



The structure of immunocompetent decidual cells in recurrent missed abortions

Struktura imunokompetentnih ćelija decidue kod ponavljanih spontanih pobačaja

Dragana Radović Janošević*, Jasmina Popović*, Miljan Krstić†, Aleksandra Tubić Pavlović*, Milan Stefanović*, Sonja Pop-Trajković*

*Clinic of Gynecology and Obstetrics, †Institute of Pathology, Clinical Center Niš, Niš, Serbia

Abstract

Background/Aim. Recurrent or habitual missed abortions (RMA) are defined as three or more consecutive abortions. In the first trimester of pregnancy habitual missed abortions occur in about 1% of population. The aim of this immuno-histochemical study of decidua in RMA of unknown etiology was to identify subpopulations of decidual lymphocytes in recurrent miscarriages and compare the distribution of immunocompetent cells in artificial abortions and RMA. **Methods.** The study included 30 women with at least 2 consecutive miscarriages in the first trimester of pregnancy. Curettements of the third missed abortion were immunohistochemically analyzed. The control group consisted of 20 women without loaded reproductive anamnesis, with the abortion for social reasons. Criteria for exclusion from the study were diagnosed uterine anomalies, positive screening for thrombophilia and women who suffered from diabetes mellitus and disorders in the function of the thyroid gland. Immunophenotyping was performed by immuno-alkaline phosphatase (APAAP) using monoclonal antibodies: CD 30, CD 45 RO, CD 56 and CD 57, CD 68. **Results.** The number of missed abortions (1,223) was on the average 9.7% of all deliveries during the test period. Among them RMA were registered in 52 (4.2%) patients and in 30 (57%) the exact etiology of abortions was not determined. RMA was most

common in the 25–34 years of age group. The largest number of RMA showed the ultrasound characteristics of missed abortion in 60% of cases and was in nulliparous patients (76.7%). The number of natural killer (NK) CD56 positive cells did not differ significantly between the types of abortion. In the decidual tissue, a number of NK CD57 positive cells was significantly higher in missed abortions compared to artificial interruptions ($p < 0.01$). In artificial termination of pregnancy there was an absolute predominance of CD45RO lymphocyte subpopulations, whereas in the RMA group there was slightly greater predominance of CD30 positive cells. The completed analysis showed a significantly higher number of CD68 positive macrophages in a decidual tissue of RMA pregnancy ($p < 0.01$). **Conclusion.** The number and phenotypic structure of NK cells are significantly different in normal pregnancy decidua and in RMA. The NK cell dominance is present in the RMA group, in favor of CD56+ and CD 57 of subpopulations with increased CD30 of T lymphocyte subpopulations. Macrophages are more numerous in the decidua of pregnancies ended in abortion, so the cause of RMA of unknown etiology in a number of cases could be dysregulation of immunocompetent cells.

Key words:

abortion, habitual; immunohistochemistry; uterus; killer cells, natural; abortion, missed; decidua.

Apstrakt

Uvod/Cilj. Ponovljeni, habitualni pobačaji (PSP) definišu se kao tri ili više uzastopnih pobačaja. Tokom prvog tromesečja trudnoće, habitualni pobačaj javlja se kod oko 1% trudnica. Cilj rada bio je da se imunohistohemijskom studijom decidue kod ponavljanih spontanih pobačaja nepoznate etiologije identifikuju subpopulacije decidualnih limfocita i uporedi distribucija imunokompetentnih ćelija decidue kod arteficialnih abortusa i kod ponavljanih spontanih pobačaja. **Metode.** Ispitivanjem je obuhvaćeno 30 žena sa najmanje dva uzastopna spontana pobačaja u prvom trimestru trudnoće. Kiretman trećeg spontanog pobačaja bio je imunohistohemijski analiziran. Kontrolu je činilo 20 žena bez opterećenja u reproduktivnoj anamnezi, sa

prekidom trudnoće iz socijalnih razloga. Kriterijumi za isključivanje iz studije bili su dijagnostifikovane anomalije uterusa, pozitivan test na trombofilije, kao i dijabetes melitus i poremećaji u funkciji štitne žlezde. Imunofenotipizacija rađena je primenom metode imunoalkalne fosfataze, (APAAP) sa korišćenjem monoklonalnih antitela: CD 30, CD 45 RO, CD 56 i CD 57, CD 68. **Rezultati.** Ukupno je registrovano 1 223 spontanih pobačaja što predstavlja 9,7% svih spontanih pobačaja. PSP ustanovljen je kod 52 (4,2%) bolesnice. Od tog broja kod 30 (57%) bolesnica sa PSP nije utvrđena tačna etiologija pobačaja. PSP je bio najzastupljeniji u starosnoj grupi 25–34 godine života. Najveći broj PSP pokazivao je ultrazvučne karakteristike izostalog pobačaja (kod 60% bolesnica). Najveći broj PSP bio je kod nulipara (76,7%). Broj *natural killer* (NK) CD56 pozitiv-

nih ćelija nije se značajnije razlikovao u zavisnosti od tipa prekida trudnoće. U tkivu decidue broj NK CD57 pozitivnih ćelija bio je značajno veći kod spontanih prekida trudnoće u odnosu na namerne prekide ($p < 0,01$). Kod namernih prekida trudnoće bila je prisutna apsolutna predominacija subpopulacije CD45RO limfocita, dok je u grupi sa PSP bila nešto veća predominacija CD30 pozitivnih ćelija. Urađena analiza pokazala je značajno veći broj CD68 pozitivnih makrofaga u tkivu decidue kod PSP trudnoće ($p < 0,01$). **Zaključak.** Broj i fenotipska struktura NK ćelija značajno se razlikuje u decidui normalne

trudnoće i kod PSP. NK ćelijska dominacija u grupi PSP je u korist CD56+ i CD57 subpopulacija uz veću zastupljenost CD30 subpopulacije T-limfocita. Makrofagi su brojniji u decidui trudnoća završenih pobačajem, tako da bi uzrok PSP nepoznate etiologije u određenom broju slučajeva mogla biti disregulacija imunokompetentnih ćelija.

Ključne reči:

abortus, habitualni; imunohistohemija; materica; ćelije ubice, prirodne; abortus, izostali; decidua.

Introduction

Recurrent or habitual missed abortions (RMA) are defined as three or more consecutive abortions. In the first trimester of pregnancy habitual missed abortions occur in about 1% of population¹.

The causes of recurrent missed abortions are multifactorial but can be divided into: embryonic, mainly due to pathological embryonic karyotype, and maternal affecting the endometrium and placenta.

Known maternal causes are anatomic, endocrine and immunological – auto- and allo- immune. Recent works talk about sperm quality as another factor that influences the frequent occurrence of abortion².

Despite knowing all the above mentioned etiological factors of recurrent spontaneous abortions, 50% of them were classified as recurrent spontaneous abortions of unknown etiology². It is believed that the largest number of them is the consequence of inadequate alloimmune response of a mother to pregnancy. Period from luteinising hormone (LH) jump to implantation to menstruation is critical for the transformation of the endometrium in terms of supporting implantation or the occurrence of menstruation. Endocrinological and the immunological systems are in a close interaction during the implantation and the maintenance of pregnancy. This communication is most striking on the endometrium of decidual pregnancy. Decidua makes a smaller part of the placenta and the only one that is of exclusively maternal origin. For this reason it does not show antigenic potential, but it is the place of significant process of establishing and maintaining gravid immunoregulation toward the fetal part of the placenta considering that it is a place of direct maternal fetal confrontation. It can be said that decidua is the place of selection, proliferation and maturation of unique population of uterine natural killer (NK) (uNK) cells^{3, 4}. Apoptosis important for the selection and favorising certain populations of immunocompetent cells also occurs in the decidua. Reorganization of decidual immunocompetent cells provides its immunosuppressive effect by regulating the activity of NK cells, which themselves undergo subpopulation recomposition under the influence of hormones and cytokines. Decidual cell populations are most important in the implantation uNK cells, macrophages, dendritic cells (DCS) and T cells. B cells and neutrophils are insignificant in decidualization and implantation. Decidual composition of immunocompetent cells effectively establishes and maintains gravidity immunomodulation and probably control and gui-

dance of placental growth^{5, 6}. If fertilization and then implantation occurs on the day 20–24 of the cycle, granular NK cells rapidly proliferate and become dominant populations of lymphoid cells achieving close contact with invading trophoblast^{7, 8}.

The tissue-specific differentiation of uNK cells is related to their function and may be a critical determinant for decidualisation or desquamation of the endometrium^{9, 10}. Despite the changes in the number of these cells, a fundamental change is subpopulation precomposition. A dominant subpopulation of NK cells of peripheral blood is CD56 dim, CD16+ and they constitute the basic population of uNK cells of the proliferative phase of the cycle. uNK cells have high level of CD56 expression, so they belong to the CD56 bright subpopulation^{7, 11}. In addition to CD56 bright, uNK cells also express killer activating and inhibitory receptors but do not express any other typical NK cell markers, such as CD16 or CD57^{7, 12}. In pregnancy, progesterone causes the reduction in the number of NK cells, activity and cytotoxicity through direct influence on NK cells or promoting TH2 cytokine activities, progesterone-induced blocking factor (PIBF) production in T lymphocytes. Also, progesterone facilitates the regrouping of peripheral NK (pNK) cells in the endometrium by expression of receptors that induce vascular endothelial growth factor (VEGF) and macrophage inflammatory protein (MIP1 β) in the endometrium. Endometrial stromal cells under the influence of progesterone produce IL15, prolactin and other factors that regulate uNK in their proliferation, differentiation and production of cytokines and other molecules that support placental trophoblastic development and promote local immunomodulation. uNK cell function is mainly related to the decision to launch the mechanism of menstruation or decidualization, control of maternal immune response to fetal allograft, degree of trophoblastic invasion and the formation of placenta – the modulation of cytokine expression during implantation.

It was long thought that T lymphocytes affect decidualisation and implantation exclusively through the TH1/TH2 balance, i.e. favourisation of TH2 cytokine immune response^{3, 6, 13}. Pregnancy is considered a TH2 cytokine-mediated event. TH1 cytokines have been associated with miscarriage and infertility. These effects of TH1 cytokines in experiments were blocked by injection of TH2 cytokines IL-10³. The conclusions of these experiments with mice were expanded to human population⁶. But the discovery of regulatory T lymphocytes with detailed elaboration of cytokine profiling of T cell subgroups showed that the

explanation is not so simple. It is evident that the TH1/TH2 hypothesis is not sufficient to explain the immune mechanisms during implantation. In human reproduction, TH1 cytokine activity is needed at several stages of pregnancy, especially during the early implantation period¹⁴. During this period, the cytokines IL1 and tumor necrosis factor alfa (TNF α) make pregnancy possible by stimulating the production of leukemia inhibitory factor (LIF) and increasing angiogenesis. It is also interesting that TH1 environment stimulates the production of TH2 cytokine¹⁴. T regulatory (Tregs) lymphocytes play a role in implantation and are necessary for the establishment of peripheral immunomodulation. They suppress autoreactive T cells¹⁵. In the context of pregnancy, Tregs are characterized as essential for the establishment of alolerance. Tregs have CD4 and CD25, these leukocytes are found in the decidua in various stages of pregnancy. The highest level in the peripheral blood is shown in the first trimester of pregnancy. Tregs cells are specific for Foxp3 marker required for their function, and in the experiments it was significantly lower in infertile women¹⁶. This supports the importance and fundamental role of Tregs from T cell population in implantation. Macrophages also play a role in implantation and decidualization. Macrophages in the endometrium in proliferative and secretory stage was fairly constant, but the number significantly increased in the decidua of early pregnancy¹⁷. Their concentration is about 45% higher in the preimplantation region of decidua which is largely achieved through redistribution of these cells from other decidual regions. Chemotaxis of macrophages and their accumulation at the site of implantation are favored by primarily trophoblast proinflammatory factors at the site of the endometrial erosion, increased levels of steroid hormones and increased concentrations of cytokines stimulated by steroid hormones¹⁸. Cytokine production of macrophages may help prepare the endometrium for pregnancy, cleaning apoptotic material of trophoblast cells which is very important because it prevents their presentation of immunocompetent cells and initiation of immune responses. Macrophages are likely to mediate the range of trophoblast invasion through TNF α and participate in the level of inflammatory reaction necessary in the early stages of pregnancy¹⁸.

A number of systems are involved in creating a favorable environment for the acceptance of allogeneic blastocysts. The three most important ones involved in it are immune cells, cytokines and adhesion molecules in the place of decidua blastocyst contact⁴.

The aim of this study was to identify subpopulations of decidual lymphocytes of recurrent spontaneous abortions by immuno-histochemical study of decidua in recurrent spontaneous abortions of unknown etiology and to compare the distribution of immunocompetent decidual cells in artificial abortion with those in recurrent spontaneous abortions.

Methods

The study included 30 women who had a history of 2 consecutive miscarriages in the first trimester of pregnancy, while the third miscarriage curettment was histopathologically

immuno-histochemically analyzed with paraffin preparations. The control group consisted of 20 women without loaded reproductive anamnesis, where the abortion was done for social reasons. Criteria for exclusion from the study were diagnosed uterine anomalies, positive screening for thrombophilia and women who suffer from diabetes mellitus and disorders in the function of the thyroid gland.

Curettings were fixed in 10% formalin during 24 hours, and thereafter moulded in paraffin. Paraffin 5-micron sections were dyed using the hematoxylin-eosin method. Immunophenotyping was performed using immuno-alkaline phosphatase as a method (APAAP) with the following monoclonal antibodies (DAKO): CD30 (marker for activated cells); CD45 RO (marker for T lymphocytes); CD56 and CD57 (markers for NK cells); CD68 (marker for macrophages).

Visualization of the reaction products was performed using the new foschsina which resulted in red deposits in the places of positive reaction. The average number was determined at 10 high-power fields (hpf).

The results were statistically analyzed by forming a database. Statistical significance was tested by commercial software using appropriate tests-Student's *t*-test and *t*-test with corrective approximate method by Cohren and Cox for small samples when necessary. The 5% level was taken as a borderline level of statistical significance.

Results

The number of missed abortions during the test period was 1,223. The percentage of missed abortions *per* year is shown in Table 1.

Out of 1,223 abortions during the test period, RMA was registered in 52 (4.2%) patients. Of that number, in 30 (57%) patients with RMA the exact etiology of abortion was not determined.

Table 1

| Percentage of miscarriages by years | | |
|-------------------------------------|---------------|---------------------|
| Year | Deliveries, n | Miscarriages, n (%) |
| 2010 | 3,189 | 301 (9.7) |
| 2011 | 3,167 | 305 (9.6) |
| 2012 | 3,133 | 304 (9.7) |
| 2013 | 3,109 | 313 (10.1) |
| Total | 12,598 | 1,223 (9.7) |

Ultrasound features of RMA of unknown etiology are shown in Table 2.

Table 2

| Ultrasonic (US) characteristics of recurrent or habitual missed abortions | | |
|---|----|-----|
| US finding | n | % |
| Missed abortion | 18 | 60 |
| Abortion spontaneus incipiens | 6 | 20 |
| Blighted ovulum | 6 | 20 |
| Total | 30 | 100 |

The largest number of RMA showed the ultrasound characteristics of missed abortions (in 60% of cases).

Frequency distribution of RMA by age groups is shown in Table 3.

RMA is most common in the 25–34 years of age group.

Parity in patients with RMA is shown in Table 4.

The largest number of RMA was in nulliparous patients (76.7%).

Immunohistochemical studies of decidua in artificial abortions and recurrent miscarriages (the presence of cell subpopulations of NK lymphocytes) are given in Table 5.

Table 3

| Distribution by the age groups | | |
|--------------------------------|----|-------|
| Age (years) | n | % |
| 25–29 | 10 | 33.3 |
| 30–34 | 11 | 36.7 |
| 35–39 | 7 | 23.3 |
| > 40 | 2 | 6.7 |
| Total | 30 | 100.0 |

Table 4

| Parity of the patients with recurrent or habitual missed abortions (RMA) | | |
|--|----|------|
| Previous deliveries | n | % |
| 0 | 23 | 76.7 |
| 1 | 6 | 20.0 |
| 2 | 1 | 3.3 |

Table 5

| Characteristics of cellular subpopulations of natural killer (NK) cells in decidual tissue | | |
|--|------------------------------|------------------------------|
| Type of abortion | NK CD56 ($\bar{x} \pm SD$) | NK CD57 ($\bar{x} \pm SD$) |
| Spontaneous | 85.03 \pm 23.3 | 95.3 \pm 26.1** |
| Artificial | 77.6 \pm 13.27 | 15.2 \pm 2.6 |

***p* < 0.01.

The number of NK CD56 positive cells did not differ significantly depending on the types of abortion (Table 5). In the decidual tissue, the number of NK CD57 positive cells was significantly higher in missed abortions compared to artificial interruptions (*p* < 0.01).

The average number of certain subpopulations of T lymphocytes in the decidual tissue (determined at 10 hpf) in RMA and artificial abortions is shown in Figure 1.

In artificial termination of pregnancy there was an absolute predominance of CD45RO lymphocyte subpopulations, whereas in the RMA group there was slightly greater predominance of CD30 positive cells (Figure 1).

The number of CD68 positive macrophages in decidual tissue in RMA and artificial abortions is shown in Figure 2.

The completed analysis showed a significantly higher number of CD68 positive macrophages in a decidual tissue of RMA pregnancy (*p* < 0.01)

Frequency percentage of leukocyte subpopulations in decidual tissue in RMA and the control group of artificial abortions is given in Figure 3.

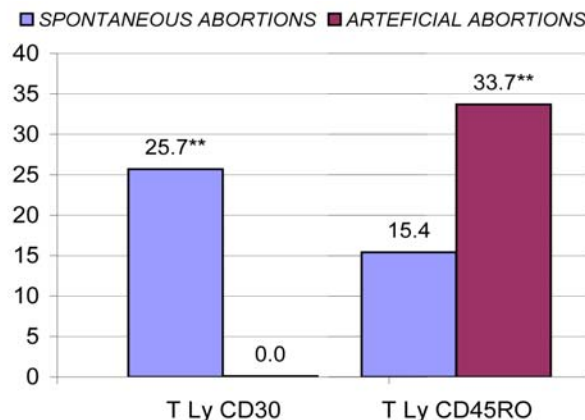


Fig. 1 – Average number of T-lymphocytes depending on the type of abortion.

Data are presented as $\bar{x} \pm SD$ (average number on 10 high-power fields – hpf); ***p* < 0.01.

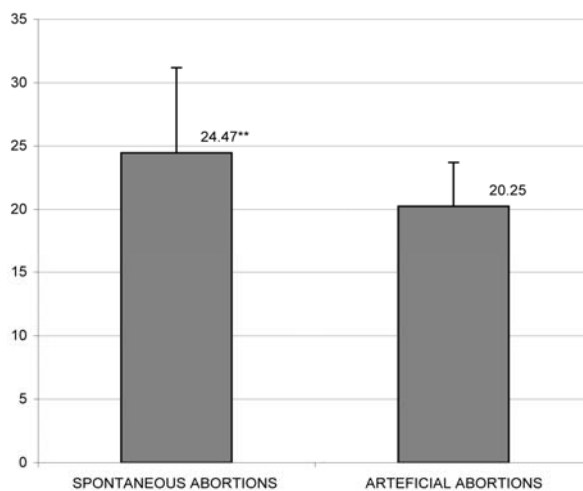


Fig. 2 – CD68 number of positive macrophages by the type of abortion.

Data are presented as $\bar{x} \pm SD$ (average number on 10 high-power fields – hpf); ***p* < 0.01.

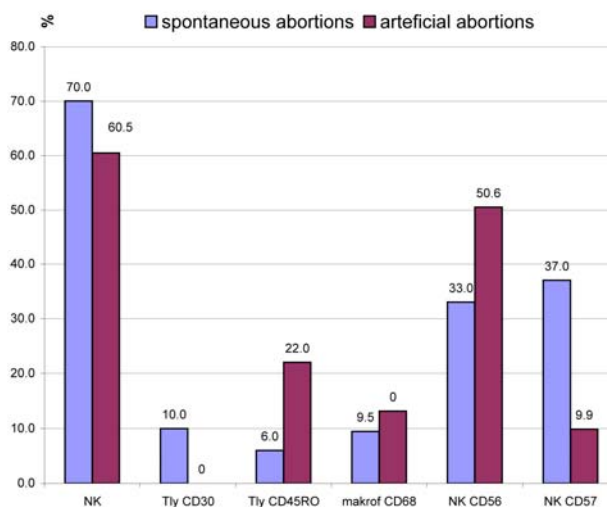


Fig. 3 – Percentage of present decidual leukocyte subpopulation, depending on the type of abortion.

Discussion

Stroma of the gravid endometrium, decidua, is an immunologically privileged position of the reproductive system of a woman with a dominant population of NK cells. Cell microenvironment, the environment in early pregnancy decidua of normal or completed by miscarriage is very important, especially in terms of intercellular contact and the success of establishing immune tolerance.

According to our results the total number of leukocytes in the decidua is 30% in artificial termination of pregnancy compared to 35% in RMA, which is a statistically significant difference. In addition to the increasing number of leukocytes, the most important results of our study show a significant difference in terms of subpopulation regrouping immunocompetent cells in the decidua of these two groups of patients. The total number of NK cells in the group of recurrent missed abortions comparing to artificial is significantly higher. If we look at the phenotypic structure in NK cells, our results show a significantly higher number of CD 56+ and CD 57+ of NK cells in the RMA group.

Our results show that CD 57 populations of NK cells occur in RMA decidua which is otherwise characteristic phenotype of NK cells of the peripheral circulation. We can say that a lot is known about the immunocellular population of decidua in normal pregnancy, but still not enough about the differences of the same population in RMA. There are several studies with the results similar to the results of this study in relation to the significant increase in the number of CD 57 cells in the decidua of pregnancies ended in miscarriage^{18, 19}. The possible mechanism of increasing the number of CD57 NK cells in the endometrium of RMA would be the activating of cytokines attacking trophoblast. In a study from 2006, Ordi et al.²⁰ investigated the presence of different subpopulations of immune cells in the decidua and endometrium in four groups of women: (I) women treated with progesterone disregarding pregnancy; (II) in extrauterine pregnancy; (III) in intrauterine pregnancy of women with RMA; (IV) the group of women with inflammatory reaction at the level of endometrium.

The results of this study show the presence of CD 56 and CD 57 cells in all the groups; especially CD 57 subpopulation was present in the group with inflammation of the endometrium in 100% of cases, and in the RMA group in 55% of cases. CD 56 is present in the group of women with progesterone therapy and RMA, but much less in the decidua of ectopic pregnancy. We can say that NK cells, producing various cytokines, directly influence trophoblastic growth and hormone production allowing decidual vascularization and implantation, and by creating immunomodulatory proteins take part in immunomodulation at the maternal-fetal site of contact.

There are several studies that have used the immunohistochemical method and flow cytometry in NK cells in women with RMA. Most of these studies speak in favor of the increasing number of CD 56 and CD 57 in NK cells. Lachapelle et al.¹¹ in a study found a similar percentage of the total number of NK cells with increased CD 16+, CD 56 dim subsets and reduced subset of CD 16, CD 56 bright NK cells. Quenby et al.⁹, in their immuno-histochemical study from

1999, found an increased percentage of CD 16+ and CD 56+ cells. Clifford et al.¹⁹ in 1999, using immuno-histochemical method, reported an increased number of CD56+ NK cells in women with RMA under 13 gestational weeks. Emmer et al.²¹ in 2002, also immuno-histochemically, obtained results on the increasing number of NK cells expressing CD 56 and CD 16 in RMA. There are two studies which show different results in terms of the number and phenotypic features of decidual NK cells. Shimada et al.²² in the 2004 study, using flow cytometry, reported no difference in the percentage of CD 56+ and CD 56, CD16 + compared to CD 56+, CD16 NK cells. Furthermore, 13. Michimata et al.¹³ in 2002, immuno-histochemically found no difference in the number of CD16 + compared to CD 56+ NK cells. There are two studies which show different results in terms of the number and phenotypic features of decidual NK cells. However, 13. Michimata et al.¹³ immuno-histochemically found no difference in the number of CD16 + compared to CD 56+ NK cells.

Our study included examination of the frequency of CD 30 and CD 45 RO T lymphocyte subpopulations in the group of intentional abortions for social reasons and in the group of habitual miscarriages. In artificial abortions we found a significantly higher number of CD45 RO T lymphocytes, and CD 30 in RMA. Studies that researched the distribution of CD45 RO T lymphocytes report that decidual lymphocytes are expressed in 90% of CD 45 antigen RO cases²⁵.

As far as the literature data on decidual CD 30 T lymphocytes are concerned, they show their increase in secretory stage of the cycle and possible hormonal influence on them²². In the case of T lymphocyte activation, immune response that leads to the loss of pregnancy is taking place in lymphoid aggregates in the vicinity of endometrial glands, where decidual T lymphocytes are grouped^{24, 25}. Our results show a slightly higher presence of macrophages in RMA decidua. The number of endometrial macrophages is partly under control of ovarian steroids and they possess estrogen and progesterone receptors¹⁸. They are also the main cells that, beside trophoblasts, synthesize prostaglandins, whose immunosuppressive role in pregnancy is known²⁶. Macrophages are the most numerous at the site of implantation, and there are 45% of them more than in other parts of decidua¹⁸.

In addition, they play an important role in the maintenance of pro- and anti-inflammatory cytokine balance³. They are also essential in the response to infective agents and in the removal of apoptotic material¹⁸. A slightly higher percentage of macrophages in RMA in our sample would indicate the presence of infection in a number of RMA. The normal phenotype of uNK cells with receptor system on their surface and cytokine production encourage the development of pregnancy by adequate angiogenesis and anti-inflammatory reaction of the decidua. This role of uNK cell phenotype CD56brightCD16- is disrupted by their lack of or exposure to inflammatory agents (viruses and bacteria). Their insufficiency recruit T cytotoxic lymphocyte subpopulations with the beginning of inflammatory pathogenesis. Also, exposure to inflammatory agents causes the conversion of cytokine NK cell production with dominant TNF- α . In such environment there is a loss of immunotolerance with inadequate angiogenesis resulting in pregnancy loss¹⁵.

Conclusion

The number and phenotypic structure of uterine NK cells is significantly different in normal pregnancy decidua and in that of RMA. Our results demonstrate NK cell dominance in the RMA group, with phenotypic structure in favor

of CD56+ and CD57 subpopulations. Also, the prevalence of CD30 subpopulation of T lymphocyte is significantly higher in the RMA group. Macrophages are more numerous in the decidua of pregnancies ended in missed abortion, so that the cause of RMA of unknown etiology in a number of cases could be dysregulation of immunocompetent cells.

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