Hyperferritinemia and markers of inflammation and oxidative stress in the cord blood of HIV-exposed, uninfected (HEU) infants

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ABSTRACT

Objectives: The purpose of this study was to evaluate markers of iron status and inflammation/oxidative stress in maternal and cord blood (CB) of HIV-infected and HIV-uninfected women as potential mechanisms for poor outcomes among HIV-exposed, uninfected (HEU) infants.

Methods: Maternal venous and cord blood (CB) specimens were obtained from eighty-seven pregnant women (45 HIV-infected and 42 HIV-uninfected) enrolled at Kalafong Hospital, Pretoria, South Africa. Iron status (serum iron, ferritin, transferrin, transferrin saturation, soluble transferrin receptor [sTfR], sTfR/log ferritin [sTfR/F index], antenatal exposure to inflammation (CB C-reactive protein, interleukin-6, haptoglobin switch-on status) and oxidative stress (total radical trapping ability of CB plasma [TRAP], and chronic oxidative stress (soluble receptor of advanced glycation end-products [sRAGE]) were assessed by laboratory studies.

Results: There were no differences in maternal hematological and iron indices except that HIV-infected mothers had decreased WBC counts (P=0.048) and increased serum ferritin (P=0.032). Ferritin levels were significantly higher in CB than in maternal blood (P<0.001) in both groups and further elevated in the CB of HEU infants (P=0.044). There was also an inverse relationship between CB sTfR/F index and sRAGE (r=-0.43, P=0.003) in the HIV-infected but not HIV-uninfected group.

Conclusions: Our study shows for the first time that ferritin is significantly elevated in CB of HEU infants. The inverse relationship between sTfR/F index and sRAGE in CB suggests that chronic oxidative stress or RAGE axis activation in HIV-infected mothers may play a role in modulating ferritin levels.

INTRODUCTION

Over ninety percent of the world's HIV-infected pregnant women live in sub-Saharan Africa and, with recent progress in preventing mother-to-child HIV transmission the vast majority of infants born to these women are uninfected.(1) There is now increasing evidence, however, that HIV-exposed, uninfected (HEU) infants are experiencing increased rates of morbidity and mortality compared to children born to HIV-uninfected mothers,(2,3) although the reasons for this remain unknown.

One hypothesis is that intrauterine differences may adversely affect the HIV-exposed infant. Maternal immune response to HIV infection and fetal exposure to intrauterine inflammation and oxidative stress are possible mechanisms.(4) HIV infection induces a state of chronic oxidative stress which plays a role in pathogenesis and disease progression. Increased free radical production, depletion of antioxidant reserves due to innate immune activation, altered iron metabolism and direct effect of HIV-1 proteins gp120 and Tat have been implicated.(5)

Among HIV-infected women, hyperferritinemia has been independently associated with increased mortality through altered immune responses and an increased predisposition to opportunistic infections.(6) The potential role of altered iron status associated with HIV infection in pregnancy and ferritin levels within the cord blood (CB) of HEU infants have not been previously explored. Soluble transferrin receptor (sTfR) directly measures cell surface iron receptors and reflects inversely the amount of iron available for erythropoiesis.(7) Conventional laboratory tests of iron status, such as serum ferritin and transferrin saturation are markedly influenced by inflammation, behaving as acute-phase reactants and making it difficult to differentiate between iron-deficiency (or overload) and anemia of chronic disease. Values of

sTfR are elevated in iron deficiency anemia but remain unchanged in states of inflammation, and therefore sTfR and sTfR/log ferritin index (sTfR/F) are useful markers in distinguishing between inflammation and chronic disease.(8)

The Receptor for Advanced Glycation-End products (RAGE) has recently emerged as an important mediator of cellular damage in conditions marked by chronic oxidative stress such as atherosclerosis, diabetes, and preeclampsia.(9) RAGE binds a variety of damaged cellular constituents such as proteins displaced from their physiologic location post-injury.(10) A truncated soluble receptor (sRAGE) has protective function by binding and clearing excess RAGE ligands. One recent study in HIV-infected subjects suggested that sRAGE may play a protective effect against metabolic disturbances and atherosclerosis associated with combined antiretroviral therapy.(11)

Another indicator of antenatal oxidative stress is the Total Radical Trapping Ability of Plasma (TRAP). TRAP reflects the additive effect of extracellular antioxidants such as soluble vitamin C and E, and circulating thiols. The advantage of TRAP is that it provides a comprehensive assessment of multiple antioxidants.(12) Haptoglobin is an acute-phase reactant protein considered near-absent at birth. Antenatal exposure to inflammation results in early haptoglobin expression ("switch on") in the fetus.(13) The purpose of this study was to explore potential mechanisms for poor outcomes among HEU infants by comparing markers of iron status, inflammation and oxidative stress in maternal and cord blood (CB) of HIV-infected and HIV-uninfected women.

METHODS

Study population. This study was conducted at Kalafong Hospital in Tshwane, South Africa between September 2010 and May 2011, and was approved by the institutional review boards of the University of Pretoria and Yale University. After giving consent, ninety-one women were initially enrolled. IRB approval was obtained to have research assistants attend the labor ward and invite women who were late in pregnancy or early labor to participate. Women older than 15 year with uncomplicated, singleton pregnancies were eligible to participate if they resided in the greater Pretoria area and intended to remain in the area. The HIV-infected group included only those who were diagnosed as positive for the first time in the current pregnancy. HIV-uninfected women were required to have evidence of a negative test completed during the pregnancy. Women with a history of psychiatric illness and those who were likely to move out of the area were not eligible.

After enrollment, all women completed short interviews to obtain socio-demographic and pregnancy-related information. HIV-infected women provided information regarding illness history, antiretroviral treatment and adherence. Additional data relating to pregnancy, delivery and CD4 count were abstracted from medical records. Previously established methods were used to establish accuracy of gestational age.(14)

According to the 2010-2011 South Africa PMTCT guidelines(15), women with CD4 <350 received combination antiretroviral therapy (tenofovir (TDF), nevirapine (NVP), and lamivudine or emtracitabine (FTC)) during pregnancy and those with CD4 >350 received antenatal zidovudine (AZT), and intrapartum single-dose NVP and AZT. Data on duration of ARV treatment and responses to treatment were not available. All infants were confirmed uninfected by HIV PCR testing done at local clinics at 6 weeks of age. Women were invited to

return with their children for follow-up interviews conducted at 3 months of age. Four children

were lost to follow-up and therefore removed from subsequent analyses, leaving 87 women and

infants in the final analysis.

Biological specimens. Maternal venous blood samples obtained at enrollment were sent

to the clinical laboratory for hemoglobin, hematocrit, white blood cell (WBC) and platelet

counts. CB was collected prior to expulsion of the placenta using a standardized protocol(16) and

blood was distributed between serum and citrated plasma tubes. The technique of direct

umbilical vein puncture precluded the inadvertent contamination of the sample with maternal

blood. This was confirmed by lack of haptoglobin immunoreactivity in CB and also higher

ferritin levels in CB compared to maternal blood. Samples were centrifuged for 10 minutes

(1,000g, 4ºC) and subsequently stored at -80ºC.

Indices of iron status. Automated analyzers were used for measurement of iron indices

including: iron (colorimetric assay, P800/Cobas modular analyzer, Roche), ferritin

(chemiluminescent assay, E170 modular analyzer, Roche) and transferrin (immunoturbidimetric

assay, P800 analyzer, Roche). Total iron binding capacity (TIBC, calculated) and percent

transferrin saturation (calculated) were determined in both maternal and cord blood serum while

sTfR (immunoturbidimetric assay, Integra400 analyzer, Roche) and sTfR/F index (calculated)

were determined only in CB serum.

Markers of antenatal exposure to inflammation and oxidative stress. C-reactive protein

(CRP), Interleukin-6 (IL-6) and sRAGE concentrations in CB were measured by validated

immunoassays (R&D Systems, Minneapolis, MN). CB haptoglobin switch-on status was

determined from levels of haptoglobin immunoreactivity (ELISA) and Western blot, as

previously published from members of our group.(17) TRAP, an indicator of non-enzymatic

antioxidant reserve and the first line of defense against free radicals, was measured as previously described.(18)

Statistical analyses. Student t-test and Mann-Whitney U test were used for comparisons of continuous data and Chi square or Fisher's exact tests were used for categorical data. Comparisons among linked maternal and CB samples were by 2-way repeated measures (ANOVA) after logarithmic transformation. Correlation analyses were performed using Spearman's rank order correlation.

RESULTS

Ninety-one women were initially enrolled but 4 were lost to follow-up and thus excluded from subsequent analyses. Our final analysis included 87 women: 42 were HIV-uninfected and 45 HIV-infected, of which 51% (23/45) were on HAART therapy. CD4 counts were available for 41/45 HIV-infected women. Among these women 11 (27%) had CD4 counts <200, 15 (37%) had CD4 counts 200-349, and 15 (37%) had CD4 counts ≥350. The mean age of HIV-infected women was slightly older (28.0 versus 25.0, P=0.045) and there were also fewer primigravidas (18% versus 43%, P=0.024). There were no significant differences between groups with respect to labor/delivery and neonatal characteristics.

Nearly all pregnancies were full term in both groups and birth weights were all >2000 grams, with 51% >3000 grams. Both groups had high rates of Cesarean section (58% HIV-infected vs. 52% HIV-uninfected). Fewer women in the HIV-infected group had premature rupture of membranes (2% vs. 12%), although not statistically significant. More than half of all women in each group were treated with antibiotics during or after delivery (64% vs. 53%).

Table. Maternal hematological/iron indices and fetal cord blood analyses of iron status, inflammation, and oxidative stress

Variable	HIV-Infected (n=45)	HIV-Uninfected (n=42)	*P* value
Maternal hematological parameters			
Hemoglobin at initial visit, *g/dL* *	11.4 ± 0.3	11.4 ± 0.2	0.974
Hemoglobin during labor, *g/dL* *	11.9 ± 0.3	11.6 ± 0.3	0.393
Hematocrit, *%* *	39.1 ± 0.9	38.9 ± 0.8	0.918
WBC count, *cells x 1,000/mm^3* *	8.7 ± 0.4	10.0 ± 0.4	0.048
Platelet count, *cells x 1,000/mm^3* *	202.1 ± 9.8	197.6 ± 10.6	0.754
Maternal indicators of iron status			
Iron, *μmol/L* ‡	14.6 [8.6-22.4]	11.7 [7.1-18.1]	0.115
TIBC, *μg/dL* ‡	15.5 [9.0-28.5]	12.0 [6.8-21.5]	0.162
Transferrin, *g/L* ‡	3.5 [3.1-4.3]	3.6 [3.3-4.2]	0.859
Ferritin, *ng/mL* ‡	40.5 [20.0-61.0]	26.0 [13.8-45.5]	0.032
Fetal indicators of iron status (cord blood)			
Iron, *μmol/L* ‡	22.4 [15.8-29.0]	21.0 [17.4-28.0]	0.580
Total iron binding capacity (TIBC), *μg/dL* ‡	47 [31.5-60.0]	44.0 [32.8-59.0]	0.678
Transferrin, *g/L* ‡	1.9 [1.7-2.0]	2.0 [1.7-2.3]	0.474
Soluble transferrin receptor [sTfR], *mg/L* ‡	6.1 [4.5-8.3]	7.0 [5.6-8.2]	0.136
Ferritin, *ng/mL* ‡	184.0 [98.8-298.0]	136.0 [78.5-240.8]	0.044
sTfR/log ferritin (sTfR/F index) ‡	2.8 [2.0-3.8]	3.2 [2.5-4.7]	0.045
Fetal indicators of inflammation (cord blood)			
IL-6, *pg/mL* ‡	4.3 [3.2-6.2]	5.3 [3.5-17.6]	0.152
IL-6 > 100 pg/mL †	3 (7)	2 (5)	1.000
CRP > 1 mg/L †	3 (7)	5 (12)	0.475
Hp switch-on status †	7 (16)	5 (12)	0.855
Fetal indicators of oxidative stress (cord blood) ‡			
TRAP, *mmols/L* ‡	0.935 [0.757-1.045]	0.880 [0.765-1.043]	0.525
sRAGE, *pg/mL* ‡	1,312 [964-2,368]	1,531 [1017-2,247]	0.841

* Data presented as mean ± standard error and analyzed by Student t-tests

‡ Data presented as median [interquartile range] and analyzed by Mann Whitney tests

† Data presented as number of subjects and (percentage) and analyzed by Chi square tests

As seen in the table, HIV-infected women had lower WBCs (8.7 vs. 10.0 x 10^9/L, P=0.048) and higher ferritin levels (40.5 vs. 26.0 ng/mL, P=0.032) than HIV-uninfected women, but there were no differences in maternal iron, TIBC, or transferrin levels. For both HIV-infected and uninfected groups, mean CB ferritin was more than four times higher than respective maternal ferritin levels. Ferritin levels were also higher in CB of HEU infants when compared to HIV-uninfected CB (184.0 vs. 136.0 ng/mL, P=0.044); however, there was no elevation in CB iron or transferrin receptor levels. In the HIV-infected group, there were no

differences in either maternal ferritin levels (P=0.69) or cord blood ferritin (P=0.73) for those

receiving HAART and those not receiving HAART. It is notable that there were no significant

differences between groups in IL-6 or haptoglobin switch-on status, and measures of oxidative

stress (TRAP & sRAGE). CRP was slightly higher in the HIV-uninfected group.

Subsequent analyses showed no significant correlation between maternal and CB ferritin

levels (r=0.24, P=0.11). Furthermore, there was no correlation between ferritin and IL-6, CRP or

TRAP in CB. However, as shown in the figure, a significant inverse correlation between sRAGE

and CB sTfR/F index (r=-0.427, P=0.004) was found in HIV-infected but not in HIV-uninfected

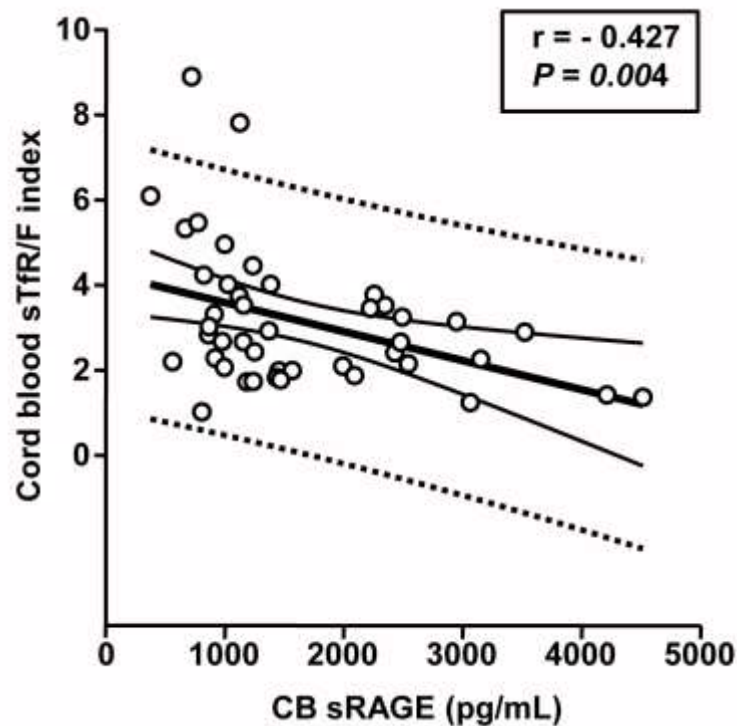subjects (r=-0.132, P=0.403) (not shown).

Figure. Relationship between soluble transferrin receptor-ferritin index and soluble RAGE in cord blood of HEU infants (n=45). The thick black line represents the linear regression line. The 95% confidence and prediction intervals are shown by the thin continuous and dotted lines, respectively. The Spearman correlation coefficient r and the level of statistical significance are shown. This significant inverse relationship was not observed in newborns of HIV-uninfected mothers.

DISCUSSION

This study demonstrates that maternal HIV infection induces a state of fetal hyperferritinemia, which is unrelated to maternal ferritin level and does not appear secondary to fetal pro-inflammatory state. We also found elevated maternal ferritin levels among HIV-infected women, which has been previously described(6) and has been shown to inversely correlate with CD4 levels, and is an indicator of poorer prognosis and increased mortality.(19)

In HIV-uninfected women, elevated maternal ferritin has been associated with higher rates of pregnancy complications such as gestational diabetes, intra-uterine growth retardation (IUGR), and neonatal sepsis.(20) We found no association between CB ferritin and maternal ferritin levels in HIV-uninfected mothers, which suggests independent sources of hyperferritinemia and is consistent with previous studies.(21) In South Africa, all mothers routinely receive iron therapy during pregnancy; however, adherence to this was not assessed.

Although the mechanism of elevated ferritin in cord blood of HEU infants remains to be elucidated, we can make some inferences. Ferritin normally reflects the storage iron compartment and sTfR reflects the functional iron compartment,(8) but in our study the other iron indices including sTfR remained within normal limits. Ferritin is also viewed as an acute phase reactant, yet CRP>1 and IL-6 were not elevated in CB of the HIV-infected group implying that the elevation in CB ferritin was not secondary to fetal inflammation. HIV infection likely does not impact the haptoglobin switch-on status as the numbers of such infants did not differ between groups.

A potentially important clue may result from the inverse correlation between CB sRAGE and sTfR/F index. sRAGE plays a role in inflammation and oxidative stress-induced tissue injury.(10) Low cord blood sRAGE levels have been reported in premature newborns exposed to

intra-amniotic inflammation and recently in IUGR independent of fetal inflammation.(22) It is

possible that in this cohort, ferritin is being produced in the placenta in response to a state of

oxidative stress, resulting in excess RAGE ligand and leading to consumption of sRAGE.

Unaffected CB TRAP levels are more consistent with a state of chronic rather than acute

oxidative stress. The CB ferritin levels elevated beyond maternal levels support a fetal or

placental source of ferritin that is independently modulated by maternal HIV infection. Although

elevated ferritin has been associated with a number of adverse effects in chronic inflammatory

conditions and malignancy, it ultimately remains unknown whether hyperferritinemia in CB has

long-term deleterious effects on postnatal development. As ferritin emerges to have bioactivities

independent of its iron storage function such as angiogenesis regulation,(23) further studies

investigating the mechanism and kinetics of hyperferritinemia in HEU infants beyond birth are

warranted.

The high rates of antibiotic administration in the study are likely a reflection of local

practice. There is no routine screening for B-hemolytic streptococci during pregnancy and all

women undergoing cesarean sections receive antibiotics. Indications for giving antibiotics were

the same for all mothers and there was no significant difference in numbers between groups

(64% HIV-infected vs. 53% HIV-uninfected).

In summary, this study demonstrated elevated ferritin levels among HIV-infected

pregnant women, a finding that has previously been associated with a poorer prognosis in non-

pregnant, HIV-infected individuals. Additionally, we report for the first time that ferritin is

significantly elevated in CB of HEU infants compared to those who are not HIV-exposed. The

finding that markers of inflammation (CRP, IL-6) were unchanged suggests that the increase in

CB ferritin is unlikely caused by inflammation. The inverse relationship between sTfR/F index

and sRAGE in CB suggests that oxidative stress may play a role in modulating ferritin levels. Further research is necessary to explain these findings and to determine whether this observation might contribute to adverse outcomes among HEU infants.

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