency during early childhood, because evidence indicates that poor iron status negatively affects cognitive functioning, and leads to long term motor impairment since it supports neuronal and glial energy metabolism, neurotransmitter synthesis and myelination.[3,4] Deficiency of iron is directly linked to the occurrence of esophageal webs in patients with Plummer-Vinson Syndrome leading to increased susceptibility to the development of esophageal cancers and intraoral squamous cell carcinomas.[5,6,7] Iron deficiency anemia is also often implicated in the etiology of recurrent aphthous stomatitis.[7]

Saliva is a complex fluid composed of a variety of organic and inorganic components at a gradient comparable with the serum.[8] Ferritin is found to be a highly specialized ubiquitous housekeeper storage protein which stores iron and releases it in a controlled fashion required for cellular proliferation and metabolic renewal.[9,10] Ferritin levels seem to reflect the magnitude of iron stores in the body and decreased or increased serum ferritin levels are used as a

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marker for anemia and iron overload disorders.[11] Agarwal and co-workers observed that saliva contains Ferritin and that its levels in saliva were much higher than the serum levels.[12] The biological system maintains the salivary Ferritin levels at a higher level, probably for the iron dependent enzymatic functions of the saliva, thus conserving the iron through saliva.[13]

The assessment of ferritin has been one of the gold standard methods in the detection of iron deficiency anemia.[14] However, it involves drawing of venous blood and carries more risk of contamination and is more prone to risk. Furthermore, this invasive procedure may not be suitable for pediatric patients. Assessment of the iron status has proven to be difficult, especially in children living in areas with high infection pressure.[15] This calls into need, the identification of a less invasive approach for assessment of Ferritin with the same sensitivity and specificity levels as ferritin assessment of blood. Changes in the levels of salivary ferritin appear to occur even before the hematological changes and hence can be used as a valuable tool in monitoring the iron status.[16] Further, the structural, histochemical and clinical changes occur before significant alterations in the red cell morphology and haemoglobin levels.[7]

Within this background, it was the objective of this study to determine the salivary Ferritin levels in children with iron deficiency anemia and assess its reliability as a predictive marker of the disease. The null hypothesis was that there is no alteration of salivary ferritin levels in children with iron deficiency anemia.

MATERIALS AND METHOD

The study comprised of 60 children of which the study group comprised of 30 individuals; aged 8-14 years with iron deficiency anemia. The cases were initially selected based on the clinical features which included significant pallor of the conjunctiva, nail beds and atrophic glossitis. A hematological examination was performed to determine the hemoglobin and serum ferritin levels to confirm the diagnosis. Based on the cut off values for hemoglobin proposed by WHO,[17] patients with levels less than 12.0g/dl were selected for the experimental group. The patients were divided into three groups: mild, moderate and severe based on cut off values of hemoglobin proposed by WHO. The control group comprised of equal number of children with hemoglobin levels above 12g/dl and without any clinical evidence of iron deficiency anemia. Subjects with pre-existing medical illness and on medications were excluded from the study.

The protocol for the present study was approved by the Ethical committee and Scientific review Board of the University. A written informed consent was obtained from participants and a predetermined data sheet was used to record the history and the clinical features were evaluated.

Collection of saliva

About 2ml of unstimulated saliva was collected from the study group and the control group. Donors were asked to refrain from eating or drinking for at least 60 minutes prior to each collection. Smoking, chewing gum and intake of beverages were also prohibited during this hour. The subjects were asked to rinse the mouth with deionized distilled water and were supplied with a sterile container. Briefly, after a rinse of the mouth with water, saliva was allowed to accumulate in the floor of the mouth for approximately 2 minutes and the subjects were asked to expectorate the unstimulated saliva into the sterile container. Following collection, the samples were aliquoted and the samples were then centrifuged at 2500 rpm for 5 minutes. The centrifugation resulted in saliva samples free of large particulate debris and reduced viscosity, thereby allowing a more accurate and reproducible analysis. The supernatant was subsequently stored at – 30°C until analysis was performed.[18]

Blood Collection

Venous blood was drawn using a 21-gauge needle with minimal tourniquet application to prevent stasis. From each subject, about 4.5 ml of venous blood from both the groups were collected with the help of iron free syringes and needles through venepunctures and was placed in iron free labelled 5ml plastic evacuated vials containing 0.5ml of ethylene Diaminetetraaceticacid (EDTA). The content was immediately mixed by gently inverting the tube 5 times and the samples were stored at -50º C until analysis. The sample was centrifuged within 30 minutes at 2,500rpm at 20°C for 15 minutes without brake. Plasma was carefully removed and transferred to a nonactivating centrifuge tube using a plastic pipette and was centrifuged again using the same conditions. Platelet-free plasma was divided into aliquots and stored at –80°C until analysis.[19]

Salivary Ferritin Analysis

The salivary Ferritin levels were analyzed using a solid phase enzyme-linked immunosorbent assay technique using a commercially available Ferritin kit (Orgentec Diagnostika GmbH, Germany).

The working solution of the Anti-Ferritin Antibody-Horseradish Peroxidase (HRP) Conjugate was prepared by diluting 480µl of HRP conjugate in 24 ml of the assay buffer before use. The wash buffer was prepared by diluting the contents of each vial (50ml) of the buffered wash solution concentrate in 450 ml of distilled or de-ionized water prior to use.

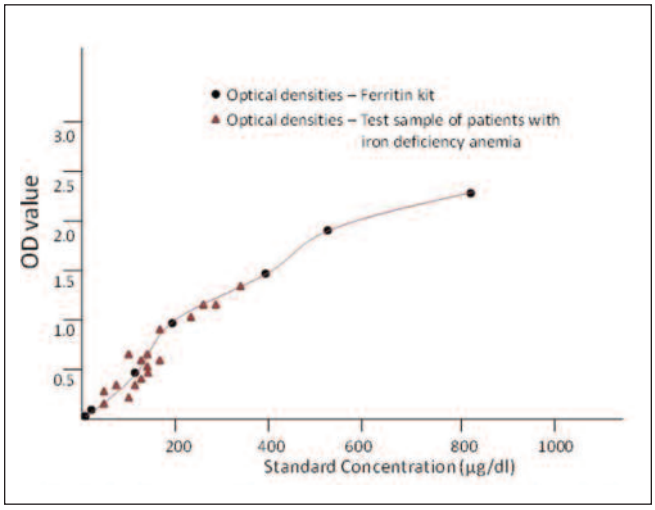
A Sterile polyvinyl chloride flat bottom 96 well microwell titration plate was removed from the sealed bags, rinsed twice with methanol and dried. The microwell plate was coated with highly purified specific anti-rabbit-ferritin antibodies and incubated for 2 hours at 37 C, followed by washing twice with blocking buffer and the plate was incubated for 30 minutes with wells filled with blocking buffer in order to deprive them of non-specific binders. The plates were then washed thrice with wash buffer, Phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) at pH 7.4. About 30 µl of each calibrator, control and the sample was dispensed into the corresponding labelled wells and incubated for 2 hours at 37C. To this, 200 µl of the Anti

Ferritin Horse Radish Peroxidase conjugate working solution diluted 1:200 was dispensed into each well using a multichannel pipette and was continuously mixed for 30 seconds followed by incubation for 45 minutes at 18-25C. The wells were washed with 300µl of diluted wash buffer and the well was tapped firmly against the absorbent paper to remove the residual water droplets. About 150 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate stabilized in buffer solution was pipetted into each well at timed intervals which resulted in the development of the blue colour. It was gently mixed for 5 seconds followed by incubation for 10-15 minutes at room temperature in the dark. The enzymatic reaction was stopped by adding 50µl of 2% sulfuric acid (stopping solution) at same time intervals to obtain yellow color. The plate was then read on a DTX 880 microwell plate reader at 450 nm within 20 minutes after the addition of stopping solution.

Assessment of Hemoglobin and Serum Ferritin

Before the onset of analysis the frozen samples were rapidly thawed at 37°C for 5 minutes. The hemoglobin levels were determined by an Automated Hemoglominometer (Serono Baker; Allentown, PA) and serum Ferritin was typically assessed in serum with enzyme-linked immunosorbent assays (ELISA) (Orgentec Diagnostika GmbH, Germany).

The mean absorbance value for each set of reference standards, controls and samples were calculated at 450nm with the microtitre well reader. A standard curve was constructed on a linear graph paper by plotting the mean absorbance obtained for each reference standard against its concentration in µg/mL, with absorbance on the Y axis and concentration on the X axis. The ferritin concentrations were calculated from the sample absorbance reading from the fitted curve (Graph 1).



Graph 1. The Calibration Curve With Mean Optical Densities along Y Axis and Calibrator Concentrations along X Axis showing the Standard Values and the Corresponding Optical Density for the Ferritin Kit and for the experimental patients

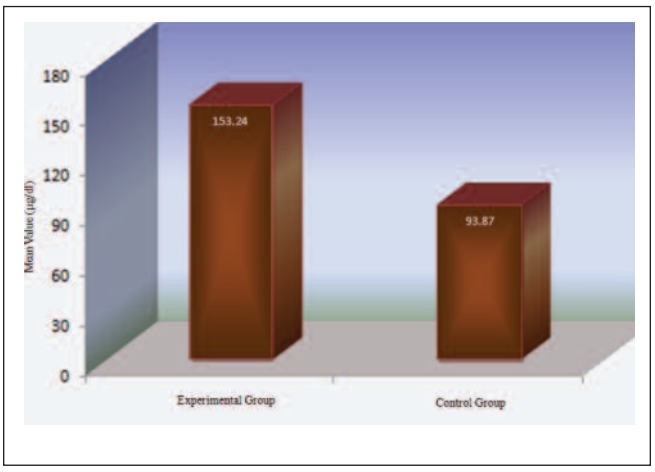
Statistical Analysis

Statistical comparison of the mean values of salivary Ferritin and serum ferritin levels was done by student t test at a significance level of 5%. All statistical analyses were performed using SPSS SAS (9.2) version.

The sensitivity and specificity was determined and the positive predictive value and negative predictive value was calculated.

RESULTS

The mean value of salivary ferritin in subjects with iron deficiency was 153.24 ± 26.58 µg/dl and the mean ferritin levels in the control subject was 93.87 ± 23.15 µg/dl as compared to a serum ferritin levels of 82.34 ± 9.6 µg/dl in normal subjects and 31.69 ± 6.28 µg/dl in iron deficient subjects. The salivary ferritin level was found to be significantly higher in subjects with iron deficient subjects compared to the controls ($p<0.001$) (Table 1). The salivary ferritins in mild moderate and severe groups were determined and the ratio of salivary ferritin to serum ferritin was determined. The ratio was significantly higher in severe group and



Graph 2. Ferritin levels in iron deficiency anemia and controls

Table 1. Gender distribution, Group statistics and Student t test in iron deficient patients and controls

Group	Number	Gender		Group Statistics (Salivary Ferritin); ng/dl			Independent Sample test
		Males	Females	Mean	Standard deviation	Standard mean error	Sig (2 tailed)
Iron deficient patients(cases)	30	9 (30%)	21 (70%)	153.24	23.583	8.650	0.000
Controls	30	13(43.3%)	17(56.6%)	93.87	26.152	5.505	0.000

Table 2. Relationship between Hemoglobin, Serum Ferritin and Salivary Ferritin

	Hemoglobin levels		Salivary ferritin ng/dl	Serum ferritin ng/dl	Salivary: serum ferritin ratio
	WHO proposed Hb values levels to diagnose anemia of cases	Hb			
Iron deficient patients (cases)	Mild (11.0-11.9g/dl)	11.2 g/dl	121.06 ± 10.23	73.24 ± 7.34	1.65:1
	Moderate (8.0-10.9 g/dl)	9.3g/dl	148.45 ± 8.13	39.35 ± 6.2	3.77:1
	Severe (<7.9 g/dl)	7.1g/dl	206.72 ± 19.87	21.64 ± 18.35	9.55:1
Controls	Normal >12 g/dl	13.2g/dl	93.87 ± 26.152	90.45 ± 8.23	1.03:1

Table 3. Sensitivity and specificity, positive and negative predictive values of patients with iron deficiency anemia

Sensitivity	Specificity	Positive predictive value	Negative predictive value
100%	88.24%	87.6%	100%

patients with mild iron deficiency anemia were found to be slightly above normal.

The sensitivity of ELISA in determination of salivary ferritin was 100% and the specificity was 88.24%. The positive predictive value and negative predictive value was 87.6% and 100% respectively.

DISCUSSION

The assessment of serum ferritin promises to be a useful tool in the evaluation of the iron stores. However, these iron markers are considerably altered by inflammation, which limits their applicability, especially in areas with a high infection pressure. Further, the technique involves drawing of venous blood and is an important consideration upto 12 years of age, when the iron deficiency is at its peak. Hence, the present study was conducted to assess the salivary ferritin in the diagnosis of iron deficiency anemia which demonstrated that there was a significant increase in the mean value of Ferritin in saliva of iron deficient children.

The study was focused on children (8-14 years) because anemia is prevalent in young age group and controlling anemia in this vulnerable age group could significantly reduce the complications in older age as they have an irreversible impairment in neurological changes and are more susceptible to candidiasis, recurrent aphthous ulcers and squamous cell carcinoma.[7, 14, 20]

The initial selection of cases was done based on clinical examination since various studies have revealed that pallor often had a correlation with the hemoglobin levels and hence can be used as an initial tool in the diagnosis of iron deficiency anemia. Also reports by the Integrated Management of Childhood Illness (IMCI) strategy developed by the World Health Organization (1997) recommended the use of palmar pallor as the initial screening tool.[21,22]

The salivary ferritin in patients with mild iron deficiency anemia based on the haemoglobin value was 121.06 with a standard deviation of 10.23. The salivary ferritin: serum ferritin was found to be 1.65: 1. This was slightly above the levels of normal patients. However, the ratio in moderate and severe was significantly higher than the normal. The sensitivity and specificity was also above 95% which highlights the diagnostic significance of this assay.

The sensitivity test is a measure of how likely the test is to come back positive in patients with iron deficiency anemia and is defined as the percentage of iron deficient patients having Ferritin positivity by ELISA. Specificity is a measure which determines how likely the test is to come back negative in patients who does not have the deficiency. Our study demonstrated a Sensitivity and specificity of 100% and 88.24% respectively with positive predictive value of 87.6%. In our study the diagnostic specificity is low, which can be attributed to the sample size and the fluctuations in the values of patients with mild iron deficiency anemia. However, when the sensitivity and specificity was determined in moderate and severe groups, the values were much higher which indicates the diagnostic ability in determination of iron deficiency anemia.

The exact mechanism by which anemia caused a rise in salivary Ferritin is not exactly known. However, it may be speculated that the iron dependent enzymatic functions of the saliva also help in the conservation of iron through saliva of iron deficient patients.[16] Other possibilities include endocytosis of ferritin by the ducts of salivary glands and its excretion into the saliva and presence of high molecular weight iron binding properties of saliva.[23,24] Internalization of Ferritin in the intercalated ducts in the form of lysosomes in the parotid duct could serve as a possible mechanism for the increased salivary levels. This mechanism hitherto has been established in rats, but evidence is not conclusive in human beings. This may also be the mechanism for alterations in the proteins in saliva before it reaches the oral cavity.[25] Furthermore saliva possesses a marked iron binding ability and the high molecular weight iron binding substance in saliva might have a function in health and disease, both because of its molecular weight and its resistance to acid peptic digestion.[24]

Another possible mechanism may also be attributed to the increased salivary manganese levels which inhibit the salivary ferritin transport leading to its retention thereby raising the salivary ferritin in iron deficiency anemia.

However, the scanty literature on this aspect does not allow us to draw an exact pathogenesis for the rise of Ferritin.[26] Activity of the enzyme arginase, in human saliva has been found to be more in serum. It is possible that iron and manganese share a common cellular transport mechanism and thus excesses of one element inhibit transport of the other.[27,28]

Further, the epithelial changes commonly found in iron deficient patients could result from the derangements in intracellular iron metabolism and deficiency of iron containing enzymes, the effects of which might be aggravated by a rapid turnover of cells. This concept is supported by Waldenstrom et al who suggested that the oral mucosal changes in iron deficiency was due to the lack of iron containing compounds in tissues rather than tissue hypoxia.[29] Iron deficiency anemia has been implicated to be the primary cause of tissue changes and the wide range of oral mucosal changes in iron deficiency suggest the importance of enzymatic alterations in Iron deficient subjects.[30]

Wide fluctuations have been observed in the iron containing enzyme, cytochrome c in the oral mucosa, which directly affects the aerobic pathway of energy metabolism in mitochondria with a reduction in the cell proliferation of the oral epithelium. Atrophic glossitis and reduction in the thickness of the epithelium of the buccal mucosa can be attributed to this reason. Due to lack of a clear relationship between tissue iron depletion and the development of oral epithelial changes, a role has been postulated for other essential nutrients and vitamins, especially folic acid, pyridoxine and vitamin B12.[7]

Decrease in the enzyme cytochrome oxidase in the buccal mucosa has been found to be evident even before the development of the symptom.[31,32] Toskes *et al* reported reduced serum folate levels in iron deficiency. The iron dependence of the enzyme formimino transferase could result in metabolic block of the folate metabolism in iron deficient subjects.[33] The reduction in cytochrome concentration is a real effect of iron deficiency which explains the reduction of both energy production and cell proliferation seen in oral epithelia under aerobic conditions.[34]

The presence of glossal pain in iron deficiency anemia was explained by Ohshima et al based on his studies on the taste abnormality and in patients with glossal pain without clinical manifestations and reported that Ferritin was decreased in some of the patients whose taste sensation returned to normal after oral iron administration, which indicated that iron deficiency may cause taste impairment. He also suggested that the metallic taste in the saliva was due to increase iron present in the saliva.[34] Osaki et al assessed the relationship between Ferritin deficiency and glossal pain and ascertained the cause of glossal pain and the oral pathophysiology in anemia and related it to the Ferritin levels and concluded that glossal pain was present in iron deficiency anemia and increase in the degree of pain was associated with an increase in the severity of anemia. He also suggested that that alteration in the Ferritin levels causes glossal pain.[35]

Iron deficiency anemia is characterized by a marked increase in the incidence of oral candidiasis. The Ferritin concentration within oral epithelial cells is directly related to their susceptibility to damage by the human pathogenic fungus, Candida albicans. It has been hypothesized that the host Ferritin is used as an iron source by this organism.[36,37]

From the evidence at hand, the salivary Ferritin assay promises to be a useful tool in the detection of iron stores. It also avoids the need for a Venipuncture and is a particularly important consideration in the young age group, when the incidence of iron deficiency is at its peak, and when it is often technically difficult to obtain several millilitres of peripheral blood. Thus salivary diagnostic tests would provide an inexpensive testing, would reduce affordability and accessibility barrier to early diagnosis and saliva based techniques could supplant current diagnostic techniques. The method also holds great value in epidemiologic surveys to determine the prevalence of iron deficiency and evaluate the efficacy of intervention programs to alleviate it.

The present study, first of its kind revealed that salivary Ferritin markedly increased in iron deficiency anemia and also highlights that it is directly related to the oral epithelial changes. It emphasizes the fact that controlling anemia in children could significantly reduce the complications in older age and would enhance intellectual and work capacity thereby improving family and community socioeconomic development. Further, it highlights the potential use of saliva as a monitoring tool for iron deficiency and thus will aid to improve the quality of life of iron deficient individuals.

CONCLUSION

Expressions of ferritin in saliva, in patients with iron deficiency anemia may be due to the enzymatic functions in the saliva and the endocytosis of ferritin which can possibly elevate the salivary ferritin. Further extensive studies and increasing the sample size, could open a new horizon in the broad field of Iron deficiency anemia, and the salivary Ferritin may be used as a reliable diagnostic marker in iron deficiency anemia and may confirm the exact mechanism by which the salivary Ferritin was increased. Studies with clinical correlation may shed more light and elucidate further, the role of Ferritin in saliva and may enable novel therapeutic measures to come.

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