Interplay of Inflammatory Markers and Vitamin D Deficiency for Development of Pregnancy-associated Anemia

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Abstract

Objectives: To estimate the serum levels of vitamin D (VD), hepcidin (Hp), interleukin (IL)-6 in pregnant women during 1st trimester and their relation with estimated serum ferritin concentration (SFC) and hemoglobin concentration (HC).

Patients & Methods: 106 pregnant women were clinically evaluated and gave blood samples at 1st trimester for ELISA estimation of serum levels of VD, Hp, IL-6 and FC. All women with pregnancy-associated anemia (PAA) received oral daily iron supplemental therapy and HC was re-estimated at 3rd trimester and HC change was calculated. Study outcomes included frequency and severity of 1st trimester iron deficiency (ID) and anemia, and hypovitaminosis D (Hypo-VD) and its relation to other studied parameters. Secondary outcome was the effect of iron supplemental therapy (IST) on frequency and severity of anemia.

Results: At 1st trimester, 41 women were anemic and 27 women had ID, while on the 3rd trimester, 56 women had anemia; 29 had persistent and 27 women had newly developed anemia. Mean HC at 3rd trimester was significantly lower than at 1st trimester and only 16 women had increased, while 90 women had decreased HC. At 1st trimester, 56 women had hypo-VD, 31 women had insufficient and 19 women had sufficient VD levels. Estimated 3rd trimester HC was negatively correlated with serum Hp and IL-6. Percentage of HC change at 3rd trimester showed positive significant correlation with serum levels of VD and SFC, while showed negative significant correlation with serum Hp and IL-6. Regression analysis defined high serum VD level as significant positive, while high serum IL-6 as significant negative predictors for improved HC. Kaplan-Meier regression analysis defined higher risk for persistent or development of anemia when serum 25-OH VD was <50 nmol/L and SFC was <22 ng/ml, and serum Hp and IL-6 levels were at ≥27.5 ng/ml and ≥40 ng/ml, respectively.

Conclusion: PAA must not be considered as ID anemia and should not be managed simply with IST. PAA is a multi-factorial condition strongly associated with hypo-VD, upregulated inflammatory cytokines and hepcidin. Wider-scale therapeutic trials for correction of hypo-VD and use of immunomodulators must be instituted to approach the ideal preventive and/or therapeutic modalities for PAA.

Keywords: Pregnancy-associated anemia, Hypovitaminosis D, Interleukin-6, Hepcidin, Iron supplemental therapy

Introduction

Pregnancy is a unique physiological process that involves intricate interplay of inflammatory and anti-inflammatory milieu, hormonal changes, and cellular and molecular events at the maternal-fetal interface (1). Anemia is a common problem in obstetrics and perinatal care and worldwide prevalence of iron deficiency anemia (IDA) ranged between 20 and 80% (2). Pregnant women are particularly vulnerable to iron deficiency (ID) due to the high iron demands...
of pregnancy and those who may benefit most from iron supplementation must be identified (3).

One point of debate concerning management of pregnancy-associated anemia (PAA) is the use of iron supplemental therapy (IST) where there is probable evidence that high intake of heme iron is associated with increased risk of manifest and gestational diabetes (4) and the frequency of glucose intolerance-related outcome of pregnant women was 13% and 11% with selective or routine iron supplements (5).

Vitamin D (VD) is known to regulate innate and adaptive immune processes at the cellular level (6), influences the maternal-fetal unit and breakdown in VD homeostasis may underlie the development of various pregnancy-associated diseases (7) as well as being associated with a plethora of adverse health effects on the offspring (8, 9). However, the role of VD status on associated inflammatory processes across pregnancy is unclear (6).

Hepcidin (Hp) which is one primary phase proteins is antibacterial peptide hormone produced in the liver (10), but it is the master regulator of systemic iron bioavailability in humans (11). Hepcidin regulates iron intestinal absorption, tissue distribution, macrophage iron release and extracellular concentration through its effects on ferroportin (Fpn)-mediated export of cellular iron (12); thus, hepcidin deficiency causes iron overload, while elevated hepcidin levels especially during inflammatory conditions causes iron restriction (13).

Regulatory T cells and T helper 17 cells are two distinct subsets of CD4+ T cells, which are mutually antagonistic in the immune response (14). The CD3+, CD3+/CD4+, and CD3+/CD8+ T-cell populations express intracellular cytokines including interferon-gamma, tumor necrosis factor-α, interleukin (IL)-4 and IL-10 (15). Regulatory T cells expand during pregnancy and are present at the fetal-maternal interface at very early stages in pregnancy (16). Several types of T cells have been associated with the pathogenesis of pregnancy-induced complications (17); including pre-eclampsia (18) and unexplained recurrent spontaneous abortion (19).

**Objectives**

This prospective observational study aimed to determine the levels of VD, Hp, IL-6 in serum of pregnant women during 1st trimester and the relation between these levels and estimated serum ferritin concentration (SFC) and hemoglobin concentration (HC).

**Design**

Prospective comparative trial

**Setting**

Tertiary referral hospital, KSA
Patients & Methods

After approval of the study protocol by the Local Ethical Committee, all pregnant women attending the antenatal care unit (ACU) during spring seasons of two consecutive years and signed written fully-informed consent to participate in the study were eligible for evaluation.

All women were asked to attend ACU fasting for clinical evaluation and to give blood sample for investigations at 1st trimester and start of 3rd trimester for re-estimation of HC. Women had chronic systemic diseases, bleeding tendency, endocrine disorders especially concerning thyroid and parathyroid glands, skeletal abnormalities, maintained on immunosuppressive therapy or therapies affecting bone marrow health were excluded from the study. Also, women had multiple pregnancy or singleton fetus but had fetal congenital anomalies were also excluded from the study.

Iron deficiency (ID) and ID anemia (IDA) were diagnosed according to IDA-working group consensus report (20) and VD status was defined according to 25-OHD concentration after Stroud et al. (21) as sufficient (≥75 nmol/L), insufficient (50-75 nmol/L) and deficient (<50 nmol/L). All women with IDA received oral iron supplemental therapy in the form of once daily oral ferrous fumarate 350 mg caps (HAEMOTON cap; Glaxo Smith Kline Co., Egypt). For all women, HC was re-estimated at the 3rd trimester and Hb deficit was calculated as HC$_{3rd}$ – HC$_{1st}$ divided by HC$_{1st}$ and multiplied by 100.

Sampling & Investigations

Venous blood samples (5 ml) were collected from the antecubital vein under complete aseptic conditions at booking time and were divided into three parts:

1. The first part was put in EDTA tube (about 1.8 mg trik EDTA/ 1 ml blood) for at once Hb conc. estimation by cyanomethemoglobin method (22).
2. The second part of the sample obtained at booking time was put in a tube containing sodium fluoride (2 mg sodium fluoride/ ml blood) to prevent glycolysis for estimation of blood glucose levels using glucose oxidase method (23).
3. The third part of the sample obtained at booking time was kept in a plane container and allowed to clot then serum was separated by centrifugation at 3000 rpm for 10 min. Serum was removed and placed in pyrogen-free Eppendorf tubes and stored at -70°C until ELISA assayed by Spectrophotometer for estimation of 25OHD, ferritin, hepcidin and IL-6 levels.
Methodology

Spectrophotometric estimation of fasting serum levels of:

a. 25OHD using the Calbiotech Vitamin D (VD) Kit (Calbiotec, A Life Science Co, USA; Catalog No. VD220B) which is a solid phase enzyme-linked immunoassay (ELISA) based on the principal of competitive binding (24).

b. Ferritin concentration (SFC) using ELISA kit from Eagle Bioscience Inc., USA (Catalogue No FER31-K01) that was used for quantitative determination of serum ferritin by the use of quantitative sandwich enzyme immunoassay technique (25).

c. Hepcidin using ELISA kit from Calbiotec, A Life Science Co, USA (Catalogue No DHP250) depending on quantitative sandwich enzyme immunoassay technique (26).

d. IL-6 using the Eagle Biosciences Human IL-6 ELISA Assay Kit (Eagle Bioscience Inc., USA; Catalogue No IL631-K01) which employs the quantitative sandwich enzyme immunoassay technique (27).

Study outcome

1. Primary outcome included the frequency and severity of 1st trimester iron deficiency and anemia, and hypovitaminosis D (Hypo-VD) and its relation to 1st trimester levels of other studied parameters.

2. Secondary outcome included the effect of iron supplemental therapy (IST) on frequency and severity of anemia.

Statistical analysis

Obtained data were presented as mean±SD, numbers and percentages. Results were analyzed using paired t-test and Chi-square test (X² test). Possible relationships were investigated using Pearson's linear regression. Data were analyzed using Regression analysis (Stepwise Method) to define the significant predictors for oncoming Hb conc. in pregnant women. Statistical analysis was conducted using the IBM SPSS (Version 23, 2015) for Windows statistical package. P value <0.05 was considered statistically significant.

Results

One hundred and twenty-three pregnant women were evaluated; 17 women were excluded and 106 were enrolled in the study (Fig. 1). Enrolment data of studied women are shown in table 1.
Table (1): Patients' enrolment data

<table>
<thead>
<tr>
<th>Data</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.1±2.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.3±7.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.8±3.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.5±2.8</td>
</tr>
<tr>
<td>Obstetric history</td>
<td>Primi 28 (26.4%)</td>
</tr>
<tr>
<td></td>
<td>Para-1 33 (31.1%)</td>
</tr>
<tr>
<td></td>
<td>Para-2 45 (42.5%)</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>Systolic 114.3±9</td>
</tr>
<tr>
<td></td>
<td>Diastolic 72±4.6</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>98.8±5.1</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD & numbers

At 1st trimester, 41 women were anemic with HC_{1st} <11 gm/dl and 27 women had iron deficiency with SFC of <15 ng/ml. Twenty-two women had IDA with HC_{1st} <11 gm/dl and 19 women had other types of anemia. On start of the 3rd trimester, 56 women had anemia; 29 had persistent anemia, while 27 women had newly developed anemia. On the other hand, 12 of women who were anemic at 1st trimester had improved iron indices on iron supplemental therapy and 38 women had persistently good iron indices since 1st to 3rd trimester (Fig. 1 & 2).

![Figure 1: Consort Flow sheet](image-url)
Despite of the frequency on non-anemic women, irrespective of being improved anemic or persistent non-anemic, the mean $HC_{3rd}$ was significantly lower compared to $HC_{1st}$ and only 16 women had increased HC, while 90 women had decreased HC (Fig. 3).
At 1st trimester, mean serum estimated serum 25-OHD was 51.47±18.6; range: 19.5-81.8 nmol/l, mean SFC was 17.2±4.2; range: 10.5-29.7 ng/ml, mean serum Hp was 22.53±5.44; range: 16.7-38.96 ng/ml and mean serum level of IL-6 was 34.82±15.1; range: 10-69 ng/ml. Women categorization according to serum 25-OHD levels defined 56 women with hypo-VD, 31 women had insufficient and 19 women had sufficient VD levels (Fig. 4).

Estimated HC$_{3rd}$ was significantly negatively correlated with serum Hp ($r$=-0.258, $p=0.007$) and IL-6 ($r$=-0.315, $p=0.001$). The percentage of change of HC$_{3rd}$ in relation to HC$_{1st}$ showed positive significant correlation with serum 25-OHD ($r$=0.394, $p<0.001$) and SFC ($r$=0.257, $p=0.008$), while showed negative significant correlation with serum Hp ($r$=-0.229, $p=0.018$) and IL-6 ($r$=-0.342, $p<0.001$).

Regression analysis defined high serum 25-OHD level as significant positive ($\beta$: 3.387, $p=0.001$), while high serum IL-6 as a significant negative ($\beta$: -2.546, $p=0.012$) predictor for improved HC$_{3rd}$. Kaplan-Meier regression analysis defined higher risk for persistent or development of anemia when serum 25-OHD was $<50$ nmol/L (Fig. 5) and SFC $<22$ ng/ml (Fig. 6), and serum Hp and IL-6 levels at $\geq27.5$ ng/ml (Fig. 7) and $\geq40$ ng/ml (Fig. 8), respectively.
Fig. (5): Kaplan-Meier regression analysis of 1st trimester serum 25-OHD levels for a cutoff for prediction of HC3rd improvement/deterioration

Fig. (6): Kaplan-Meier regression analysis of 1st trimester serum SFC levels for a cutoff for prediction of HC3rd improvement/deterioration
Discussion

The current study detected high frequency of anemia among enrolled pregnant women where about 38.7% were anemic, 25.5% had iron deficiency and 20.8% had IDA. Multiple previous studies detected similar figures for disturbed iron indices among pregnant women (2, 28, 29, 30). The reported figures indicated discrepant hematological milieu in these women and point to the possibility that multiple pathogenic mechanisms may underlie the development
of pregnancy-associated anemia (PAA). In support of this assumption, IST for anemic women, since 1st till 3rd trimester, improved HC in only 29.4%. Moreover, at the 3rd trimester about 41.5% of women who were not anemic deteriorated and became anemic despite of having within normal range SFC at 1st trimester.

As another support of the assumption of multiplicity of mechanisms underlying for PAA development; estimated HC3rd showed negative significant correlation with 1st trimester serum Hp and IL-6 levels that were positively correlated with the extent of HC3rd deterioration. On the other hand, the extent of HC3rd improvement was positively related to 1st trimester levels of serum 25-OHD and SFC. Regression analysis defined high serum 25-OHD level as significant positive, while high serum IL-6 as a significant negative predictor for HC3rd improvement and Kaplan-Meier regression analysis defined appropriate cutoff point for 1st trimester serum levels of 25-OHD, SFC, Hp and IL-6 for prediction of HC3rd and extent of its change. These findings point to the interplay of multiple factors, other than ID, for development or progression of PAA.

The current study reported a frequency of hypo-VD of 52.8%, VD insufficiency rate of 29.2% and VD sufficiency rate of 18%. These figures indicated a high prevalence of hypo-VD among pregnant women. Similarly, Lee et al. (31) out of their series of pregnant women found 71.7% patients exhibited vitamin D deficiency, while 21% and only 7.3% had insufficient or adequate VD levels, respectively.

In line with the detected relation between VD status and iron indices, Thomas et al. (32) detected a positive correlation between maternal 25OHD and hemoglobin at both mid-gestation and at delivery and attributed it to both a direct and an indirect effect mediated by erythropoietin. Thereafter, Yuan et al. (33) reported significantly lower 25OHD concentrations in anemic women than controls with significantly increased risk of anemia with decreasing serum 25OHD concentrations in a dose-dependent manner up to 80% increase in anemia risk in women with 25OHD concentrations <50 nmol/L. In support of the role of VD for maintenance of normal hematological milieu, Michalski et al. (34) reported that low vitamin D status may be linked to the reduced HC, and Nikooyeh & Neyestani (35) strongly suggested an association between low circulating concentrations of 25-OHD and anemia. Such role could be attributed to the findings that hypo-VD contributes to decreasing bone marrow local production of calcitriol with increasing calcium membrane permeability, so erythropoiesis declines (36); these changes as evidenced by In vitro studies occur at mRNA and protein levels (37).

The obtained results indicated that inflammatory status as manifested by high serum Hp and IL-6 inversely affects the hematological indices and their estimated levels at 1st trimester could predict extent of change in HC3rd. These findings go in hand with Lee et al. (38) who detected high ferritin and Hp concentrations at 12th week of gestation and attributed these high levels to a direct effect of IL-6 because its levels at delivery were 1.6-fold higher than...
mid-gestation levels with a positive association between IL-6 and both Hp and ferritin. Moreover, Cutone et al. (39) found increased IL-6 and IL-1β is associated with up-regulation of cytosolic ferritin and down-regulation of ferroportin (Fpn) leading to intracellular iron overload leading in vivo to higher host susceptibility to infections and iron blood deficiency, thus causing and anemia of inflammation (AI).

Moreover, the reported weak response to IST could be attributed to the finding that anemia of inflammation is unrelated to the lack of iron, but to iron delocalization: cellular/tissue overload and blood deficiency (40). In line with the obtained data and proposed assumptions, Paesano et al. (41) found bovine lactoferrin which possesses high sequence homology and identical functions with human lactoferrin, inhibits bacterial growth and biofilm (40) and exerts an anti-inflammatory activity against IL-6, thus up-regulating Fpn and transferrin receptor 1 (TIR1) and down-regulating ferritin (Ftn), pivotal actors of iron and inflammatory homeostasis (42), thus inhibiting intracellular iron overload and re-establishing iron homeostasis with increased availability of iron for erythropoiesis (40).

The relationship between inflammation and development of anemia was attributed to the findings that inflammatory cytokines shorten erythrocyte lifespan by activating macrophages, prioritize leukocyte production in the marrow, and induce hepcidin to increase plasma transferrin saturation and the concentration of non-transferrin-bound iron (43).

The contradictory effect of VD and Hp on the extent of HC change throughout pregnancy could be attributed to the Hp regulatory action on iron homeostasis through its effects on ferroportin (Fpn)-mediated export of cellular iron through binding to Fpn and inducing its internalization (12), thus VD could have a modulatory effect on hepcidin/Fpn pathway inducing more iron release, thus increasing SFC, to be available for more synthesis of hemoglobin, so increasing Hb conc. These findings and assumption supported that previously reported by Bacchetta et al. (12) who documented that VD is a potent regulator of the hepcidin-Fpn axis in humans and Azizi-Soleiman et al. (44) who found VDD is associated with higher hepcidin level in the body. Smith & Tangpricha (45) attributed the regulatory effect of VD on hepcidin/Fpn axis to a direct suppression of hepcidin mRNA transcription.

Conclusion

Pregnancy-associated anemia must not be considered as iron deficiency anemia and should not be managed simply with iron supplements. PAA is a multi-factorial condition strongly associated with hypo-VD, upregulated inflammatory cytokines and hepcidin. Wider-scale therapeutic trials for correction of hypo-VD and use of immunomodulators must be instituted to approach the ideal preventive and/or therapeutic modalities for PAA.
References


