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Vitamin A Level and Hepcidin Gene Expression in Children with Iron Deficiency Anemia.

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ABSTRACT

Iron deficiency anemia (IDA) and vitamin A deficiency affect more than 30% of population worldwide .Vitamin A deficiency may impair iron metabolism, but the mechanism of this action is still unclear. This study aimed to assess serum vitamin A in iron deficient anemic children and its correlation with iron status and hepcidin gene expression before and after low dose iron supplementation. This interventional study included 38 patients aged from 5-13 years diagnosed as iron deficiency anemia compared with 37 age and sex matched controls. Vitamin A was measured using high performance liquid chromatography, Complete blood count, iron indices, serum hepcidin and hepcidin gene expression before and after 8 weeks of iron supplementation were assessed. Our study found that after 8 weeks of low dose iron supplement serum iron, transferrin saturation%, serum ferritin, body iron content, and hepcidin gene expression fold change were significantly increased. Serum vitamin A was significantly decreased in IDA children (P=0.033), and negatively correlated with hepcidin (r= -0.54, P=0.03).We concluded that after low dose of oral iron therapy for 8 weeks in IDA, there was improvement of iron status with slight hematological improvement and reduction in serum vitamin A.

Keywords: Serum vitamin A, Hepcidin gene expression, Children, Iron deficiency

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INTRODUCTION

Iron deficiency anemia (IDA) is considered a major health problem among school children in our country and other developing countries. Iron deficiency (ID) can cause growth retardation, increased incidence of infection and reduced life vitality. Moreover, it can cause many complications during child birth period in females [1].Globally, 50% of cases of anemia are due to ID [2].

Vitamin A deficiency (VAD) involves about 190 million children globally which cause night blindness, xerophthalmia, infection and iron deficiency anemia [3]. Iron deficiency anemia and vitamin A deficiency were found to be closely related in both humans and animals [4].

Worldwide, Iron deficiency anemia and Vitamin A deficiency involve more than 30% of population. Infants, children and pregnant women are the most risk groups for these deficiencies [5].

Many studies showed that, the metabolism of vitamin A and iron are closely related [6-7] Roodenburget al., found that vitamin A deficiency affects hematopoiesis by influencing iron tissue mobilization and absorption [8].Shi et al., found that iron deficiency could affect vitamin A nutritional status by influencing absorption ,storage and transportation of vitamin A[9] , while Tanumihardjo, found that iron supplementation improves iron status and could induce vitamin A reduction. The mechanism of enhancing effect of iron on vitamin A reduction isn't known [10].

Hepcidin, is a 25-amino acid peptide hormone produced in the liver discovered in 2001, which is a central regulator of systemic iron metabolism .The initial role of hepcidin is to control the iron amount by controlling the absorption of iron from the intestine and release of iron into our circulation from spleen macrophages and enterocytes[11-12].

Hepcidin gene expression is stimulated by iron, obesity and chronic inflammation to limit iron availability, while hepcidin gene expression is inhibited by iron deficiency, hypoxia and erythropoietin to increase iron availability for erythropoiesis[13].

It's known that vitamin A have a regulatory role on many genes in addition to its antioxidant effects. Research on rats suggested that vitamin A may regulate the biosynthesis of hepcidin gene as its deficiency may increase hepcidin gene expression and inturn change in iron hematostasis[14].Human studies especially in children are lacking.

Intervention programs for treatment and prevention of IDA by using traditional iron supplementation and food fortification appear to be insufficient for solving this problem.

Failure of previous programs may be due to complexity of many factors, vitamin A deficiency is one of them [15].Other factor is the absorption of a large oral dose of iron (Traditional dose) will cause an increase in plasma iron, which in turn will cause an increase in hepcidin, which in turn will interfere with iron absorption of the next dose of iron [16]. So our study aimed to assess the effect of low dose oral iron supplement on level of serum vitamin A and to study the response of hematological parameter ,iron status and hepcidin gene expression to this dose in iron deficiency anemia.

MATERIALS AND METHODS

This interventional study was conducted in Pediatrics Clinic in the Centre of Excellence in National Research Centre, Egyptian the period from March to September 2020.The study included 38patients aged from 5-13 years diagnosed as iron deficiency according to criteria of WHO,2001[17] (serum ferritin ≤30 mg/l, transferrin saturation ≤16%,mean corpuscular volume (MCV)≤73 fl and corpuscular hemoglobin concentration(MCHC) ≤32 g/l), and anemic (hemoglobin level≤ 11 g/dL) adopted by Egypt Demographic and Health Survey[18].

Laboratory investigations were done in the form of complete blood count (CBC), serum iron, total iron binding capacity, and serum ferritin and those who were eligible for the study were included. Exclusion criteria were: concurrent infection (C reactive protein (CRP)> 5), chronic inflammatory diseases (rheumatic disease, inflammatory bowel disease), parasitic infestation, thalssemia traits α & β (they were ruled out by reticulocyte count % <2% and HB electrophoresis (A_2<4%), lead toxicity, iron,

vitamin A supplementation and blood transfusion during the last 6 months. Thirty seven healthy children age and sex matched were enrolled in the study as a control group.

All the included children were subjected to full history taking with stress on dietetic history, iron or vitamin A supplementation during last 6 months. Manifestation of iron deficiency (fatigue, leg cramps on climbing stairs, poor school performance, cold intolerance and reduced resistance to infection) were looked for. Full clinical examination was carried out to detect signs of iron deficiency as pallor of the mucous membranes, koilonychia, glossy tongue, atrophy of the lingual papillae and angular stomatitis. Also, we stressed on manifestation of vitamin A deficiency as night blindness, xerophthalmia and recurrent infection.

Five ml of venous blood were withdrawn under aseptic conditions; withdrawal from 10 to 11am (to avoid diurnal variation), 2ml were collected into EDTA vacutainer tubes (one ml for CBC & reticulocyte count and the other for hepcidin gene expression), the other 3ml were collected into plain tubes and centrifuged, serum samples were stored at -80^{oC} for further laboratory investigations.

Laboratory Investigations:

The following were assessed to all patients and controls: complete blood count (using automated hematology analyzer SysmxXN100**(Sysmex America Inc)**,reticulocyte count (using automated hematology analyzer Sysmx XN100i**(Sysmex America Inc)**,
CRP(using IMMUNOSPEC REFE 29-056)**(Bioquote Ltd, UK)**, serum iron and total iron binding capacity (TIBC) (using Olympus AU400(Autoanalyzer , Japan).Serum ferritin (SF)(Biocheck, Inc;Cat. No. EC-1025)BioCheck, Inc. Transferrin saturation % was calculated using the following formula[17](TS%= serum iron /TIBC ×100).Serum soluble transferrin receptor (sTfR) by ELISA (Cat. No: E0281Hu) (BioVendorInc). Serum hepcidin by ELISA (CatNo: E1019 Hu)**(Bioassay Technology Lab, China).**

Body total iron content(BTIC)(mg/kg) was calculated from the following equation BTIC=[-log(sTfR/SF)-2.8229]/0.1207.TFR-F index was calculated from the following equation TFR-F indexsTfR(mg/L)/LogSF(ug/L) [19].

Molecular study of hepcidin gene expression using reverse transcriptase polymerase chain reaction:

Ribonucleic acid (RNA) was extracted using QIAamp RNA Blood Mini kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Samples were extracted on the same day. The final RNA concentration was determined using a spectrophotometer (Nanodrop 2000, Therom Fisher, Walthm, USA) and RNA purity was verified by an average A260/ A280 ratio of 1.98 (range, 1.97–2.01).

RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) using a high-capacity cDNA reverse transcription kit (Applied Biosystems®, Branchburg, New Jersey, USA) in a final volume of 20 µl. Negative control samples were included in each set of reactions. Reactions were incubated at 25°C for 10 min, followed by 37°C for 120 min and final denaturation at 85°C for 5 min. The reaction was carried out in the Bio-Rad Thermal Cycler (Life Science Research). cDNA was stored at −20°C.

Real-time polymerase chain reaction:

Gene expression of hepcidin was measured using TaqMan® Amplification System (Applied Biosystems®, Branchburg, New Jersey, USA). All samples were run in a final reaction volume of 20 µl. The reaction mix was combined using 10 µl TaqMan® Universal PCR Master Mix, 3 µl of cDNA, 6 µl of Dnase free water and 1 µl of specific primers and probes 20x20 (Applied Biosystems®, Branchburg, New Jersey, USA). Expression of hepcidin gene was normalized using the Glyceraldhyde 3-phosphate dehydrogenase (GADPH) housekeeping gene. The PCR run was carried out using the thermal profile 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min on the (Roror -Gene Q – Qiagen -Germany).

Assessment of vitamin A using high performance liquid chromatography (HPLC):

Vitamin A was estimated by HPLC method as described previously by Hussein et al. [20] Sample was extracted by ethanol and hexane, dissolved and filtered by hydrophilic PVDF.

The filtrate was then injected onto a reversed phase column (C18-25cm,5µm particle size) and UV detector was set at 325 nm. The concentrations in samples were obtained from the standard curve.

IDA group was treated with low dose of oral iron syrup (10 mg/day for8 weeks) in a trial of testing the therapeutic efficacy (according to Moretti et al., Schrier and Hamed et al.,)[21,22,23].with avoidance of hazards. Each 5 ml is composed of 57.51 mg ferric ammonium citrate equivalent to 10 mg elemental iron. Treated children were followed up every 4 weeks for 8 weeks. Information was obtained regarding any complaints and adverse effects on each follow-up visit.

Full history taking, complete physical examination and all laboratory investigations were repeated one day after completing iron therapy for 32 children (6 missed patients didn't come for follow up).

The guardians of all patients and controls were informed about the content & type of the study and informed written consents were obtained from all of them. The study was approved by the ethical committee of National Research Center number (18096) according to the Institutional Committee for the Protection of Human Subjects and adopted by the 18th World Medical Assembly, Helsinki, Finland.

Statistical analysis

Data were collected, revised, coded and entered to the statistical package SPSS version 18 for Windows; SPSS Inc., Chicago Illinois, USA .The data was normally distributed as tested by Kolmogorov-Smirnov and Shapiro-Wilk for normal distribution. Continuous variables were expressed as means ± Standard deviation (SD). Independent t-test was used to compare means of variables for2 unrelated groups. Paired sample t-test was used to compare means of 2 variables (pre and post-iron therapy) within a group. Pearson correlation was used to compute the pair wise associations for a set of variables. The p-values ≤ 0.05 were taken as significant.

RESULTS

Our study included 38 children with IDA (24 males and 14 females) compared with 37 healthy children as control (21 males and 16 females).The mean age of the children with IDA and control were 9.07±2years (ranged from 5 to 13 years) and10.41±2.14 years (ranged from 6 to 14 years) respectively .Laboratory data of the studied groups were presented in table 1, it showed significant reduction in hemoglobin (HB),hematocrit (HCT), MCV, red blood cell distribution width(RDW%), transferrin saturation%, serum iron, and serum ferritin in IDA children. Serum hepcidin was lower in IDA group but not statistically significant. Hepcidin gene expression was significant lower in IDA group. There was no significant difference in serum vitamin A level between both groups.

After low dose oral iron therapy (table 2) IDA group showed improvement in HB, HCT, and MCV but not statistically significant. CRP showed no change after oral iron therapy.

Iron indices (serum iron, transferrin saturation %, and serum ferritin) were significantly increased (P =0.001, 0.001, 0.001respectively) .The body iron content changed from the iron deficit in tissues (negative value)-5.90±5.796to iron surplus in stores (positive value) 1.29±4.28(P=0.008).Serum hepcidin increased but was not statistically significant while hepcidin gene expression was significantly elevated(P=0.025).On other hand serum vitamin A level was significantly decreased(P=0.033).

Table 3 & figure 1 showed that after iron supplementation serum vitamin A was negatively correlated with serum hepcidin (r= -0.54, P=0.03).

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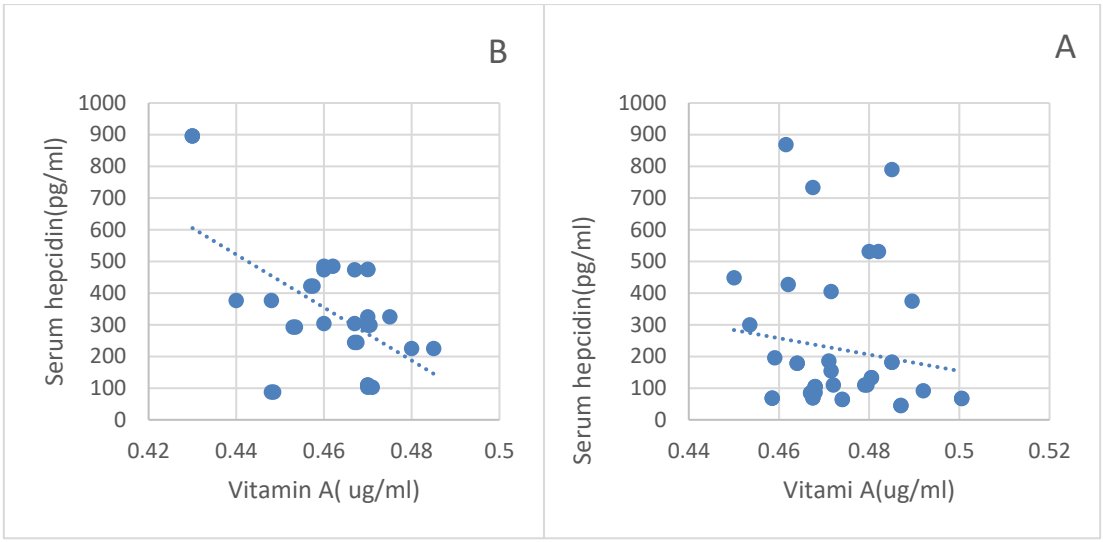

Figure 1: Correlation between serum vitamin A and serum hepcidin before(A) and after (B)low dose oral iron supplement .

Table 1: Demographic and Laboratory data of the studied group

	Control (No= 37) Mean ±SD	Patients (No= 38) Mean ±SD	P value
Age (years)	10.41±2.14	9.07±2	0.06
Wight (kg)	33.8±7.96	29.11±6.01	0.406
Height (cm)	135±18.08	127.19±22.18	0.225
Body Mass Index	33.8±7.96	29.11±6.01	0.406
CRP(mg/dl)	0.4±0.2	0.39±0.1	0.3
Reticulocyte count (%)	1.2±0.5	1.3±0.4	0.4
HB (gm/L)	12.42±0.98	10.89±1.28	0.001
HCT%	35.98±2.49	31.63±6.62	0.013
MCV (fl)	76±5.17	67.46±8.83	0.001
MCH (pg)	26.21±2.38	22.69±3.63	0.001
MCHC (g/dl)	34.15±1.89	31.5±5.54	0.064
RDW%	12.16±1.3	14.47±2.32	0.002
Serum iron (ug/dl)	92.53±10.4	33.54±8.21	0.001
Serum TIBC (ug/dl)	314.86±38.62	340.86±44.71	0.62
Transferrin saturation%	29.50±8.10	12.21±7.88	0.001
Serum ferritin (ng/dl)	118.86±27.44	24.20±2.42	0.001
Serum sTfR1 (mg/L)	0.52±0.76	0.5±0.89	0.9
TFR –F index	0.26±0.38	0.28±0.41	0.92
Serum hepcidin (pg/ml)	409.73±69.5	272.01±242.84	0.16
Hepcidin gene expression fold change	3.01±0.83	1.83±1.37	0.02
Serum Vitamin A (ug/ml)	0.47±0.017	0.472±0.011	0.5

BMI; Body mass index, CRP : C reactive protein , HB: hemoglobin, HCT: hematocrit
MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red blood cell distribution width, TIBC: total iron binding capacity, sTfR1: soluble transferrin receptor I
P-value ≤ 0.05 is significant / Data expressed as mean ± standard deviation

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Table 2: Comparison of hematological parameters, iron indices and serum vitamin A level before &after iron supplementation.

	Before iron therapy Mean ±SD	After iron therapy Mean ±SD	P value
HB (gm/L)	10.89±1.28	11.35±0.95	0.26
HCT%	31.63±6.62	34.46±2.62	0.16
MCV (fl)	67.46±8.83	68.42±8.66	0.74
MCH (pg)	22.69±3.63	22.68±3.09	0.99
MCHC (g/dl)	31.5±5.54	30.51±8.63	0.66
RDW%	14.47±2.32	14±2.11	0.64
CRP(mg/dl)	0.39±0.1	0.4±0.2	0.3
Serum iron (ug/dl)	33.54±8.21	82.89±40.08	0.001
Serum TIBC (ug/dl)	340.86±44.71	256.35±76.25	0.17
Transferrin saturation%	12.21±7.88	35.62±21.73	0.001
Serum ferritin (ng/dl)	24.20±2.42	136.13±83.06	0.001
Serum sTfR (mg/L)	0.5±0.89	0.97±1.3	0.38
TFR –F index	0.28±0.41	0.47±0.67	0.46
Body iron content(mg/kg)	-5.90±5.796	1.29±4.28	0.008
Serum hepcidin (pg/ml)	272.01±242.84	373±233.86	0.18
Hepcidin gene expression fold change	1.83±1.37	2.83±0.88	0.025
Serum Vitamin A (ug/ml)	0.472±0.011	0.463±0.013	0.033

HB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red blood cell distribution width,CRP:: C reactive protein, TIBC: total iron binding capacity, sTfR: soluble transferrin receptor, P-value ≤ 0.05 is significant / Data expressed as mean ± standard deviation

Table 3 Correlations between laboratory parameters and vitamin A level in iron deficiency anemia before and after treatment.

Variables	**vitamin A** (before treatment)		**vitamin A** (after treatment)	
	r	P-value	r	P-value
HB	-.018	0.9	-.018	0.9
Serum iron	0.30	0.36	0.3	0.36
Serum TIBC	- 0.24	0.41	- 0.24	0.4
Transferrin saturation%	0.36	0.27	0.36	0.27
Serum ferritin	- 0.36	0.17	- 0.34	0.19
TFR –F index	-0.3	0.9	-0.3	0.9
Body iron content	-0.14	0.60	-0.11	0.66
Serum hepcidin	-0.5	0.05	-0.54*	0.03
Hepcidin gene expression	- 0.12	0.7	- 0.12	0.71

HB: hemoglobin, TIBC: total iron binding capacity, sTfR: soluble transferrin receptor

DISCUSSION

Many studies have supported the concept that vitamin A has a role in iron metabolism and, in turn IDA, both in human and in rats. Some researchers suggested that persons with vitamin A deficiency were more prone to develop IDA that was reversed when vitamin A was taken in adequate doses [1].

Numerous mechanisms may explain the role of vitamin A on anemia through its effect on iron hematopoiesis, metabolism, or both and increased resistance to infection, however the exact mechanism is still unclear yet [24].

Our study showed that at the base line, there was no significant difference in serum vitamin A between patients and controls. After 2 months of iron supplementation serum vitamin A was reduced significantly in IDA group in comparison to the patients before treatment (P =0.033),that agreed with Tanumihardjo and Wierindiaet al.,[10&24]. Wierindia et al., explained this reduction by redistribution of vitamin A from plasma to the liver or increased vitamin A requirements because of accelerated erythropoiesis, they recommend vitamin A measurement to avoid its deficiency during iron treatment[24].Results of animal studies showed different effects of iron on the intrahepatic balance between cellular uptake and mobilization of vitamin A[14].

Calculated body iron content (indicator for iron absorption)is used to assess the prevalence of IDA and the impact of iron therapy and fortification trials, instead of the conventional markers(serum ferritin and sTfR) ,as it is not affected by malnutrition and inflammation[5].In our study body iron content was increased significantly after iron therapy (P =0.008) without correlation with vitamin A. indicating that VAD had no effect on iron absorption. but would rather affect iron mobilization mechanisms.

VA play a role as ligand-regulated transcription factor form any genes expression, but its role in hepcidin gene expression is unclear [25].Arruda et al., found that VAD rats had increased hepatic hepcidin gene expression, higher concentration of iron in spleen and increased oxidative status than control rats [14].On contrary, Da Cunha et al., found that VAD rats had a lower hepatic hepcidin gene expression concentration than controls [26].Our study showed no correlation between vitamin A and hepcidin gene expression.

In our study negative correlation was found between serum vitamin A and serum hepcidin(r= -0.54, P=0.03), that agreed with Cruz-Góngora[27].Also ,Citelli et al., found increased hepatic hepcidin in VAD , suggesting that VAD may affect hepatic iron mobilization as well the transcription factors of protein genes involved in the iron bioavailability[6].

After daily iron supplementation for 8 weeks, there was slight improvement in HB, MCV, and MCH compared to the patients before treatment ,while serum iron, transferrin saturation%, serum ferritin , body iron content, and hepcidin gene expression fold change were significantly increased (P =0.001, P = 0.001, P = 0.001, P = 0.008, P = 0.02 respectively). The little improvement in Hb synthesis may be due to short period of iron therapy (8 weeks) , low dose of iron supplement or lack of iron mobilization due to VAD as reflected by significant increase in ferritin rather than for HB synthesis. However Hamed et al., reported that, low dose iron therapy for 8 weeks could be sufficient to improve antioxidant status and DNA damage together with correction of the hematologic indices. This may indicate that iron treatment alone in absence of micronutrients like vitamin A, cannot efficiently complete the HB synthesis process. Thomas et al., found that vitamin A influences erythrocyte incorporation of iron by influencing iron transport to the bone marrow, iron uptake by the bone marrow and/ or erythropoiesis. It is commonly assumed that 80% of absorbed iron is incorporated into erythrocytes after 14 day. The incorporation rate varies with vitamin A status [28]

A study by Zimmerman et al., found that in children deficient in iron and VA, supplementation with vitamin A mobilizes iron from its stores to support increased erythropoiesis, an effect likely mediated by increase in circulating erythropoietin [23]. In addition to the effect of vitamin A on erythropoiesis, it is believed that VA can suppress the infectious process due to its antioxidant properties, which inturn cause more improvement of HB during the course of treatment.

Hassan and Osama showed that after 12 weeks of iron treatment, one third of their studied children remained anemic. When multi vitamins including vitamin A were added, they became non anemic [29]. Biswas found that vitamin A supplementation along with usual iron therapy was beneficial in long term outcome in children with IDA [1].

Also Al –Mkhlafi found significant impact of vitamin A on iron status among IDA children [30], while Chen et al., did not find any effect of combined therapy of iron and vitamin A in improving anemia [5].

The limitation of our study was the lack of combined therapy of vitamin A and iron to study its effect on erythropoiesis.

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CONCLUSION

We concluded that after low dose of oral iron therapy for 8 weeks in IDA, there was improvement of iron status, reduction in serum vitamin A and slight hematological improvement. Further large study with different iron doses for longer periods with and without vitamin A supplementation is needed to clarify the exact effect of iron on vitamin A level.

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