When Less Is More – Pipelle Endometrial Sampling for Quantification of Uterine Natural Killer Cells in Patients With Recurrent Implantation Failure or Habitual Abortion

Lenka LAPIDES[1,2], Ivan VARGA[1], Martin KLEIN[1], Lenka RYBÁNSKA[2], Viera BELUŠÁKOVÁ[2], Pavel BABÁL[3]

[1]Institute of Histology and Embryology, Faculty of Medicine, Comenius University in Bratislava, Bratislava, Slovakia, [2]ISCARE, Reproduction Clinic, Gynaecology & Urology, Bratislava, Slovakia, [3]Institute of Pathology, Faculty of Medicine, Comenius University in Bratislava, Bratislava, Slovakia

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Summary

Despite recent advancements in reproductive medicine, recurrent implantation failure and habitual abortion remain ongoing issues. One of the most important aspects of successful implantation is the intricate immune response and regulation necessary for the acceptance of the hemiallogenic embryo. The most numerous immune cells in the decidua are uterine natural killer cells (uNK). Studies suggest that changes in the uNK count and physiology may be responsible for the aforementioned pathological conditions. Thus, testing for uNK may provide valuable insights into their pathogenesis. The study compared Pipelle endometrial sampling with conventional curettage to find out whether the less invasive Pipelle method is a viable alternative of tissue collection. Tissue samples from 14 patients obtained by both methods were examined. The average size of tissue samples obtained with Pipelle was 17 mm^2, samples obtained with curettage had on average 34 mm^2. Using immunohistochemical visualization of CD56 (NK cells) and granzyme B antigens (serine protease-expressing activation state of NK cells), it was found that the average total count of CD56 / mm^2 was 115 for Pipelle and 120 for curettage, respectively. The study also proved a correlation between granzyme B positivity and identification of NK cells clusters. The results indicated that Pipelle endometrial sampling seems a suitable method of tissue harvesting for the purpose of uNK cells examination. Pipelle endometrial sampling is safe, cost-effective and can be performed on an outpatient basis without the need of anesthesia or analgesia. Several issues remain yet to be solved: how to standardize the subsequent uNK testing, how to interpret the results and finally yet importantly, how to use this knowledge in personalized treatment protocols.

Key words

Endometrium • Uterine NK cells • Recurrent implantation failure • Habitual abortion • Pipelle endometrial sampling • Curettage

Corresponding author

Ivan Varga, Institute of Histology and Embryology, Faculty of Medicine, Comenius University in Bratislava, Špitálska 24, 813 72 Slovakia. E-mail: ivan.varga@fmed.uniba.sk

Introduction

Infertility is defined as the inability to achieve clinical pregnancy following at least 12 months of regular unprotected sexual intercourse. Approximately 48 million couples and 186 million individuals are affected with infertility globally [1]. Reproductive medicine is one of the most progressive medical fields. However, recently there has been a slight stagnation in treatment modalities in particular problems like recurrent implantation failure (RIF) or recurrent abortion caused by an endometrial factor [2]. Moreover, RIF is still an imprecisely defined and multifactorial disorder lacking a robust scientific basis [3]. More research of these particular diagnoses is needed especially in the context of reproductive immunology, while at the same time, it is necessary to start deliberating over innovative immunomodulatory and immune-suppressive therapies [4].

The thorough understanding of immune mechanisms during implantation is essential. The intimate association between maternal and placental tissues creates

an interesting immunological paradox. Placental tissue contains paternal antigens, but under normal circumstances, the hemiallogeneic embryonic tissue and future placenta are not attacked by the maternal immune system. During decidualization, uterine leukocytes dramatically increase in number and account for at least 15 % of all cells in the decidua from early pregnancy until parturition. Moreover, their differential is unusually distinct from that found in the blood, since the vast majority (70 %) is represented by NK cells. Monocytes (15 %), and T- lymphocytes (10 %–15 %) comprise only the minority of immune cells [5,6]. Each immune cell type has an indisputable role in accepting the hemiallogenic embryo. There are cells important for the induction of immunological tolerance like dendritic cells, macrophages and especially embryonic/fetal macrophages called Hofbauer cells [7,8]. On the other side, telocytes (recently described interstitial cells) enhance decidualization [9,10].

NK cells are one of the most important cells of the human immune system. There are two different subgroups of NK cells: NK cells in the peripheral blood and NK cells in the endometrium, referred to as uNK cells. The main function of NK cells in the peripheral blood is to secure cytotoxic immunity protecting the human body from infection and uncontrolled spread of malignant cells. uNK cells may have completely opposite effect on ongoing pregnancy.

uNK cells, originally described as mononucleated granulated cells, were discovered by Paul Weill in human endometrial stroma and decidua exactly one century ago in 1922 [11,12]. Later, these immune cells were scrutinized again by Herwig Hamperl [13], one of the most prominent representatives of German pathology of the 20th Century, who called them K cells (Körnchenzellen), though the eponym „Hamperl cell" was also used [14]. uNK cells are the main immune cells at the maternal–fetal interface, because uNK cells have the potential to orchestrate the overall immune response and, either directly or indirectly, influence trophoblast invasion and vascular remodeling. These functions highlight the importance of uNK cells in supporting successful pregnancies [15,16]. Women with idiopathic recurrent miscarriage show, according to some studies, elevated uNK cell count [17] and/or elevated peripheral NK cell count [18].

Surprisingly, the evaluation of uNK cells is not recommended in ESHRE Guidelines presently [19], though it needs to be mentioned that the latest study on uNK cells cited in the Guidelines is from 2013. Nine years years, the discussion is still ongoing, what implies that more conclusive research is necessary in order to reconsider the current recommendations, and thus implement the uNK cell testing in the future revised version of the Guidelines. Currently, there are three main sampling methods available for the purpose of subsequent examination of the endometrial "immune microenvironment". The most invasive of the three is endometrial curettage which may require general anesthesia [20,21]. The less invasive is uterine lavage coupled with flow cytometry to examine the composition of the endometrial immune microenvironment [22]. However, the downside of this method is the absence of information on the endometrial cytoarchitecture of the uNK cells. The third method is Pipelle endometrial sampling, which can be performed on an outpatient basis without the necessity of general anesthesia. However, this method is rarely used mainly due to concerns that the harvested tissue sample is too small and thus not sufficiently representative for efficient examination by a histopathologist.

This study was designed to compare the efficiency of Pipelle endometrial sampling vs. conventional dilation and curettage as the method of choice of tissue harvesting for the purpose of further uNK cells examination in patients with RIF or habitual abortion (HA). We chose immunohistochemistry as the method of uNK cells examination. It was hypothesized that the Pipelle endometrial sampling is a suitable alternative to diagnostic curettage as a method of harvesting a representative sample for subsequent histopathological examination of the number and location of endometrial uNK cells in a selected group of infertile women.

Material and Methods

Patients and endometrial samples

Endometrial samples were collected from 14 patients with RIF or HA. Patients with RIF were, according to the ESHRE definition, defined as follows – a failure to implant 2 good quality embryos by patients younger than 37-years-old and a failure to implant 3 good quality embryos by patients older than 37-years-old. Patients with HA were defined according to the ESHRE definition as patients who had 2 and more abortions during the first 12 weeks of pregnancy [19]. Samples were collected between Day 19 and 21 of the menstrual cycle by Pipelle endometrial sampling and subsequently by endometrial curettage. The specimens were processed by the routine formalin-fixed paraffin embedded technique,

5-µm thick histological sections were stained with hematoxylin and eosin for the purpose of histopathological assessment of the endometrium. The measurement of the size of the specimens was performed morphometrically by two-dimensional image analysis using ImageJ 1.38 freeware (National Institute of Health, San Diego, CA, USA).

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. 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, we used antibodies against CD56 (for NK cells) visualized with immunoperoxidase staining technique using dark-brown product and granzyme B antibodies (serine protease-expressing activation state of NK cells) visualized with alkaline phosphatase using red product. We used plasma cell CD 138 staining to rule out chronic endometritis. All chemicals were purchased from Agilent technologies, USA and processed using EnVision FLEX Visualization System and Autostainer plus (DAKO, Glostrup, Denmark).

Uterine cell counting and interpretation

Positive cells were counted manually by an experienced histopathologist. The same histopathologist counted uNK in tissue samples obtained by curettage and Pipelle endometrial sampling. Positive cells were counted in three different microscopic fields of 1 mm^2 at 200x magnification. The results were expressed as the average number of uNK cells/mm^2. We used reference ranges expressed in Table 1 [23].

Table 1. Reference ranges o uNK cells according to [23]

Assessment	Reference range CD56 cells / mm^2 (200x magnification)
normal	40 – 299
slightly elevated	300 – 599
elevated	> 600

Results

We tested the representativeness of the non-invasive Pipelle endometrial sampling. From 14 patients we obtained a histological sample of the endometrium by curettage as well as by aspiration with Pipelle. Histologically, cells had late proliferative and early secretory phase changes. There was no difference in the assessment of the menstrual cycle between the two methods. The average size of tissue samples obtained with Pipelle was 17.08 mm^2. Samples obtained with curettage had on average 34.27 mm^2. In the slides stained with HE, we identified diffusely dispersed small lymphocytes in the stroma, sometimes forming small clusters, occasionally in the proximity of endometrial gland (Fig. 1).

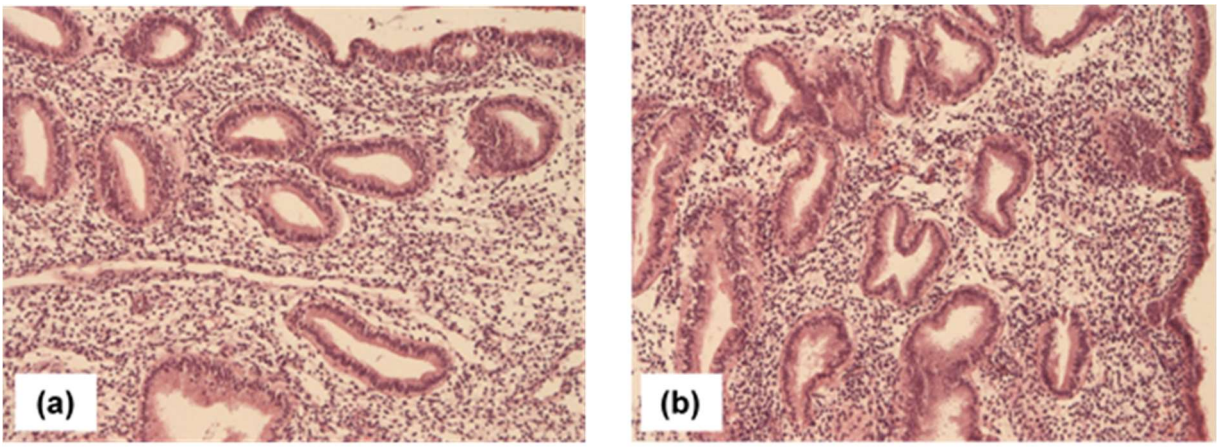

Fig 1. Histological pictures of the endometrium obtained by Pipelle sampling (**a**) and curettage of the uterine cavity (**b**) show identical results. HE, 100x.

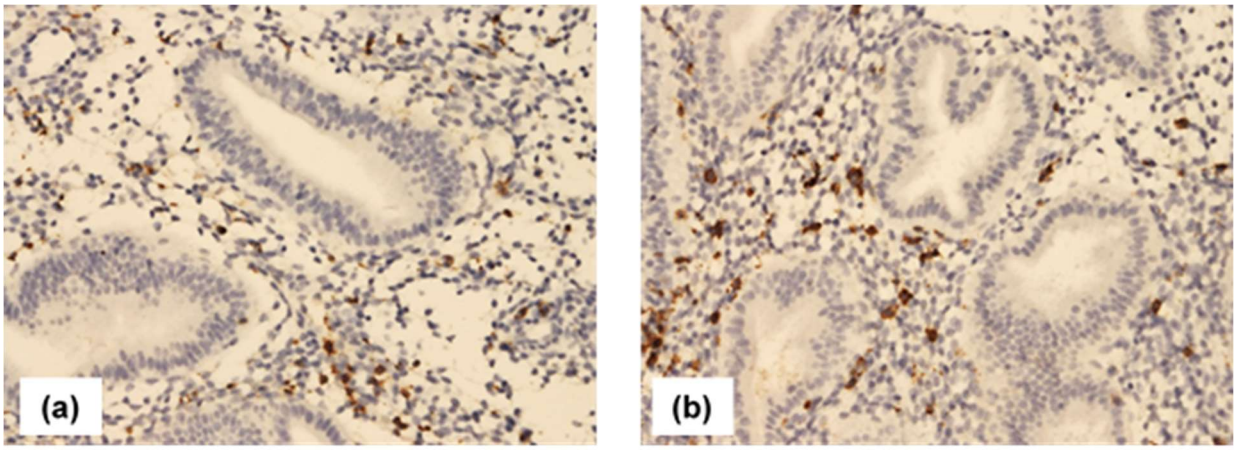

Fig 2. Immunohistochemical detection of uNK cells (brown color) in the endometrium obtained by Pipelle sampling (**a**) and curettage of the uterine cavity (**b**) shows comparable results. Immunoperoxidase technique, CD56, 200x.

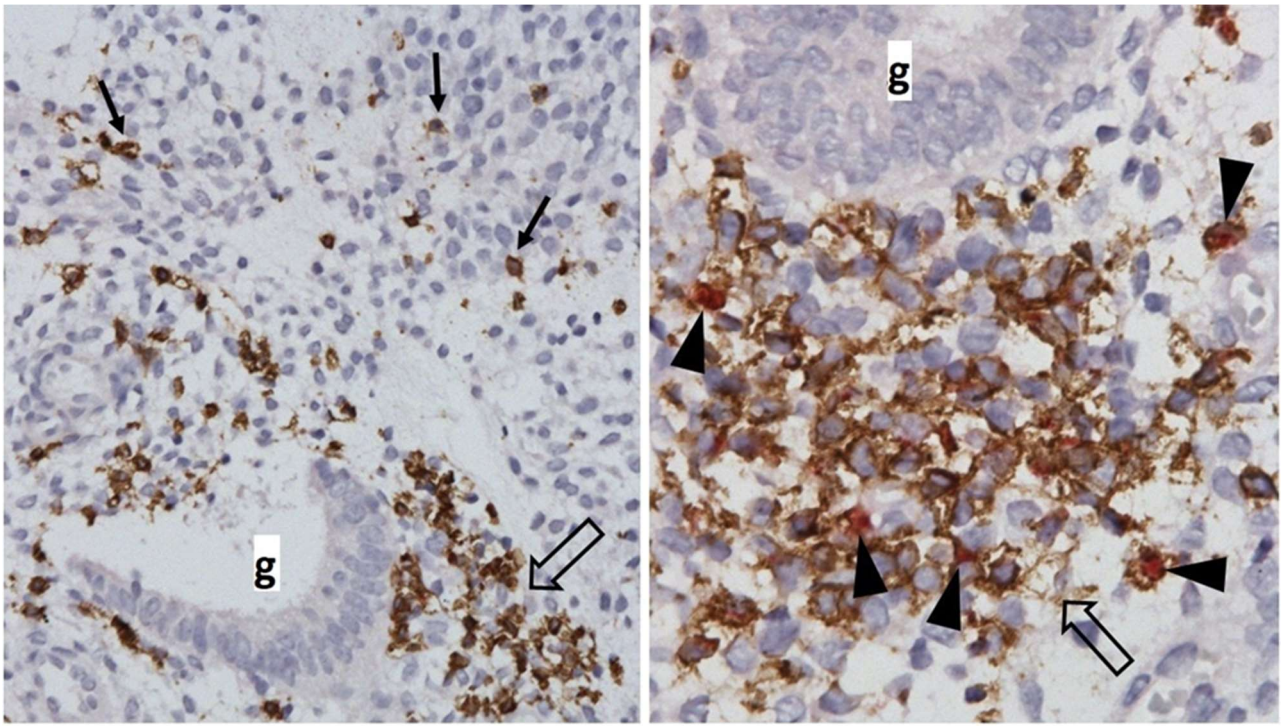

Fig 3. Representative photomicrograph of visualized uNK cells in tissue obtained by Pipelle endometrial sampling. Thin arrows (→) point to sparse infiltration of uNK cells and large hollow arrows (⇨) show dense accumulation of uNK cells. Black arrowheads (►) in (b) point to red coloured cells stained with granzyme B. "g" represents endometrial glands. 200x magnification (**a**), 400x magnification (**b**).

Immunohistochemical staining identified most of these lymphocytes as CD56-positive uNK with brown membrane staining (Figs 2 and 3).

There were differences in the counting results of CD56-positive cells in specimens obtained by the two different methods. However, there were no discrepancies in the categorization of the cases based on the number of uNK into the three classification groups (Table 1). We proved, that endometrium samples taken with Pipelle provide enough sufficient representative material – 50 % of curettage sample (Table 2). The average total count of / mm^2 was for Pipelle 115 and 120 for curettage. We identified one patient with low uNK count using both methods, and one patient with marginally decreased uNK. In this cohort of patients there were no patients with higher uNK count. 12 patients showed normal uNK count using

Table 2. Histological samples from 14 patients with the history of RIF or HA obtained by curettage as well as aspiration with Pipelle. Table shows size correlation of samples and compares CD56 count between both methods.

	Pipelle				Curettage				% Pipelle/ Curettage	
No	**size (mm^2)**	**CD56 / mm^2**	**% granzyme B**	**clusters**	**size (mm^2)**	**CD56 / mm^2**	**% granzyme B**	**clusters**	**CD56**	**size**
1	4.1	57.3	60	(+)	14.1	81.0	70	+	66.3	33.3
2	3	91.3	60	+	6.1	82.0	60	+	111.3	49.2
3	2.1	58.6	30	-	27.0	53.7	20	-	109.1	8.5
4	15.1	105.0	30	+	20.1	121.7	30	+	86.3	76.6
5	13.1	59.3	30	-	12.2	42.0	40	-	141.2	112.7
6	26.1	95.7	50	(+)	32.1	116.0	20	+	82.5	83.8
7	12.1	292.0	20	-	26.7	195.3	30	+	149.5	46.4
8	11.1	10.0	20	-	22.8	24.0	20	(+)	41.7	50.4
9	2.1	42.0	50	-	7.6	40.7	50	-	103.2	31.6
10	17.7	195	0	-	72.6	205	0	-	95	24
11	37.6	40	50	-	66.3	45	50	+	89	57
12	45.7	110	80	-	94.3	65	60	-	169	48
13	9.8	135	80	-	63.6	180	80	-	75	15
14	39.6	325	15	-	14.3	435	30	+	75	277

both methods. CD56 count was in 6 cases nearly identical (86-111 %), other 8 cases showed some differences, but the results belonged to the same group (normal or low), so the diagnostic or therapeutic consequences would have been the same. Furthermore, we proved a correlation between granzyme B positivity and identification of NK cells clusters.

Discussion

This study confirms that Pipelle endometrial sampling is, compared to conventional dilation and curettage, an equally representative method of endometrial sampling for subsequent examination of uNK count, as well as other endometrial populations of immune cells. Though unlike curettage, this non-invasive method has multiple advantages – it can be performed on an outpatient basis without the necessity of anesthesia or analgesia.

For a successful implantation to occur, the endometrium must undergo decidualization during the implantation window. Decidualization is best defined as a sequence of changes in the endometrium involving vascular remodeling, morphological alteration, immunological modulation and other processes mainly within the endometrial stroma necessary for an embryo to thrive. It is a reaction to the implanting blastocyst, but can start even before it [24]. To achieve a successful implantation of the embryo, a transient immune switch is necessary to achieve local tolerance. uNK of the endometrial stromal compartment are crucial in this regard, but are also important in other aspects of successful embryo implantation and further embryo development. uNK influence vascular remodeling by inducing the secretion of angiogenic factors affecting the pre-existing spiral arteries. They also promote placental growth through production of angiogenic immunotropic cytokines [25]. uNK are also involved in trophoblast invasion. Interestingly, they also produce different growth factors, indicating their role in the growth and development of the embryo [26]. Moreover, as reviewed by Sojka *et al.* [27], specific subsets of uNK may "remember" previous pregnancies, so in next pregnancy they are primed to effectively perform their implantation-related tasks and other roles in the maintenance of a successful gestation. It is known that primigravidae are at higher risk of pregnancy-associated complications, including miscarriage, so perhaps uNK can provide a clue for better understanding of this phenomenon.

Testing of uNK is most commonly performed using immunohistochemical visualization and subsequent manual counting by a histopathologist. Here it is necessary to stress one of the most prominent potential drawbacks, which is the subjectivity of such counting [16]. Other issues may arise, for instance a lack of standardization of the area selection for assessment, not to mention potential differences in tissue processing protocols. There is also a problem with the lack of consensus in regard to cut-off values and reference ranges. The main areas which have to be standardized in order to provide reproducible results are: tissue collection, tissue fixation and processing and quantification [28]. uNK can be also measured using flow cytometry. This technique is advantageous, because it can determine uNK in different stages of development [29]. However, as mentioned earlier, flow cytometry provides no information on uNK in the context of their morphological relations to surrounding tissue components. In order to establish the uNK testing as a reliable method, several issues have to be addressed in further research.

Apart from the already mentioned standardization problems with the given approach, it is also necessary to justify and standardize the implementation of diverse biopsy (curettage, lavage, Pipelle) and examination methods (immunohistochemistry, flow cytometry). Next, the exact period of the menstrual cycle during which the samples should be taken has to be established. Considering that uNK are not the only immune cells present during implantation, one needs to define the exact interactions among endometrial immune cells. In this context it is necessary to mention epithelial and stromal cells producing various cytokines, which regulate uNK physiology. Worth mentioning is the Th1/Th2 model of T helper cells differentiation. For a successful implantation and further embryo development to occur, the local immunity has to be switched from Th1 to Th2 phenotype in a timely and orderly manner. This Th phenotype-switching directly influences uNK. If Th1 differentiation persists and the Th2 differentiation fails to ensure, uNK acquire cytotoxic activity similar to that of the peripheral NK and start to target cells of the trophoblast, recognizing them as foreign [25]. Moreover, there are other subsets of T cells which intricately interact during pregnancy including Th9, Th17, Th22 and Tfh cells. Therefore, a complex immune profiling is necessary in order to prevent RIF and other related conditions [30].

Discussing the comparison between Pipelle endometrial sampling and conventional curettage in different diagnoses, we chose only a few of those papers published lately, since we have almost 30 years' worth of studies on the topic at our disposal. Abdelazim *et al.* [31] compared the diagnostic accuracy of these two methods in 140 patients with abnormal uterine bleeding. The authors concluded that Pipelle endometrial sampling provides many benefits including safety, cost-effectiveness, avoidance of general anesthesia, and finally yet importantly, it is a highly specific and sensitive outpatient procedure for the diagnosis of hyperplasia and/or neoplastic processes of the endometrium. Similar findings were published by Sanam *et al.* [32], who likewise compared these two methods in patients with abnormal uterine bleeding. The numerous advantages of Pipelle endometrial sampling over the conventional curettage, namely in the terms of sampling adequacy, low failure rate, duration and cost, provided a cumulative case for its introduction as a feasible alternative. To avoid any biases, Piriyev *et al.* [33] designed a double-blind study, which resulted in almost identical findings emphasizing the advantages of the Pipelle method. Gungorduk *et al.* [34] evaluated the accuracy of the two approaches in different endometrial conditions. Even though the sensitivity for detecting hyperplasia and atypia was similar between the two, conventional curettage was significantly more sensitive in the evaluation of endometrial atrophy. On the other hand, a recently published paper by Hwang *et al.* [35] concluded that dilation and curettage are superior in reflecting the actual diagnosis of patients with endometrial hyperplasia when compared to specimens from hysterectomies. Nevertheless, the bottom line is that Pipelle endometrial sampling has more advantages than drawbacks and should be considered an adequate alternative to the traditional approach.

Conclusions and further directions for research

Patients with RIF or HA need recommendations depending on their problem. Probably the best answer is personalized medicine with standardized testing and therapy guidelines. In this study, we confirmed that Pipelle endometrial sampling is an equally sensitive alternative to dilation and curettage as a method of tissue harvesting for the purpose of uNK testing. Unlike curettage however, it has multiple advantages – it can be performed on an outpatient basis without the need of anesthesia or analgesia. The issues which need to be addressed in further research can be summarized as follows:

- How can we improve and standardize the uNK

cell testing?

- How to approach patients with low uNK cells and elevated uNK cells?
- Which patients tend to be therapy resistant?
- How to personalize the treatment, length of the treatment, the dosage for patients with elevated uNK cells? Which are the alternatives to corticosteroids and intralipid infusions?
- How to focus on patients with low uNK cells? How to confirm the effect of endometrial scratching before embryo transfer as well the importance of sexual intercourse after performing transfer-activating uNK cell activity?

To fully understand the uNK physiology and to apply the knowledge necessary for the development of treatment strategies for infertile couples, a close cooperation between histology, immunology, reproductive medicine and many other fields is inevitable.

Conflict of Interest

There is no conflict of interest.

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