Abstract. – AIM: To study the behaviour of the peripheral lymphocyte subsets in foetuses affected by growth restriction.

PATIENTS AND METHODS: Thirty consecutive pregnant women with an ultrasound diagnosis of foetal growth restriction were included in this study (group A) while 30 women with a physiologic pregnancy were recruited as control group (group B). The diagnosis was performed during the ultrasound of the third trimester and confirmed at birth. Blood samples were drawn after the ultrasound of the third trimester for all patients. The analyzed populations were: WBC, total lymphocytes, CD2+, CD3+, CD4+, CD5+, CD8+, CD19+, CD56+, HLA-DR+, CD45+, CD3+HLA-DR+, CD4+CD3+, CD3+CD8+, CD2+CD56+, CD19+CD5+, ratio (CD4+CD3+)/CD3+CD8+.

RESULTS: The percentage and absolute value of the NK cells was higher in the group A [(20.90 vs. 15.09)% , p = 0.0005; (419.55 vs. 341.40) Ul/μl, p = 0.0005]. This trend was confirmed by the CD2+CD56+ natural killer (NK) subset [(18.84 vs. 13.42) Ul/μl, p = 0.0005]. Instead, the CD4+ percentage value was lower in the group A [(41.15 vs. 44.84)% , p = 0.03] through the CD4+CD3+/CD3+CD8+ ratio was not significantly different.

CONCLUSIONS: Our findings reinforce the concept of pregnancy as a controlled systemic inflammatory state that if altered can have adverse consequences for the mother and the foetus.

Keywords: Natural killer, Foetal growth restriction, Lymphocyte subsets.

Introduction

Foetal growth restriction (FGR) is defined as growth less than the 10th percentile for gestational age. It is an important syndrome that exposes the foetus and the newborn to high risk of perinatal mortality and morbidity1. Unfortunately, a definite cause of FGR is not identified in the 40-50% of the cases2, although the dysregulation of the maternal immune system has been repeatedly hypothesized as a possible cause since immunologic factors – such as cytokines, natural killer (NK) cells, activated macrophage and lymphocytes – have been shown to be associated with several pregnancy complications such as recurrent spontaneous abortion (RSA), preeclampsia and preterm delivery3-5. In particular, it is demonstrated that the maternal immunologic state is maintained by local secretion of T helper-2 (Th2) cytokines while the pregnancy complications such as spontaneous miscarriage, preterm delivery and preeclampsia, seem to be associated with a predominance of T helper-1 (Th1) reactivity in the mother6. These cytokines activate the NK phenotype cells (CD56+CD16+) and transform them into LAK (lymphokine activated killer), powerful cells able to damage the trophoblast6. Then, physiologic pregnancy needs a balanced production of Th1 and Th2 cytokines; indeed, several authors reported a relation between FGR and Th2 response reduction (IL-4, IL-5, IL-10, IL-13) and Th1 response increase (IL-2, INF-γ, TNF-β, TNF-α, IL-3) and suggested that the NK cells might be involved in the modulation of these responses7. Nevertheless, the behaviour of the lymphocyte subsets has been assessed in the endometrium quantifying the percentage and absolute value of the uterine NK. However, it would be interesting to investigate this behaviour also in circulating blood lymphocytes, thus, allowing the use of this experimental information in a clinical setting. Based on the above considerations, the purpose of the present study is to test the possible relationship between the peripheral lymphocyte subsets and the FGR.

Patients and methods

We conducted a prospective study enrolling 60 patients admitted to the Obstetric Unit of Ferrara University from January 2011 to January 2012. Thirty consecutive pregnant women with a diagnosis of FGR were included in this study (group A) while 30 women with a physiologic pregnancy were recruited as control group (group B).
Exclusion criteria: heavy smokers, history of RSA, maternal anaemia, pluriparity, gestational diabetes mellitus, uncertain date of the last menstruation, ethnic group other than Caucasian, foetal malformations.

The diagnosis of FGR was performed during the ultrasound of the third trimester (30th-32nd week of gestation) and confirmed at birth (identified by a weight below the 10th percentile). The ultrasound evaluation of the foetal growth was performed by measuring of the biparietal diameter, femoral length, head and abdominal circumference compared to reference values. The presence of an abdominal circumference below the 10th percentile was essential to diagnose the IUGR. Ultrasound measurements were taken by the same operator (F.V.) to avoid inter-observer interference.

Blood samples were drawn after the ultrasound of the third trimester for all patients. Fresh samples were analyzed in the Laboratory of Hematologic Unit of Ferrara University. Local ethical committee approval was obtained. The patients agreed to sign an informed consent.

**Immunophenotype assay**

Blood count was performed using ADVIA® 2120 haematology system (Siemens, Tarrytown, NJ, USA). One hundred microliters of blood were incubated at room temperature with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated monoclonal antibodies to various lymphocytes cell surface markers. Conjugated anti-CD2 FITC, anti-CD3 FITC, anti-CD3 PE, anti-CD4 FITC, anti-CD5 PE, anti-CD45 FITC, anti-CD56 PE, anti-HLA-DR PE monoclonal Mouse anti-Human antibodies were used for analyzing different lymphocytes subsets. After incubation with the antibodies, cells were lysed using fluorescence activated cell sorting (FACS) Lysing Solution, then washed with Cell WASH PBS and resuspended in 0.5 ml of phosphate buffered saline (PBS). Lymphocytes were analyzed with FACS Calibur™.

**Flow citometry**

The lymphocytes were gated on FSC (Forward-scattered light) versus SSC (Side-scattered light) histogram. CD45 FITC fluorescence of this population was displayed on a one-parameter histogram. The following fluorescences were put on a two-parameter histogram: CD2 FITC and CD56 PE, CD3 FITC and HLA-DR PE, CD3 FITC and CD8 PE, CD4 FITC and CD3 PE, CD19 FITC and CD5 PE. The analyzed populations were: WBC, total lymphocytes, CD2+, CD3+, CD4+, CD5+, CD8+, CD19+, CD56+, HLA-DR+, CD45+, CD3+HLA-DR+, CD4+CD3+, CD3+CD8+, CD2+CD56+, CD19+CD5+, ratio (CD4+CD3+)/(CD3+CD8+).

**Statistical analysis**

The software SPSS v.20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. “Non parametric” Mann-Whitney test was used to analyse the result between study subjects and controls. *p* value < 0.05 was considered statistically significant.

**Results**

Three patients of the group A were excluded because at delivery their infants had a weight over the 10th percentile for gestation. Therefore, the study population was composed by 57 patients (27 for the group A; 30 for the group B). Maternal age was not different between the two groups [respectively 32.66 (±2.84) y, 32.70 (±3.21) y], while there was a strong difference in gestational age [respectively, 37.00 (±1.27) y, 39.30(±0.91) y] and in the birth weight [2078 (±208.24) g, 3120 (±218,93) g, respectively]. In analyzing the results in Table I, we found a different behaviour of the NK cells between the two groups, indeed both the percentage and the absolute value were significantly higher in group A. This trend was confirmed by the NK subset (CD2+CD56+) that was higher in group A. The behaviour of the T lymphocytes was interesting. In particular, CD4+ and CD4+CD3+ lymphocytes were significantly higher in the group B. However, the different behaviour of CD4+ lymphocytes did not change the CD4+CD3+/CD3+CD8+ ratio that was higher – but not significantly - in group B. This different behaviour is also graphically clear (Figure 1).

**Discussion**

Placentation is a dynamic process that is dependent on invasive foetal trophoblasts migrating through the decidualized endometrium. This process induces a local maternal immune response that is characterized largely by cells of the innate arm, such as decidual NK cells and macrophages. NK cells play a fundamental role in the innate immune response for their ability to secrete cytokines and kill target cells without prior sensibilization. Although the role of these cells in
the decidua is unknown, experiments in the mouse\textsuperscript{10,11} have demonstrated that NK stimulation markedly impacts pregnancy, resulting in rapid pregnancy loss. On the contrary, other authors\textsuperscript{12,13} have demonstrated a decrease in specific decidual leukocyte subsets in pregnancies complicated by pre-eclampsia and FGR. However, most studies about the lymphocytic behaviour in FGR are based on the analysis of the uterine NK cells. These experimental data are not particularly helpful in a clinical setting because it is unthinkable to perform a placental biopsy to all pregnant women as a screening test for FGR. Conversely, if these data were verified on blood lymphocyte subsets, they would have greater clinical significance and also practical consequences.

The analysis of the circulating NK cells has produced interesting results in the study of the infertility and RSA so far. For instance, Karami et al\textsuperscript{14} found that the percentage of CD56\textsuperscript{+} cells and the level of peripheral blood NK cell cytotoxicity increased in RSA patients and women with IVF failure. Similar results were found by other authors\textsuperscript{15,16}. Therefore, our aim was to study the peripheral lymphocytic population in women with FGR.

Our results are statistically and graphically clear (Figure 1). The percentage and concentration of NK cells and its subset are significantly higher in the women with FGR (Table I). These findings do not confirm the data of Williams et al\textsuperscript{12} about the leukocyte populations in the biopsies obtained by placental bed of the women with pre-eclampsia. The authors explained that the decreased numbers of CD56\textsuperscript{+} cells in FGR might be involved in decreased vascular adaptation to pregnancy during placentation. Furthermore, they stated that a possible limitation of the study was the placental bed biopsies that may not be representative of the whole area. However, our results agree with previous data of Stallmach et al\textsuperscript{17} and

\begin{table}
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\hline
\textbf{Group A (n. 27)} & \textbf{Group B (n. 30)} & \textbf{p value} \\
\hline
CD2\textsuperscript{+} (%) & 84.67 (±3.53) & 83.69 (±5.05) & NS \\
CD3\textsuperscript{+} (%) & 75.84 (±5.81) & 76.55 (±2.99) & NS \\
CD4\textsuperscript{+} (%) & 41.15 (±7.39) & 44.84 (±4.05) & 0.03 \\
CD5\textsuperscript{+} (%) & 72.33 (±3.85) & 77.73 (±4.05) & NS \\
CD8\textsuperscript{+} (%) & 36.46 (±6.12) & 34.40 (±7.95) & NS \\
CD19\textsuperscript{+} (%) & 8.53 (±2.46) & 9.86 (±4.39) & NS \\
CD56\textsuperscript{+} (%) & 20.90 (±5.00) & 15.09 (±2.91) & 0.0005 \\
HLA-DR (%) & 23.40 (±7.77) & 24.54 (±11.90) & NS \\
CD45\textsuperscript{+} (%) & 97.20 (±1.79) & 97.25 (±2.09) & NS \\
CD3HLA-DR (%) & 10.16 (±6.31) & 9.58 (±7.60) & NS \\
CD4\textsuperscript{+}CD56\textsuperscript{+} (%) & 41.15 (±7.39) & 44.84 (±8.45) & 0.03 \\
CD3\textsuperscript{+}CD8\textsuperscript{+} (%) & 30.56 (±6.12) & 29.80 (±8.81) & NS \\
CD2\textsuperscript{+}CD56\textsuperscript{+} (%) & 18.84 (±4.81) & 13.42 (±2.58) & 0.005 \\
CD19\textsuperscript{+}CD8\textsuperscript{+} (%) & 1.73 (±0.94) & 2.05 (±1.06) & NS \\
CD4\textsuperscript{+}CD3\textsuperscript{+}CD8\textsuperscript{+} (%) & 1.42 (±0.59) & 1.72 (±0.80) & NS \\
LYMP (%) & 20.66 (±5.34) & 23.40 (±7.81) & NS \\
WBC (Ul/µl) & 994.00 (±1928.38) & 10051.00 (±3005.96) & NS \\
LYMP (Ul/µl) & 1984.44 (±345.51) & 2332.00 (±1117.36) & NS \\
CD56 (Ul/µl) & 419.55 (±155.18) & 341.40 (±146.59) & 0.004 \\
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\end{tabular}
\caption{Different behaviour of the lymphocyte subsets between the two groups.}
\end{table}

\textbf{Figure 1.} Graphical representation of the different lymphocyte percentage between the two groups.
Bachmayer et al\textsuperscript{18} who reported increased numbers of CD56\textsuperscript{+} cells in pre-eclampsia decidua compared with controls, thus, confirming that the pregnancy complicated by FGR – in opposition to physiologic pregnancy – is characterized by a shift away from Th2 type and bias toward Th1 type immune response\textsuperscript{19}.

The analysis of the other lymphocyte populations agrees with previous evidence demonstrating the decrease of the CD4\textsuperscript{+} cells\textsuperscript{20} although the CD4\textsuperscript{+}CD3\textsuperscript{−}/CD3\textsuperscript{+}CD8\textsuperscript{+} ratio was not different between the two groups. This finding should be analyzed considering the behaviour of these cells in the physiologic pregnancy where the ratio decreases in first trimester and increases in the third. On the contrary, in our data the ratio decreases in the third trimester of pregnancy suggesting an increased cytotoxic lymphocyte activity in a specific condition such as the FGR.

The B lymphocytes behaviour (CD19\textsuperscript{+}) was not different between the two groups; it disagrees with previous study that suggested an increased CD19\textsuperscript{+} value in women with FGR in comparison to women with normal pregnancies\textsuperscript{20,21} and indicated even a negative correlation with birth weight\textsuperscript{11}.

Our findings seem to be the experimental demonstration of the hypothesis postulated by Sargent et al\textsuperscript{22}, namely that aberrant NK cell activation, both locally in the decidua and systematically in maternal blood, might be the cause of preeclampsia.

**Conclusions**

Our data reinforces the concept of pregnancy as a controlled systemic inflammatory state that if altered can have adverse consequences for the mother and the foetus.

**Conflict of interest**

The Authors have no conflict of interests of declare.

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