Hematological Alterations and Impaired Thymic Function in Newborns of HIV-Infected Mothers Receiving Antiretroviral Drugs

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PII: S097475591200623
ABSTRACT

Objectives: To investigate the effects of antiretroviral (ARV) drugs on hematological parameters and thymic function in HIV-uninfected newborns of HIV-infected mothers.

Study design: Cross sectional study.

Setting: Chiang-Mai University Hospital, Chiang-Mai, Thailand.

Participants/Patients: 49 HIV-uninfected and 26 HIV-infected pregnancies.

Methods: Cord blood samples of newborns from HIV-uninfected and HIV-infected mothers were collected. Hematological parameters were measured using automatic blood cell count. T-cell receptor excision circles (TRECs) levels in cord blood mononuclear cells (CBMCs), CD4⁺ and CD8⁺ T-cells were quantified using real-time PCR.

Main Outcome Measures: Effect of ARV drugs used for prevention of HIV-mother-to-child transmission (MTCT) on hematological parameters and thymic function.

Results: Newborn of HIV-infected mother tended to have lower mean levels of hemoglobin than those of HIV-uninfected mother (137 ± 22 vs 146 ± 17 g/L, \(P = 0.05\)). Furthermore, mean of red blood cell (RBC) counts and hematocrit and median of TRECs in CD4⁺ T-cells in the newborns of the former were significantly lower than those of the latter [3.6 ± 0.7 vs 4.8 ± 0.6 x 10¹² cells/L, \(P <0.001\); 0.40 ± 0.07 vs 0.46 ± 0.05 L/L, \(P <0.001\) and 0.53 (IQR: 0.03-5.76) vs 13.20 (IQR: 2.77-27.51) x 10⁻³ pg/μL, \(P = 0.02\), respectively].

Conclusion: The finding suggested that the ARV drugs altered hematological parameters and impaired thymic function (TRECs CD4⁺ T-cells) in HIV-uninfected newborns of HIV-infected mothers.

Key Words: Antiretroviral drugs, Hematology, HIV, Newborn, Thymic function.
INTRODUCTION
Almost half of the estimated 40 million people living with HIV are women of childbearing age [1]. The risk of these women to transmit HIV to their infants is 15-25% when no precautions are taken [2]. The HIV-mother-to-child transmission (MTCT) rate has dramatically reduced to be less than 2% if antiretroviral (ARV) drugs of highly active antiretroviral therapy (HAART) regimen were administrated to the HIV-infected woman during pregnancy and labor as well as to her infants [3, 4]. The previous studies showed that Zidovudine (ZDV) which is a potent inhibitor of bone marrow function is associated with hematological abnormalities not only in mothers, but also in newborns, because this drug can cross the placental barrier and negatively affect fetal erythropoiesis [5-8]. Moreover, ZDV-based HAART commonly associated with a greater negative impact on hematologic parameters than ZDV-free regimens [9]. The adverse hematologic effects of ARV drugs have been reported in HIV-uninfected infants, especially in their early life [10]. The frequently adverse hematologic effects found are anemia, neutropenia, lymphocytopenia and thrombocytopenia [7, 11-13].

The thymus is a primary source of naïve T-cells and plays a key role in establishing and maintaining a peripheral T-cell pool [14]. Thymus reaches its maximum volume by one year of age. After that its size as well as its function decline [15]. A production of naïve T-cells by the thymus can be quantified by measuring T-cell receptor excision circles (TRECs), a DNA fragment formed during T-cell development. These DNA fragments do not replicate during mitosis and are thus diluted during cell division [16]. Previous studies demonstrated that both HIV-proteins and some antiretroviral drugs inhibited progenitor cells and thymic functions, as indicated by the frequency of TRECs [17-19]. However, an evaluation of hematological and immunological toxicity in newborn exposed to maternal ARV drugs administered during pregnancy has been limited. The aims of this study were to measure and compare hematological parameters and TRECs levels in HIV-uninfected newborn of HIV-infected mother receiving ARV drugs for prevention of HIV-MTCT with those of normal control newborn.

METHODS
Study population
This study was conducted at Chiang-Mai University Hospital, Chiang-Mai, Thailand. The protocol was approved by the Faculty of Medicine Ethics Committee, Chiang-Mai University, Chiang-Mai, Thailand. All pregnant women participated in this study had signed a written informed consent. To obtain the subjects, the exclusion criteria for the study were set as follow: women with twin or multiple births, infected with other microorganisms, used of psychopharmaceutical drugs, illicit drugs, alcohol and tobacco during gestation. From March to December 2011, 26 HIV-infected and 49 HIV-uninfected pregnant women were enrolled. These HIV-infected women received ARV drugs [ZDV plus Lamivudine (3TC) and Lopinavir/Ritonavir (LPV/r)] during pregnancy and labor every 12 hours.
with adding of ZDV every 3 hours during labor and delivered vaginally or elective caesarean section. The following data were collected from all women: age, gestational age at delivery and mode of delivery. For the HIV-1 infected women, the following additional data were collected: antiretroviral prophylaxis (type and timing), CD4+ T-cell counts (cells/µL) during pregnancy and plasma HIV-1 RNA viral load measured in a week before delivery (log₁₀ copies/mL). All women in our study were given iron and folate supplementation as recommended by the Thai National Guidelines for Pregnancies [20]. Diagnosis for HIV-1 infection in infants born to HIV-1 infected mothers was performed at one and four months of age using DNA PCR (AmpliCor® HIV-1 DNA assay version 1.5, Roche Molecular Systems Inc., USA).

**Isolation of cord blood mononuclear cells (CBMCs)**

Cord blood samples were drawn from clamped umbilical vein within 5-10 minutes after delivery into ethylenediamine tetraacetic acid anticoagulation (EDTA) tubes (BD Vacutainer™, Franklin Lakes, NJ, USA). The sample tubes were then shipped to the hematology laboratory, Faculty of Associated Medical Sciences, Chiang-Mai University within 3 hours. Upon arrival, hematological parameters were measured using an automated blood counter (Sysmex KX-21; Sysmex Corporation, Kobe, Japan). Cord blood mononuclear cells (CBMCs) were isolated using Ficoll-Hypaque gradient (IsoPrep, Robbins Scientific, Sunnyvale, CA, USA). Cells were then aliquoted and stored in liquid nitrogen until used.

**Separation of CD4+ and CD8+ T-cells**

CD4+ and CD8+ T-cell separation was performed from CBMCs of the 15 HIV-uninfected newborns of HIV-infected mothers and only 12 HIV-uninfected newborns of HIV-uninfected mothers. Frozen CBMCs were thawed and washed twice in cold phosphate-buffered saline solution. CD4+ and CD8+ T-cells were separated using a magnetic cell separator (EasySep®, STEMCELL Technologies, USA) according to manufacturers’ instructions. The separated CD4+ and CD8+ T-cells cells were counted on hemacytometer under light microscope using Turk’s solution.

**DNA preparation and quantification of TRECs**

DNA was extracted from 1.5 x 10⁶ cells of CBMCs, separated CD4+ and CD8+ T-cells using the NucleoSpin® kit (Macherey-Nagel, KG., Duren, Germany) according to manufacturers’ instructions and was stored at -20°C until used. TRECs analysis was performed by quantitative real-time PCR as described by Ometto et al. [21] with slightly modification. The DNA amplification was carried out in a 25 µL reaction mixture containing 5 µL DNA sample or sterile distilled water as a no template control, 1x real-time PCR Master Mix (Thermo Scientific ABsolute™ QPCR ROX Mix, Surrey, UK), 400 nM each primer (forward, 5’-CACATCCCTTTCAACCATGCT-3’; reverse, 5’-GCCAGCTGCAGGGTTTAGG-3’; GenBank sequence accession number DQ858179.1) and 200 nM of the fluorogenic probe (5’-ACACCTCTGTTTTTGTAAAGGTGCCCAC T-3’) conjugated with FAM (6-carboxyfluorescein) at the 5’-end, and TAMRA (6-carboxytetramethylrhodamine) at the 3’-
end. The PCR primers and the fluorogenic probe were specifically designed for the detection of human TRECs. The amplification was performed in a Rotor-Gene 6000™ (Corbett Research; Mortlake, New South Wales, Australia). The mixture was preheated at 95°C for 15 min, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. A cycle threshold (C_T) is defined as the PCR cycle at which an increase in the fluorescence above the baseline signal is first detected. The C_T value is inversely related to the copy number of the target sequence. TRECs concentrations were calculated from a standard curve of a plasmid clone containing TRECs which run in parallel with the test. All samples and TRECs plasmid were run in duplicate. TRECs level in CBMCs was presented as concentration of TRECs per 1.5 x 10^6 CBMCs while those in CD4+ and CD8+ T-cell was presented as concentration of TRECs per cell.

**Statistical analysis**

The data are presented as median, mean and standard deviation (SD). Statistical analyses were performed using SPSS software package (Statistical Package for the Social Sciences 11.0, Chicago, IL, USA). Characteristics and hematological parameters were compared between 2 groups of newborns of HIV-infected and uninfected mothers using Compare Means with Independent Samples t test and Fisher’s exact test while levels of TRECs between the two groups were compared using Mann-Whitney test. The level of significance for all analyses was set at 0.05.

**RESULTS**

**Participants and clinical data**

The clinical data of participants are shown in Table I. Mean of maternal ages and gestational ages at delivery were similar between HIV-infected and uninfected women. All HIV-infected mothers received ZDV plus 3TC and LPV/r starting at the average of gestational age at 21 weeks. Most of HIV-infected and uninfected women delivered vaginally (65% and 85%, respectively). Means of birth weight of the two groups have no significantly differences. Mean CD4+ T-cell count of HIV-infected mother was 517 cells/µL. The HIV RNA viral loads measured at one week before delivery of HIV-infected women were less than 40 copies/mL and none of all newborns born to HIV-1 infected mothers were HIV-infection.

**Hematological alterations in HIV-uninfected newborns of HIV-infected mothers**

Mean levels of white blood cell (WBC) counts, absolute neutrophil counts, absolute lymphocyte counts and platelet counts in newborns of HIV-infected and uninfected mothers did not differ significantly (Table II). There was a trend of lower hemoglobin levels in newborns of HIV-infected mothers than those of HIV-uninfected mothers. Moreover, means of red blood cell (RBC) counts and hematocrit in newborns of HIV-infected mothers were significantly lower than those of HIV-uninfected mothers. On the other hand, newborns of HIV-infected mothers showed higher mean levels of red cell indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH)
and mean corpuscular hemoglobin concentration (MCHC) than those of HIV-uninfected mothers (Table II).

**Decrease of TREC CD4 T-cell in HIV-uninfected newborns of HIV-infected mothers**

No significant difference in median of TREC levels in CBMCs (Fig. 1a) and in CD8 T-cell (Fig. 1b) between newborns of HIV-infected mothers and uninfected mothers. However, TREC levels in CD4 T-cell (Fig. 1c) in newborns of HIV-infected mothers were significantly lower than those of HIV-uninfected mothers.

**DISCUSSION**

Antiretroviral drugs administered to HIV-infected mothers and their offspring diminished the risk of HIV-MTCT to lower than 2% [3, 4]. Despite the fact that infant born to HIV-infected mother receiving ARV drugs remain uninfected, hematological parameters have been shown to be impaired [11, 12]. The current study showed that ARV drugs (ZDV plus 3TC and LPV/r) administered to HIV-infected mother for prevention of HIV-MTCT altered the hematological parameters of newborns as indicated in the decrease of RBC counts, hemoglobin and hematocrit levels and the increase of MCV, MCH and MCHC. Furthermore, the thymic function of these newborns was also impaired as indicated in the decrease of TREC CD4 T-cell. The previous study showed that maternal derived HIV-proteins diffusing across the placental barrier during pregnancy could reduce thymic function [19, 22]. In addition, both HIV-proteins and ARV drugs are known to inhibit progenitor cell function [17, 18]. However, in present study, the effects of HIV-proteins on thymic function might be less than those of ARV drugs since maternal viral loads in all HIV-infected mothers measured at one week before delivery were less than 40 copies/mL.

Our data are reassuring, ARV prophylaxis dose seem to significantly reduce hematological indices because the mean MCV in newborns of HIV-infected mother was significantly higher than those of HIV-uninfected mothers. Moreover, some newborns (31%) of HIV-infected mother had MCV higher than the normal upper limit value (120 fL). In present study, all HIV-infected mother received ZDV and 3TC which have been report for the induction of macrocytic anemia [10, 23]. Antiretroviral drugs are routinely prescribed during the second trimester, in which hematopoiesis and lymphopoiesis are active, i.e., hepatic hematopoiesis and lymphopoiesis, spleen development, thymic education and bone marrow development. The administration of ARV drugs during the critical window of hematopoiesis and lymphopoiesis may affect the generation of these precursors [12]. Therefore, an impaired of hematopoiesis and lymphopoiesis may have contributed to the hematopoietic alteration and the reduction of thymic output, respectively. The decrease of CD4 TREC levels observed in the present study was consistent with the previous study by Clerici et al. that showed CD4/45RA/62 (naive lymphocytes) in HIV-uninfected newborns of HIV-infected mothers received ZDV for prevention of HIV-MTCT were significantly lower than those of newborns of HIV-uninfected mothers [22]. In contrast, Kolte et al. showed that thymic size but not thymic
function (TRECs CD4+ T-cell) in HIV-uninfected newborns of HIV-infected mothers received ARV drugs [ZDV/ plus 3TC and LPV/r or Nevirapine (NVP)] for prevention of HIV-MTCT was significantly lower than those of HIV-uninfected mothers [24]. These two different observational results can be explained that there was a difference in the study populations. While our cohorts were newborns, Kolte’s cohorts were children with age of 15 months, that was probably when the side effect of ARV drugs resolved. Moreover, the maternal ethnicities between the two groups of children were different [24]. There are many parameters that have been shown to be associated with the hematologic variables such as maternal ethnicity, drug use, maternal CD4+ T-cell count at delivery, mode of delivery and also infant gestation age, birth weight and sex [25, 26]. In a current study, these factors are controlled by matching of maternal ethnicity, maternal age at delivery, gestational age, mode of delivery, fetal sex and birth weight between the test group and control group.

WBC counts, absolute neutrophil counts, absolute lymphocyte counts and platelet counts in newborns of HIV-infected mothers were similar to those of HIV-uninfected mothers (Table II). These results were consistent with the previous study by Bunders et al. that showed the levels of WBC counts, absolute neutrophil counts, absolute lymphocyte counts and platelet counts measured at birth in HIV-1/ARV-exposed infants were not different from those in matched comparison group. However, a lower WBC counts, absolute neutrophil counts in HIV-1/ARV-exposed infants were observed at 5 weeks of age while a lower level of hemoglobin in these infants were observed at birth and 5 weeks of age [27]. Thus, further studies are needed to evaluate how long the hematological alteration and impaired thymic function persist.

The present study has a limitation in the limited volume of cord blood collected thus levels of TRECs in CD4+ and CD8+ T-cells could be analyzed in only 12 and 15 samples of newborns of HIV-uninfected and infected mothers, respectively. Moreover, it was impossible to analyze the levels of TRECs in memory or naïve CD4+ and CD8+ T-cell sub-populations (CD45RO+ and CD45RA+), which are the immune resources. Although, the hemoglobin and hematocrit in newborns of HIV-infected mothers were significantly lower than those of HIV-uninfected mothers. We also found that, mean levels of these two hematological parameters in both groups were lower than normal range levels (hemoglobin 165-215 g/L and hematocrit 0.48-0.68 L/L). These lower levels might have caused from the hematologic genetic disorders such as thalassemia and G-6-PD deficiency frequently found in Thai population [28]. However, the hematologic genetic disorders were not used as a variable factor in our study.

In summary, our study indicates that ARV drugs (ZDV plus 3TC and LPV/r) for prevention of HIV-MTCH altered the hematological parameters and impaired thymic function (TRECs CD4+ T-cell) in newborns of HIV-infected mothers. These phenomena may impact the quality of life including growth, development, vaccination responses and susceptibility to infections of infants. Therefore the long-term effects of these drugs in larger population are needed to be clarified.
Acknowledgements:
The authors thank all HIV-infected and uninfected mothers who participated in this study. We gratefully appreciate the help and assistance of physicians, nurses and technicians of Maharaj Nakorn Chiang-Mai Hospital, Chiang-Mai, Thailand.

This study was supported by the Thailand Research Fund and the Commission on Higher Education. It was also supported in part by the National Research University Project under Thailand's Office of the Higher Education Commission.

Contributors: RW and NP: patient enrolment, data acquisition, data analysis, laboratory analysis and drafting of manuscript; PS and DK: data analysis and interpretation and critical revision of the manuscript; PO, PSV and SP: concept and design, data acquisition, data analysis and interpretation and critical revision of the manuscript. All the authors were involved in preparation of the manuscript.


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FIG. 1 T-cell receptor excision circles (TRECs) levels of newborns of HIV-infected and uninfected mothers. (a) TRECs in CBMCs, (b) TRECs CD8⁺ T-cell, (c) TRECs CD4⁺ T-cell. Data are presented median (horizontal line), 25th and 75th (box) and 5th and 95th percentile (whisker lines). TRECs levels in CBMCs were analyzed from 26 and 49 newborns of HIV-infected and uninfected mothers, respectively while TRECs CD8⁺ and CD4⁺ T-cell were analyzed from 15 and 12 newborns of HIV-infected and uninfected mothers, respectively.
TABLE I  CHARACTERISTICS OF HIV-INFECTED AND UNINFECTED MOTHER AND THEIR NEWBORNS. THE DATA ARE EXPRESSED AS MEAN ± SD (RANGES).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HIV-infected mother (n = 26)</th>
<th>HIV-uninfected mother (n = 49)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at delivery (years)</td>
<td>30 ± 7 (17-42)</td>
<td>27 ± 6 (15-42)</td>
<td>0.08*</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>38 ± 2 (33-40)</td>
<td>38 ± 1 (34-41)</td>
<td>0.58*</td>
</tr>
<tr>
<td>Gestational age at ARV prophylaxis initiation (weeks)</td>
<td>21 ± 5 (14-27)</td>
<td>Not Relevant</td>
<td></td>
</tr>
<tr>
<td>CD4+ T-cell count during pregnancy (cells/µL)</td>
<td>517 ± 188 (186-859)</td>
<td>Not Relevant</td>
<td></td>
</tr>
<tr>
<td>HIV RNA load measured at one week before delivery (copies/mL)</td>
<td>&lt;40</td>
<td>Not Relevant</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery VD : CS</td>
<td>17 : 9</td>
<td>39 : 10</td>
<td>0.24#</td>
</tr>
<tr>
<td>Gender of newborn Male : Female</td>
<td>18 : 8</td>
<td>24 : 25</td>
<td>0.08#</td>
</tr>
<tr>
<td>Birth weight of newborn (g)</td>
<td>2,873 ± 461 (2,050-3,910)</td>
<td>3,029 ± 412 (2,250-3,950)</td>
<td>0.18*</td>
</tr>
</tbody>
</table>

TABLE II  HEMATOLOGICAL PARAMETERS OF NEWBORNS OF HIV-INFECTED AND UNINFECTED MOTHERS. THE RESULTS ARE SHOWN BY MEAN ± SD (RANGE).

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Newborn of HIV-infected mother (n = 26)</th>
<th>Newborn of HIV-uninfected mother (n = 49)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x 10⁹ cells/L)</td>
<td>13.0 ± 5.0 (3.5-24.3)</td>
<td>14.6 ± 5.6 (5.3-35.1)</td>
<td>0.24</td>
</tr>
<tr>
<td>Absolute neutrophils (x 10⁹ cells/L)</td>
<td>7.4 ± 2.6 (2.4-12.3)</td>
<td>6.5 ± 2.7 (0.8-11.5)</td>
<td>0.27</td>
</tr>
<tr>
<td>Absolute lymphocytes (x 10⁹ cells/L)</td>
<td>4.8 ± 2.9 (2.1-12.8)</td>
<td>5.8 ± 3.1 (2.8-21.2)</td>
<td>0.19</td>
</tr>
<tr>
<td>RBC (x 10¹² cells/L)</td>
<td>3.6 ± 0.7 (1.7-4.9)</td>
<td>4.8 ± 0.6 (3.7-6.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>137 ± 22 (71-166)</td>
<td>146 ± 17 (104-180)</td>
<td>0.05</td>
</tr>
<tr>
<td>Hematocrit (L/L)</td>
<td>0.40 ± 0.07 (0.21-0.51)</td>
<td>0.46 ± 0.05 (0.36-0.54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>113 ± 10 (95-130)</td>
<td>95 ± 9 (75-110)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>38.4 ± 4.3 (30.7-49.4)</td>
<td>30.48 ± 4.1 (21.2-36.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>339 ± 16 (305-380)</td>
<td>319 ± 20 (271-356)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet counts (x 10⁹/L)</td>
<td>318 ± 92 (157-511)</td>
<td>287 ± 64 (181-422)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

VD = Vaginal delivery, CS = caesarean section; *Compare Means with Independent Samples t test; #Fisher’s exact test