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Significant correlation between regulatory T cells and vitamin D status in term and preterm labor

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ABSTRACT

Background and aim: Vitamin D insufficiency and deficiency have been associated with an increased risk of adverse pregnancy outcomes. Also, vitamin D is known to play a role in promoting the function of regulatory T-cells (Tregs). Tregs play an important role in suppressing the immune response during pregnancy. Our study aimed to investigate Tregs phenotypes in preterm and tem laboring women and its association with vitamin D level.

Methods: This cross-sectional study included 82 pregnant women, divided into 46 term and 36 preterm laboring women in addition to 30 healthy non-pregnant women. The percentage of $CD4^+CD25^+Foxp3^+$Treg cells and their composition of four different Treg subsets were evaluated using flow cytometric analysis. Also, serum vitamin D levels were measured by ELISA.

Results: The percentage of the $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs were significantly decreased in term and preterm laboring women compared to the non-pregnant controls. The percentage of $CD45RA^+$ Tregs, was significantly increased in term laboring women than preterm laboring women and non-pregnant women. Also, term labor women had increased proportion of HLA-DR^{high}Tregs. Preterm labor women had significant increased proportion of HLA-$DR^{negative}$ Tregs compared to term labor women. The overall prevalence of vitamin D deficiency and vitamin D insufficiency was higher in preterm than term laboring women and non-pregnant women. Significant positive correlations were found between serum level of 25 (OH)D and percentage of $CD4^+$ $CD25^+$ $Foxp3^+$Tregs and percentage HLA-DR^{high}Tregs among term and preterm laboring women with vitamin D deficiency.

Conclusion: There is a strong association between the percentage of Treg phenotypes and vitamin D level in term and preterm labor women with vitamin D deficiency. Also, the onset of term and preterm labor is associated with changes in the composition of the total Treg pool with different Treg subsets which in turn may be responsible for immunologic mechanisms that associated with labor induction.

1. Introduction

During pregnancy, the maternal immune system is always confronted with fetal alloantigens. The immunological mechanisms that prevent the rejection of the fetus are still understood. The fetus is normally accepted by the maternal immune system despite expression of paternal alloantigen (Marzi et al., 1996; Wilczynski, 2005). This is partly explained by a switch in T helper (TH)-cell cytokine balance, from the aggressive cell-mediated and proinflammatory effects of Th1-like cytokine to Th2-like cytokine. Also, regulatory T cells (Tregs) are involved in the maintenance of tolerance during pregnancy (Arenas-Hernandez et al., 2016; Clark, 2016).

Normally, the fetus grows and develops very well with ongoing pregnancy, until the onset of spontaneous labor. Sometimes pregnancy ends as a premature birth. Up till now, it is not well known if the immunological rejection processes participate in the induction of irresistible preterm labor. Tregs are increased in pregnant women compared to non-pregnant women (Luciano et al., 2014). The number of Tregs cells was increased in early pregnancy peaking in second trimester and declining towards the end of pregnancy and post-partum (Somerset et al., 2004; Heikkinen et al., 2004).

The decrease in Tregs during the normal course of pregnancy may be due to the migration of these cells from the peripheral blood into the decidua (Miyara et al., 2009), to prevent the acute allogeneic response

Abbreviations: Treg, Regulatory T cells; FOXP3, Forkhead box protein; 3MFI, mean fluorescent intensity

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towards the fetus. The dysregulation of Tregs involved in the pathogenesis of complications, such as gestation-associated hypertensive diseases and preterm intrauterine activation (Baecher-Allan et al., 2006; Steinborn et al., 2008).

Treg cells are comprising approximately 5%–10% of CD4 + T cells they characterized by the expression of the CD25 surface marker and the transcription factor Forkhead box protein 3 (Foxp3) (Sakaguchi, 2005; Zahran et al., 2018; Hetta et al., 2015; Mehta et al., 2016). Tregs are divided based on their expression of CD45RA. $CD45RA^+$ Tregs are naive or resting Tregs, while CD45RA- Tregs cells which fully functional effector Tregs. CD45RATregs include a considerable proportion of cells which positive for HLA-DR expression. These $HLA\text{-}DR^+$Tregs are highly differentiated and have the most of the suppressive activity of the total Treg pool (Sasaki et al., 2007; Kisielewicz et al., 2010).

Vitamin D deficiency is a global problem occurs in pregnant women with global reports citing that 20%–80% of pregnant women may suffer from vitamin D inadequacy (Mulligan et al., 2018). Vitamin D insufficiency has been negatively associated with multiple immune-mediated diseases which is believed to be attributed to its numerous immunomodulatory properties (Cantorna et al., 2015). Vitamin D is able to skew the T cell compartment into a more anti-inflammatory and regulated state, with inhibition of Th1 and Th17 cells and promotion of Th2 and Tregs. The frequency of $Foxp3^+$Treg cells can be increased by high concentrations of the active form of vitamin D (Urry, 2012).

Little information exists about the composition the total Treg population during labor. The aim of this study was to study Tregs number phenotypes in preterm and tem laboring women and its correlation with vitamin D level.

2. Methods

This cross-sectional, case-control study was conducted in the department of Obstetrics and Gynecology, faculty of medicine, Assiut University, Egypt and approved by the Assiut University ethics committee. It was conducted in accordance with the Declaration of Helsinki. Laboring women at the early stages of labor and another non-pregnant control woman were invited to participate in the study. A written informed consent in accordance with Assiut University Ethical Committee guidelines was taken from all study subjects.

They were divided into 3 groups; Group 1, Control group, included 30 healthy non-pregnant women. They were relatives of the preterm and term labor women, free from common chronic viral infections, autoimmune diseases and not currently taking any immunosuppressive drugs or vitamin D supplements. Group 2 included 46 term “37 weeks gestation or more" laboring women. Group 3 included 36 preterm “less than 37 weeks’ gestation" laboring women. Women were excluded from the study if they have PROM (preterm rupture of membranes), DM, SLE, antiphospholipid syndrome, Preeclampsia, chronic hypertension, twins or any other condition that may cause Preterm labor.

Blood sample were obtained from each woman, the samples was obtained from the laboring women at the onset of labor. We calculated gestational age in weeks at the time of birth based on the best obstetrical estimate using the date of last menstrual period with confirming first trimester ultrasounds.

2.1. Assessment of vitamin D status

Serum levels of 25(OH) D (vitamin D), a combination of 25(OH) D2 and 25(OH) D 3, were determined using ELISA kit (Catalog No: VD220B, Calbiotech, Spring Valley, CA USA). For our analysis, we classified women into three groups that defined Vitamin D status; vitamin D deficiency women[25(OH)D < 20 ng/ml], vitamin D insufficiency women[25(OH)D 20–29 ng/ml], and vitamin D sufficiency women[25(OH)D > 29. ng/ml] (Hollis, 2005).

2.2. Flow cytometric detection of regulatory T cell phenotypes

For all participants in this study, Phycoerythrin (PE) conjugated anti CD25, fluoroisothiocyanate (FITC)-conjugated anti Foxp3 and peridinium-chlorophyll-protein (Per-CP) conjugated anti CD4, allophycocyanin (APC)-conjugated anti HLA-DR and APC-conjugated anti CD45RA were used to detect Tregs and their phenotype. The monoclonal antibodies were from Becton Dickinson (BD) Biosciences, San Jose, CA, USA, expect CD25 (IQ Product the Netherland) and anti Foxp3 (Bioscience, USA)

One hundred µl of blood sample was incubated with 10 µl of anti CD4, anti CD25 and anti CD45RA or anti CD4, anti CD25, anti HLA-DR in separate tubes. After incubation for 20 min at room temperature in the dark, red blood cells lysis and washing with phosphate buffer saline (PBS) were done. Fixed solution was added to fix the cells followed by incubation for 10 min. After that, the cells were washed with PBS, and then permelized solution and 10 µl of Foxp3 were added to each tube and incubated for 20 min at room temperature. After washing, the cells were resuspended in PBS, and analyzed by FACSCalibur flow cytometry with CellQuest software (Becton Dickinson Biosciences, USA). An isotype negative control was done with each sample. Forward and side scatter histogram was used to delineate the lymphocytes (R1). Then CD4+ cells were gated. Total $CD4+CD25+$, $CD4+CD25^{+low}$, $CD4+CD25^{high}$ and $CD4+CD25^{high}$ Foxp3+ Tregs was evaluated as a percentage of CD4+ cells. The expression of Foxp3+ on $CD4+CD25^{+high}$ cells was expressed as geometric mean of fluorescence intensity (MFI). The expression of CD45RA and HLA-DR was evaluated on $CD4+CD25^{high}$Foxp3+ Tregs as shown. $CD45RA^+$Tregs, $HLA\text{-}DR^{+high}$Tregs, $HLA\text{-}DR^{+low}$Tregs and $HLA\text{-}DR^{negative}$Tregswere evaluated as a percentage of $CD4+CD25^{high}$ Foxp3+ Tregs (Fig. 1).

2.3. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD) for continuous variables and percentages for categorical variables. One Way ANOVA were used to analyze continuous variables. The *P* value is considered statistically significant when less than 0.05. Pearson’s correlation was used to correlate the studied parameter. Chi-square test was used to compare the frequency of vitamin D status between pregnant and non-pregnant women.

3. Results

There was no significant difference between groups in the mean age. Also, there was a significant difference in the gestational age between term and preterm labor women ($P < 0.0001$). However, no significant difference in parity between term and preterm labor women ($P = 0.18$) as shown in Table 1.

3.1. Lymphocytes subsets in laboring women and the controls

Table 1 show lymphocytes subsets in laboring women and the controls The results show that the total lymphocytes and $CD4^+$ T-helper lymphocytes were significantly reduced in term (Group 2)and preterm laboring women (Group 3) than the non-pregnant control women (Group 1), with no significant difference between term (Group 2)and preterm laboring women (Group 3).We did not find any significant differences in the percentage of $CD4^+CD25^+$ and $CD4^+$ $CD25^{low}$ T cells within the total $CD4^+$ T cell pool between the three groups. There was an only significant difference in the percentage of $CD4^+$ $CD25^{high}$ T cells between the non-pregnant women (Group1) and term laboring women (Group2). While, a highly significant decline was detected in the percentage of $CD4^+CD25^{high}$Foxp3Tregs in the laboring women than the non-pregnant women and the lowest level was found in