Uterine Natural Killer Cells in the Context of Implantation: Immunohistochemical Analysis of Endometrial Samples from Women with Habitual Abortion and Recurrent Implantation Failure

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Summary

Infertility affects approximately 48 million couples globally. Despite the enormous progress of the methods of reproductive medicine that has been made since the first test-tube baby was born in 1978, the implantation rate of day-3 embryos is only around 15-20 % and 30 % of day-5 embryos. Numerous strategies aim to improve implantation rates and prevent repeated implantation failure. However, there is no specific general recommendation leading to satisfying results. One of the many risk factors relevant in this regard is the uterine immunological make-up, mainly the uterine Natural Killer (uNK) cells. They orchestrate the overall immune response during implantation by influencing trophoblast invasion and vascular remodeling and throughout pregnancy, uNK cells are also the main immune cells at the maternal–fetal interface. Previously, uNK count has been correlated with various fertility issues including idiopathic recurrent miscarriage. The present study used endometrial samples collected from 256 patients with recurrent implantation failure (RIF), habitual abortion (HA) and idiopathic sterility. Samples were collected between day 19 and 21 of the menstrual cycle mainly by Pipelle endometrial sampling. The samples were fixed in formalin for 24 hours and further processed for immunohistochemistry using anti-CD56 to visualize this antigen marker of uNK cells. Immunohistochemical counting was performed to assess the low, normal, or elevated count of uNK cells. According to the one-way ANOVA test, the age of our patients did not have any influence on the count of uNK cells. With Spearman correlation analysis, we found statistically significant correlation (p-value 0.05) of -0.133 between prior miscarriage and lower uNK cell count. Using the same analysis we found statistically significant correlation (correlation 0.233 with p-value 0.01) between number of uNK cells and activation status. Patients with higher uNK cells were more frequenty diagnosed with endometriosis (p-value 0.05, correlation 0.130). Patients with an immunological factor of sterility (defined by a clinical immunologist) had a lower chance of gravidity (-0.203 with p-value 0.01). Based on our results, we can confirm that there is a correlation between RIF, HA, idiopathic sterility, endometriosis, and immunological factor of sterility (uNK cell count). The true predictive value with regard to fertility outcomes needs to be addressed in future research.

Keywords

Uterine NK cells • Recurrent implantation failure • Habitual abortion • Immunohistochemistry • CD56

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Introduction

Infertility affects approximately 48 million couples and 186 million individuals globally. Each year there are nearly 2.4 million ongoing *in vitro* fertilization (IVF) cycles internationally. It is estimated that since Louise Brown's birth in 1978, over 8 million IVF babies have been born around the world. Despite the enormous progress of the methods of reproductive medicine that has been made since then, the implantation rate of day-3 embryos is 15-20 % and 30 % of day-5 embryos [1]. The most important factor for successful treatment is the

quality of the transferred embryo, which is dependent on the oocyte and sperm quality, which are mostly non-modifiable.

Numerous strategies aim to improve implantation rates and prevent repeated unsuccessful implantation, however there is no specific general recommendation leading to satisfying results. Frequent risk factors leading to recurrent implantation failure (RIF) could be either maternal: genetic, anatomical abnormalities (e.g. uterine septa, myomas, endometrial polyps, intrauterine adhesions), immunological (e.g. HLA, Natural Killer (NK) cells), infections, hematological, or male factors as well as many others. The probable risk factor determines diagnostic (e.g. like ERA test, Win-test, microbiome, endometrial immune profiling) and therapeutic approach like endometrial scratching, endometrial flushing, antibiotics and various others [2]. Comprehensive understanding of implantation mechanisms slowly but convincingly leads to personalization of diagnostic and therapeutic approach based on uterine immune profiling.

Understanding the immune mechanisms during implantation is essential. The optimally balanced immune response at the maternal–fetal interface plays a deciding role in endometrial receptiveness of the semi-allogeneic embryo during the window of implantation. In the mid-luteal phase nearly all immune cells belonging to our adaptive immunity leave the endometrium. At the same time, innate immune cells e.g. uterine leukocytes (macrophages, especially embryonic/fetal macrophages called Hofbauer cells, uterine NK cells (uNK) cells) invade the endometrium and dramatically increase in number thus representing at least 15 % of all cells in the decidua [3,4]. Adaptive immune system is controlled mainly by regulatory T cells (Tregs), which are a subgroup of suppressor CD4+ T cells. Their role is to secure immune tolerance, coordinate inflammation and support vascular adaptation [5]. Innate immune system is impacted by Th-1/ Th-2 cytokines balance. Differentiation of local immune cells in the beneficial or deleterious pattern is dependent on Th-1/Th-2 preeminence. In a Th-1 dominant environment, macrophages differentiate into deleterious M-1 macrophages, uNK cells into lymphokine-activated killer cells, dendritic cells into deleterious DC-1, and T cells into deleterious Th-17 cells. All these cells become able to target and kill the embryo. On the contrary, in a Th-2 dominant environment, macrophages differentiate in M-2 macrophages settling adhesion, uNK cells become angiogenic and immunotropic, dendritic cells differentiate in DC-2 providing an effective communication and T cells into Tregs to promote local tolerance [6].

NK cells can be found in the human spleen, lymph nodes, blood, lung, liver, gut and endometrium. Their function is determined by their specific location [5,7]. uNK cells do represent up to 70 % of decidual leukocytes. 90 % of uNK cells are represented by $CD56^{superbright}$ CD16- phenotype [8].

uNK cells, originally described as mononucleated granulated cells, were discovered by Paul Weill in human endometrial stroma and decidua a century ago [9,10]. Later, these immune cells were scrutinized again by Herwig Hamperl [11], one of the most prominent German pathologist of the 20th century, who called them K cells ("Körnchenzellen"), though the eponym „Hamperl cell" is also used [12]. The origin of uNK cells is not exactly clear. There are two hypotheses: an older one, which supposes their origin from peripheral NK cells and a new hypothesis which suggests that uNK cells are tissue resident [13]. Though they are supposed to have only minimal cytotoxicity, they can become cytotoxic mainly during the proliferative phase of the menstrual cycle, preventing microbial infection [5]. As the menstrual cycle proceeds, the cytotoxic function is getting weaker and their count is increasing. This process is probably activated by sex hormones mainly through the influence of progesterone [14]. Some authors suggest that if fertilization does not occur they will undergo apoptosis before the next cycle as the level of progesterone decreases [5]. During pregnancy, uNK cells are the main immune cell population at the maternal–fetal interface [15].

uNK cells orchestrate the overall immune response during implantation by influencing trophoblast invasion and vascular remodeling. They surround spiral arteries and produce angiogenic growth factors like vascular endothelial growth factor (VEGF) or stromal cell derived factor (SDF). These released factors can enhance or inhibit the invasion, highlighting the importance of uNK cells in supporting successful pregnancies [16,17]. Moreover, uNK cells take part in the recognition of fetal trophoblast by producing receptors which recognize embryonic HLA (human leukocyte antigen). Interestingly, around 20-30 % women with idiopathic recurrent miscarriage or RIF show, according to some studies, elevated uNK cell count [18] and/or elevated peripheral NK cell count [18,19].

The present study investigated a RIF cohort. The main aim was to immunohistochemically evaluate the count of uNK cells with possible clinical translation to personalized endometrial immune profiling.

Material and Methods

Patients and endometrial samples

Endometrial samples were collected from 256 patients with RIF, habitual abortion (HA) and idiopathic sterility. In our study were included patients with RIF, who were <37-years-old and failed to implant ≥2 blastocysts and who were ≥37-years-old and failed to implant ≥3 blastocysts. The patients with HA were defined as those with the history of 3 or more abortions after the pregnancy was confirmed clinically and biochemically. Patients with extrauterine pregnancy were excluded. In the case of fresh transfer, 5 days old blastocysts quality one and two were transferred. For frozen embryo transfer, 6 day old blastocysts quality one and two were used. Protocols for fresh transfer were medicated with estrogen and progesterone and frozen embryo transfer were either native cycles or medicated similarly as fresh transfers.

Patients had following main sterility factors: andrological (abnormal sperm count/abnormal progressive movement), ovarian (age factor/low AMH <1), polycystic ovaries, congenital uterus anomalies (e.g. *uterus bicornis*), genetic factors, myomas, endometriosis (including adenomyosis), central (e.g. hypogonadism) and immunological (immunological alteration diagnosed by reproductive immunologist through blood sampling e.g. elevated NK cells in the peripheral blood or elevated embryotoxic cytokines).

Samples were collected between day 19 and 21 of the menstrual cycle dominantly by the Pipelle endometrial sampling, followed by the formalin fixation for at least 24 hours.

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethical Committee of the ISCARE, Reproduction Clinic, Gynaecology & Urology in Bratislava, Slovakia, where the tissue samples were obtained. The informed consent was obtained from all patients.

Immunohistochemical staining

Tissue sections were examined after immunohistochemical staining. The 5 μm thick tissue sections were boiled in citrate buffer for antigen retrieval. Afterwards, primary anti-CD56 antibodies for uNK cell detection were applied and subsequently visualized with diaminobenzidine yielding a brown color using EnVision FLEX Visualization System and Autostainer plus (Agilent DAKO, USA).

Uterine cell counting and interpretation

The counting of positive cells was performed manually by an experienced histopathologist. Positive cells were counted in three different microscopic fields of 1 mm^2 at 200x magnification based on the following ranges:

- Low uNK cells ≤ 40 CD56 cells / 1 mm^2 200x
- Normal 41-299 CD56 cells/1 mm^2 200x
- Elevated uNK cells ≥300 CD56 cells / 1 mm^2 200x

Results

Our initial cohort included 256 patients aged 25 to 50, (the mean age 37,7 years). The mean time of trying to get pregnant was from 2015 until January 2022. We excluded 79 patients who did not fulfill our inclusion criteria - count of transferred embryos, count of abortions, no history of extrauterine pregnancy, no clear information about previously transferred embryo quality. We were monitoring several variables: age, sterility factors, time of treatment, data about transferred embryos, data regarding pregnancy outcomes, data regarding biopsies, endometrium histology, count of uNK cells, count of plasma cells to rule out inflammation, and activation status. Using a statistical analysis, we could find the following correlations:

Undertaking the one-way ANOVA test, age of our patients did not have an influence on the count of uNK cells. With the Spearman correlation analysis, we found statistically significant correlation (p-value 0.05) of -0.133 between prior miscarriage and lower uNK cell count. Using the same analysis we found statistically significant correlation (correlation 0.233 with p-value 0.01) between number of uNK cells and activation status. Patients with higher uNK cells were found to have endometriosis more frequently (p-value 0.05, correlation 0.130). Patients with an immunological factor of sterility (defined by a clinical immunologist) had a lower chance of gravidity (-0.203 with p-value 0.01).

Discussion

The results of the present study indicate that the endometrial and decidual microenvironment of immune cells is an important factor determining fecundity and fertility success and the risk of developing fertility-related conditions like RIF, HA or idiopathic sterility. uNK cells as key players in this regard, were found to be low in

number in patients with the abovementioned conditions, indicating that uNK cells have an essential role in maintaining normal embryo-friendly uterine microenvironment. However, by putting the present results in context, many issues arise that have to be addressed before the routine examination of uNK cells is implemented into clinical practice. Most importantly, its clinical value and predictive power in regard to pregnancy outcome have to be substantiated.

The principal issue is that studies on uNK count and its role in RIF, HA and other fertility-associated conditions report conflicting results. They can be broadly divided into three main categories based on the main findings. Research teams report that detrimental effects are caused by either low, or high uNK count. The third category includes those studies that haven´t found any association between uNK count and fertility problems whatsoever. Starting with the latter category, Donoghue *et al.* [20] performed an immunohistochemical examination of uNK cells in patients with RIF and found no significant difference in uNK count compared to women with implantation success. The authors also highlighted a problem that the methods of measuring uNK cell count are inconsistent, and thus hard to reproduce. Similar results were reported by Michimata *et al.* [21] who conducted the immunohistochemical profiling of multiple leukocyte populations in the endometrium including CD56+ and CD16+ uNK cells. There was no difference between the quantity of uNK cells, and other studied leukocytes compared to controls. Therefore, they concluded that pregnancy outcomes cannot be established based on immunophenotype and quantitative parameters of uNK cells or any of the studied immune cells for that matter. Bohlmann *et al.* [22] also used immunohistochemistry combined with RNase protection assays. Same as the previous research group, CD56+ uNK cells were not the only immune cell population examined. The bottom line was that patients with the history of two or more idiopathic abortions had similar immune make-up including uNK cells as healthy control subjects. The category of high count of uNK cells and its relation to fertility issues includes a work by El-Azzamy *et al.* [23] who performed an immunohistochemical investigation of CD56+ CD16+ uNK cells in mid-secretory phase endometrium of patients with recurrent pregnancy losses. They found a significantly higher count of uNK cells and correlated it with faulty vascular transformation - a process integral for proper decidualization of the endometrium. Zhao *et al.* [24] chose an innovative methodology which implemented the evaluation of immune cell clustering, not merely the assessment of uNK cell density. Compared to fertile controls, immuohistochemically detected CD56+ uNK cells were significantly increased in density. Moreover, they were found to be clustered with CD68+ macrophages. These findings indicate that the interaction of uNK cells with other immune cell populations should also be investigated as their cross-talk seems to be another significant variable that can influence fertility outcomes. On the other hand, while the higher count of uNK cells can be significantly increased in patients with the history of recurrent miscarriage compared to controls, it might have little predictive value regarding the subsequent pregnancy outcome. Such conclusion was published by Tuckerman *et al.* [25] who performed a retrospective study of endometrial biopies from 87 women with unexplained recurrent miscarriage. Despite the higher uNK cell count, from the 51 who got pregnant following the biopsy, 19 misscarried and 32 delivered a live newborn. Thus no prediction would has been successful based merely on the previously detected higher uNK cell density. Finally yet importantly, the third category of studies correlating fertility-related condtitions with low uNK cell count can be represented by the results of the present study. Similarily, Babayeva *et al.*´s [26] immunohistochemical study also investigated samples of patient with the history of RIF and found statistically significant decrease in uNK cell count in the RIF study group.

All in all, the above-discussed studies clearly show that simply by measuring the density of uNK cells in a biopsy sample of patients with the history of fertility-associated problems whether it is performed using immunohistochemistry, or flow cytometry, no definitive predictions can be made. Therefore, it is highly warranted to combine the quantitative assessment of uNK cells with their functional evaluation, e.g. the examination of cytokine profiles. This approach was adopted by Fu *et al.* [27] who combined immunohistochemical density evaluation of uNK cells with cytokine secretion profiling. From the quantitative perspective, the authors observed a low count of uNK cells in decidual samples of patients with recurrent spontaneous abortion. In addition to their decreased density, uNK cells were also abnormally distributed. In healthy controls, uNK cells had uniform distribution, while in the study group, uNK cells were clustered in concentrated areas of the decidua. The cytokine profile was also abnormal contributing to the pathological immune microenvironment. Th_{17} cells were recognized as the main antagonist in this scenario. The

authors found that uNK-mediated Th_{17} supression is the key factor in promoting immune tolerance during pregnancy, since the proinflammatory Th_{17} cells are known to be highly deleterious in relation to normal microenvironmental maintenance in the decidua. Therefore, in case of the numerically diminished and/or malfunctioning uNK cells, Th_{17} cell action can become dysregulated, enabling Th_{17} cells to "wreck havoc" in the decidua leading to pregnancy loss. Wang *et al.* [28] also presented a function-focused approach to uNK cell examination. They studied the expression of the killer cell immunoglobulin-like receptor (KIR) responsible for the uNK killing activity in patients with recurrent miscarriage. The principal finding was that the decrease in KIR expression is involved in the pathogenesis of recurrent miscarriage. The authors also suggested that KIR evaluation might be implemented as a diagnostic approach.

Another pitfall of studies on uNK role in fertility-associated conditions is that patient cohorts often lack strict definition. According to ESHRE, patients with RIF are defined as those who are younger than 37-years-old and fail to implant 2 good-quality embryos and those who are older than 37-years-old and fail to implant 3 good-quality embryos. Patients with HA are defined as those who had 2 and more abortions during the first 12 weeks of pregnancy [29]. However the exact definition of RIF or HA is controversial. The above used ESHRE definition for RIF is the most commonly applied. However, some authors use another definition which classifies RIF as two previously failed transfers [30], while others define RIF as at least four previously failed embryos transfers [31]. Moreover, the exact definition of a "good-quality embryo" is also subjective and depends on intraobserver, interindividual and intercenter differences. Therefore, the standardization of objective embryo quality assessment is of utmost importance [32]. Without such protocols, it is very difficult to distinguish whether the embryo failed to implant because of its inferior quality despite being evaluated as "good-quality" or due to other reasons (e.g. abnormalities of uNK cells). A problem with interpretation may also arise when therapeutic interventions for RIF are being implemented soon after two failed transfers, what could reasonably be just a statistical misfortune. It is supposed that a proportion of these possibly false diagnoses could be around 46 % [33].

Conclusions

The principal issues with the uNK evaluation in patients with RIF, HA and other fertility-associated conditions that need to be addressed in future research can be summarized as follows:

- Lacking standardized quantification protocols for uNK cell counting
- Inconsistent correlations between uNK cell count and fertility outcomes
- Inconsistent methodology of uNK cell detection (immunohistochemistry vs. flow cytometry)
- Inconsistent definition of RIF and HA
- Predominance of quantitative (focusing on uNK cell count) over qualitative and functional studies on uNK cells (pro-inflammatory or anti-inflammatory action, KIR expression etc.)
- Lacking standardized protocols for objective good-quality embryo evaluation

Conflict of Interest

There is no conflict of interest.

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