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Urinary Hepcidin in Diagnosis of Iron Deficiency Anemia

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ABSTRACT

Iron Deficiency Anemia (IDA) is a common health problem among children especially in developing countries. Traditionally, symptoms such as pallor, as well as evidence collected from l complete blood pictures (CBC) and a serum ferritin tests have been among standard clinician and laboratory methods of diagnosing IDA. More recently, advanced research has focused on the use of hepcidin as a main regulator of iron absorption and metabolism as a measure of degree of anemia. Hepcidin has been detected in both serum and urine. We aimed in this study to evaluate the use of urinary hepcidin in the form of a hepcidin creatinized ratio in diagnosis of IDA. In this case, control study, 45 IDA children aged 6-10 years and another 45 healthy non-anemic children aged 6-10 years were recruited. Laboratory tests, including CBCs, serum ferritin and urinary hepcidin levels (by ELISA) were conduct. Significantly lower hemoglobin levels, MCV, MCH, ferritin, serum iron, hepcidin and Hepcidin creatinine ratios were detected in cases of anemia compared to controls. The only exceptions were RDW percentages that were significantly higher in cases compared to controls. In our study, urinary levels of hepcidin (ng mg^{-1} creat) showed a significantly positive correlation with HB, MCV, MCH and serum iron and ferritin levels. On the other hand, urinary hepcidin level showed a significantly negative correlation with RDW percentages. A Receiver Operating Characteristics (ROC) curve was used to detect the cutoff for urinary hepcidin levels. This cut off was to be used to differentiate IDA patients, from healthy children. The cutoff point differentiating IDA from healthy children was concluded to be at 1.3 ng mg^{-1} creat. The area under the ROC curve was 0.7; sensitivity was 91% and specificity of the cutoff point was 51%. We find that urinary hepcidin correlated positively with serum ferritin levels in children and that it was significantly lower in children with IDA. Urinary hepcidin thus seems to be a simple, non-invasive and sensitive parameter for the estimation of serum iron and iron stores.

Key words: Urinary hepcidin, ferritin, IDA, children

INTRODUCTION

Iron is an essential element for nearly all living organisms. Iron is a key component of oxygen storage and transporting proteins, such as hemoglobin and myoglobin, as well as many enzymes that catalyze oxidation-reduction reactions necessary to generate energy and produce various metabolic intermediates for host defense (Anderson *et al.*, 2007).

Despite tightly regulated mechanisms of uptake and distribution, iron homeostasis is often disrupted by inadequate iron supply (e.g., insufficient dietary iron intake, blood loss, parasites) and increased iron demand (e.g., rapid growth, hypoxia) (Fretham *et al.*, 2011).

In nationally representative surveys in Egypt, IDA was reported to be 49 % in children under five Egypt demographic and health survey (El-Zanaty and Way, 2005) and 46.6% in adolescents aged 11-19. Rural children were more likely to be anemic than urban children (51 and 44%, respectively) and children in rural Upper Egypt had the highest anemia levels (58%) (El-Zanaty and Way, 2009).

Despite the significant prevalence of this problem, commonly used tests of iron status have always had limitations. Ferritin has been used as an indicator of iron stores but its levels are also elevated in patients with coexisting inflammation. Soluble transferring receptor (sTfR) levels are seen to reflect tissue iron deficiency, but they are also influenced by erythropoietic activity (Kroot *et al.,* 2011). In addition, transferrin saturation levels may be affected by inflammation and undergo diurnal variation (Zimmermann and Hurrell, 2007).

Hepcidin-25, a 25-amino acid peptide hormone produced in the liver, is a central regulator of systemic iron metabolism (Lee and Beutler, 2009). Hepcidin, down-regulates duodenal iron absorption and macrophage iron release by modulating cellular iron export via ferroportin. The dysregulation of hepcidin production is associated with a variety of iron disorders (Nemeth and Ganz, 2009).

Hepcidin levels are reduced in patients with IDA. Therefore, blood or urine hepcidin levels could potentially be used in the determination of iron requirements and be an accurate indicator of physiological IDA (Kemna *et al.*, 2008).

Urinary hepcidin levels correlate well with hepatic hepcidin mRNA. Hepcidin-25 is the only isoform, which has a dominant role in iron regulation. Urine tests were used as an alternative for serum assays because (I) it is less affected by diurnal variation and (II) the non-invasive nature of sampling (Cherian *et al.*, 2008).

The aims of our study were to evaluate the use of urinary hepcidin-25 levels as a diagnostic test for IDA in children and to determine a useful a correlation between urinary hepcidin levels and serum ferritin levels in children.

MATERIALS AND METHODS

Forty-five patients with iron deficiency anemia were included in this case control study. They were composed of 28 males and 17 females. Their ages ranged from 6-10 years with a median age of 6.9 years. Another 45 healthy children were included in the control group. This group included 19 males and 26 females. Their ages ranged from 6-10 years with a median age of 6.8 years. This study was performed in the period between Jan 2014 and June 2014. Children of this study were recruited from different schools in the Damietta governorate by quota sampling.

Inclusion criteria

Case group: The 45 children were selected based on hemoglobin levels ≤ 11.5 gm dL^{-1} and ferritin levels ≤ 20 ng mL^{-1}.

Control group: The 45 healthy children were selected based on a hemoglobin level > 11.5 mg dL^{-1}.

All children with conditions that might affect serum ferritin were excluded from this study. This Included patients suffering from malignancies or infections, or collagen, renal or liver diseases Children who had received iron therapy or blood transfusion the 3 months prior to the study were also excluded.

A detailed history was collected for all study subject. This was composed of a (a) Personal history, including name, age, gender, residence, number of family members; (b) History of the

present illness, evident pallor, infection, drug intake, bleeding disorder and/or mental status (abnormal behavior, school performance) and (c) Past history; including nutritional, developmental history, history of parasitic infestations (by asking about infestation by parasites or history of anti- helminthic treatment) and history of chronic diseases (such as, renal failure, cardiac disease and pulmonary diseases).

Cases and controls were also subjected to a thorough clinical examination, including a general examination with a careful search for pallor, any brittle nails or spooning of nails or a sore tongue. Anthropometric measurements were taken, including weight and height measurements. Abdominal examination was performed, with a focus on detecting any organomegaly.

Blood samples were withdrawn for measurement of a complete blood count, serum ferritin and serum iron. Freshly voided urine samples were collected mid-stream in the morning and measurements of hepcidin levels by ELISA were determined.

Samples were centrifuged for 20 min at 1000×g. Particulates were removed and samples were stored in aliquot at -20ºC for later use. Repeated freeze/thaw cycles were avoided. The kits used were from Uscn Life Science Inc. This kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to measure the level of human hepcidin (Hepc) in samples.

Using the kit, hepcidin (Hepc) was added to a monoclonal antibody enzyme well, which was pre-coated with human hepcidin (Hepc) monoclonal antibodies and incubated. Next, hepcidin (Hepc) antibodies labeled with biotin were added and combined with streptavidin-HRP to form an immune complex. This was then intubated and repeated washing was done to remove the uncombined enzyme. Detection reagents A and B were added. The color of the liquid changed to the blue and-due to the effect of acid-to a final yellow.

The chroma of color and the concentration of the human substance hepcidin (Hepc) of samples were positively correlated. According to standards' concentration and the corresponding OD value, the standard curve of the linear regression equation was calculated and the OD values of the sample were applied to the regression equation to calculate the corresponding sample's concentration. Urinary hepcidin levels were correlated and normalized to urinary creatinine, Hepcidin concentrations which were thus calculated as pg mL^{-1} have been expressed as ng mg^{-1} creat.

Statistical analysis: Data processing and parametric statistical analysis of results were performed using SPSS software (version 20). Quantitative data were presented as mean and Standard Deviation (SD) and range was calculated. For comparison between groups, the student sample (t) test was used. Stepwise multiple linear regression analyses were completed to determine the factors significantly associated with iron status. Correlations and linear regression analyses to examine the relationships between CBC data, serum iron, serum ferritin and urinary hepcidin were performed. The p value≤0.05 was considered significant for interpretation of results.

RESULTS

The mean age of the studied patients and the controls were (6.9±1.1 years) and (6.8±1.1 years) respectively with a p = 0.48. Mean weights were (22±3.45) and (22.9±3.01) in the studied patients group and controls, respectively with a p = 0.18. Mean heights were (120±5.2) and (121±4.7) in the studied patients group and controls, respectively with a p<0.36. These numbers indicated that there was no significant difference between cases and controls with respect to age, weight and height (Table 1).

Table 1: Comparison between cases and controls regarding age, weight and height

Group	Cases (n = 45)	Controls (n = 45)	T -test	p-value
Age (years)				
Mean±SD	6.9±1.1	6.8±1	0.7	0.48
Range	(6-10)	(6-10)		
Weight (kg)				
Mean±SD	22±3.45	22.9±3.01	1.3	0.18
Range	(17-34)	(18-30)		
Height (cm)				
Mean±SD	120±5.2	121±4.7	0.9	0.36
Range	(115-138)	(115-134)		

Table 2: Comparison between males and females regarding serum ferritin and urinary hepcidin

Groups	Cases	Controls	Eta square	p-value
Serum ferritin (ng mL^{-1}) Mean±SD				
Males	12.7±4.6	33.7±12.7	0.05	0.04*
Females	13.4±4.8	37.6±14		
Urinary hepcidin (ng mg^{-1} creat) Mean±SD				
Males	1.0±0.8	1.5±1.2	0.3	<0.001*
Females	2.4±1.7	4.6±2.5		

Mean levels of serum ferritin were (12.7±4.6 ng mL^{-1}) and (33.7±12.7 ng mL^{-1}) in male patients and the control group, respectively. Mean serum ferritin levels were (13.4±4.8 ng mL^{-1}) and (37.6±14 ng mL^{-1}) in female patients and the control group, respectively with a p = 0.04. Mean levels of urinary hepcidin were (1±0.8 ng mg^{-1} creat) and (1.5±1.2 ng mg^{-1} creat) in male patients and the control group, respectively. Urinary hepcidin was (2.4±1.7 ng mL^{-1}) and (4.6±2.5 ng mL^{-1}) in female patients and the control group, respectively with a p<0.001. These numbers indicated that there was a significant difference between males and females in our study with respect to serum ferritin and urinary hepcidin as they were significantly lower in males than females (Table 2).

Mean hemoglobin levels were 10.9±0.9 and 12.4±0.6 g dL^{-1}% in patients and the control group, respectively with a p<0.001*. Mean MCVs were (68±6.4fl µm) and (75.8±4.3 fl µm) in patients and the control group, respectively with a p<0.001*. Mean MCHs were (23±3.1 and 26±1.9 fL pg) in patients and the control group, respectively with a p<0.001*. Mean MCHCs were (32±1.2 and 34±1.7 pg cell^{-1}) in patients and the control group, respectively with a p = 0.014. Mean RDWs were (14.8±0.9%) and (12±0.5%) in patients and the control group, respectively with a p<0.001*. Mean serum iron levels were (90±31 µg dL^{-1}) and (114±31 µg dL^{-1}) in patients and the control group, respectively with a p<0.001*. Mean urinary hepcidin levels were (0.8±0.6 ng mg^{-1} creat) and (1.6±1.3 ng mg^{-1} creat) in patients and the control group, respectively with (p<0.001*). All this figures appear to indicate that there was a significant decrease in hemoglobin, MCV, MCH, ferritin, serum iron, hepcidin and hepcidin creatinine ratio in cases compared to controls. Only RDW percentages were significantly higher in cases compared to controls (Table 3).

A significant correlation between hemoglobin and urinary hepcidin levels was found (Pearson's Rho = 0.41; p = 0.001). A significant correlation between MCV and urinary hepcidin was also found (Pearson's Rho = 0.3; p = 0.001). Another significant correlation between MCH and urinary hepcidin level was found (Pearson's Rho = 0.25; p = 0.005).

A significant correlation between RDW and urinary hepcidin level was found (Pearson's Rho = 0.38; p = 0.001). Similarly, a significant correlation between serum iron and urinary hepcidin levels was found (Pearson's Rho = 0.22; p = 0.005). Finally, a significant correlation between ferritin and urinary hepcidin levels was found (Pearson's Rho = 0.40; p = 0.001). These numbers pointed

Table 3: Comparison between cases and controls regarding laboratory data

Groups lab parameters	Cases	Controls	Test statistics	p-value
Hb% (g dL^{-1})				
Mean±SD	10±0.9	12.4±0.6	14.5	<0.001*
MCV (fL μm)				
Mean±SD	68±6.4	75.8±4.3	5.4	<0.001*
MCH (fL pg)				
Mean±SD	23±3.1	26±1.9	4.5	<0.001*
MCHC (pg cell^{-1})				
Mean±SD	32±1.2	34±1.7	2.5	0.014
RDW (%)				
Mean±SD	14.8±0.9	12±0.5	13.5	<0.001*
Serum iron (μg dL^{-1})				
Mean±SD	90±31	114±31	3.5	<0.001*
Ferritin level (ng mL^{-1})				
Mean±SD	12±4.7	36±14.3	10.2	<0.001*
Hepcidin creatinine ratio (ng hepcidin mg^{-1} creatinine)				
Mean±SD	0.8±0.6	1.6±1.3	3.7	<0.001*

*Significant at p<0.001

Table 4: Correlation between urinary hepcidin levels and laboratory data

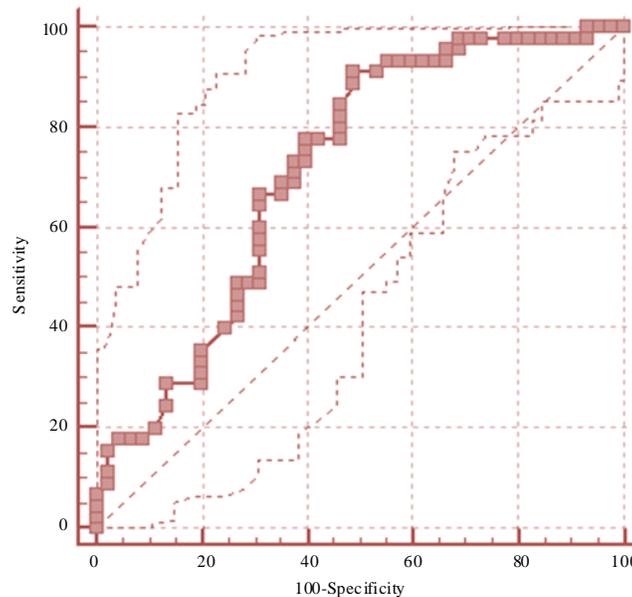| Lab data	Pearson's (r)	p-value
Hb (g dL^{-1})	0.4	0.001*
MCV (fL μm)	0.3	0.001*
MCH (fL pg)	0.25	0.005*
RDW (%)	0.38	0.001*
Serum iron (μg dL^{-1})	0.22	0.005*
Ferritin (ng mL^{-1})	0.4	0.001*

*Significant at p<0.001

Fig. 1: ROC curve for urinary hepcidin in diagnosis of IDA

to a significant positive correlation between urinary hepcidin levels and hemoglobin levels, MCV, MCH, serum iron and ferritin levels. They indicated also a significant negative correlation between RDW and hepcidin levels (Table 4). At cut value of ferritin≤20 ng mL^{-1}. The Area Under Curve (AUC) was 0.7 with cut off value of urinary hepcidin at 1.3 ng mg^{-1} creat., which seems to differentiate IDA patients from healthy children. Specificity was 51% and sensitivity was 91% (Fig. 1).

DISCUSSION

In our study, there was a significant difference between male and female serum ferritin and urinary hepcidin concentrations, being significantly, lower in males than females. These findings agreed with those of Cangemi *et al.* (2013) who found that hepcidin was significantly higher in post pubertal normal females than in normal males. Interestingly, the negative correlation between serum ferritin and sex hormones was of value in terms of hematopoiesis. Erythrocytosis is significantly related to sex hormones-especially testosterone, as the iron is incorporated into erythrocyte by testosterone and the process is under the strong regulation of hepcidin. On the other hand, ferritin and transferrin levels are significantly related to iron metabolism and are also under the control of hepcidin.

Regarding urinary hepcidin levels, they were significantly lower in cases of ID when compared to the control group. This agreed with the results of a study conducted by Sanad and Gharib (2011) who found that hepcidin levels were significantly lower in ID and IDA. They also reported a more significant reduction in its level with the progress in severity of ID.

In our study, urinary levels of hepcidin (ng mg^{-1} creat) showed significant positive correlation with hemoglobin, MCV, MCH, serum iron and ferritin levels. On the other hand, urinary hepcidin levels showed significant negative correlation with RDW percentages. This fell in line with the study conducted by Sanad and Gharib (2011) and Choi *et al.* (2012) found a high correlation between serum hepcidin and serum ferritin levels (r = 0.6) and also there were a high correlations with other iron parameters. Muller *et al.* (2012) also found that iron status, erythropoiesis and anemia correlated with the levels of the mature 25 amino-acid form of hepcidin in sick preterm neonates (Muller *et al.*, 2012). The strong correlation between hepcidin and ferritin compared with other iron-status indicators was also observed, suggesting that hepcidin may be a useful indicator of iron stores.

Iron metabolism and erythropoiesis are inextricably interconnected. Erythrocytes requires iron incorporation into the heme group to carry oxygen. Without adequate iron, the maturation of erythrocytes is impaired, resulting in microcytic hypochromic anemia. Most of the iron for erythropoiesis comes from catabolism of senescent RBCs by macrophages in the reticuloendothelial system, with effective erythropoiesis counter balancing the loss of aged blood cells. Hepcidin, expression is down regulated by erythropoietic stimuli such as anemia and hypoxia, the latter of which may play a role in iron regulation in patients with anemia accompanied by ineffective erythropoiesis (Zhao *et al.*, 2013).

Comparisons of data across different independent studies can be challenging due to the variations in cutoff values, methods of survey and laboratory analyses, population sample size and age ranges of study subject.

A Receiver Operating Characteristics (ROC) curve was used to detect the cutoff point of urinary hepcidin levels that would properly differentiate patients with ID from healthy children. The cutoff point differentiating ID from healthy children was determined to be 1.3 ng mg^{-1} creat. The area under the ROC curve was 0.7, meaning that sensitivity was 91%. Specificity at this cutoff point was 51%. The positive predictive value was determined at 65% and negative predictive value 85%. Test specificity was found to be 87%, while sensitivity was calculated at 70% at ferritin $\leq$15. In addition, test specificity was determined to be 97% and sensitivity 48% at ferritin$\leq$9. All highly supports the idea that the test is highly sensitive at the cut value of ferritin we used and is highly specific with progress in iron deficiency.

Jonker *et al.* (2013) found that the optimal cut-off for hepcidin to predict bone marrow iron deficiency derived from the ROC-curve was 0.5 nmol L^{-1}. Using this cut-off, serum hepcidin

sensitivity and specificity estimates the may be used to detect bone marrow iron deficiency were 66.7 and 49.5%, respectively (Jonker *et al.*, 2013).

Sanad and Gharib (2011) were the first, who tried to find an appropriate cut value point for urinary hepcidin to use in the diagnosis iron deficiency in children. They determined it be at 0.08 nmol mm^{-1} for third stage iron deficiency which is associated with a decline in hemoglobin concentrations below thresholds and a concomitant decrease in all iron parameters[9]. At that cut off, sensitivity and specificity were 96 and 100%, respectively.

In our study, we had a different size of sample populations as we had a larger control group. We also had a different type of hepcidin kit. All this makes different results acceptable.

We should consider the significant difference between males and females with regards to urinary hepcidin concentration. Many patients who are disease free could be told the possibility that they have the disease and could then be subjected to further complementary investigations (Lalkhen and McCluskey, 2008).

However a positive urinary hepcidin test may necessitate additional confirmatory testing and result in unnecessary burdens associated with cost for the overall health care system. Urinary hepcidin assay provides an indirect measure of the circulating hormone level and allows the development of a potential non-invasive means of diagnosing IDA. This could become particularly useful for children, as a screening test. Also, the higher pre-analytical variability associated with urine specimens compared to serum is a potential limitation of this approach. Further evaluation of urine hepcidin for non-invasive monitoring of iron status in children is necessary (Muller *et al.,* 2012).

CONCLUSION

We conclude that urinary hepcidin correlated positively with serum ferritin levels in children. decreasing in children with in IDA.. Urinary hepcidin is a simple, non-invasive and sensitive parameter of serum iron and iron store estimation.

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