

Peripheral Blood Natural Killer Cell Assay in Infertile Women

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Abstract: We have developed a simple assay for the detection of peripheral blood natural killer cells (NK) metabolic activity based on tetrazolium reduction reaction after the cultivation of isolated NK cells with sperm cells. We have adapted a reliable, inexpensive and easy to prepare method in conjunction with the EZ4U system, while target NK cells' isolation was solved using Dynabeads® immunomagnetic technology. The intended use of the introduced assay is the detection of pathological NK cells activity in immunological female infertility. The results of our pilot study showed differences in the metabolic activity of peripheral blood NK cells between fertile and infertile women. Additional analyses are necessary to determine the sensitivity and specificity of the introduced test in the immunological diagnostics of infertility.

Introduction

Natural killer (NK) cells, identified by the surface marker CD56, are a very important type of cell in the human body. They form a subtype of lymphocytes that play an important role in the immune system's host response in destroying or lysing infectious and malignant cells. They receive their name from the fact that they are produced naturally by the body, and their sole purpose is to search for and destroy harmful cells.

In some women, NK cells can overreact to a potential pregnancy resulting in infertility [1, 2, 3]. Specifically, NK cells could view the sperm or embryo as a harmful agent and decide to take action. The normal amount of CD56+ NK cells in the blood circulation ranges from around 5–12% to levels of 18% or higher and are associated with poor reproductive outcomes [4, 5]. However, critics point out that the NK cells of the uterine mucosa differ from the NK cells present in the blood and that the activity of the NK cells is more important than the amount [6, 7].

NK cells express inhibitory and stimulatory receptors. Tolerance is acquired by NK cells during their development. Normal target cells (including sperms) are protected from being detected by NK cells when signals delivered by stimulatory ligands are balanced by inhibitory signals delivered by MHC class I molecules. In “autoimmune configuration” of the maternal immune system, the expression of stimulatory ligands is augmented; as a result, stimulatory signals overcome the inhibitory signal, resulting in target cell attack (induced self-recognition). Therefore, the balance between inhibitory and stimulatory signals is important for sperm tolerance [8].

We have developed a simple assay for the detection of peripheral blood NK cells metabolic activity based on tetrazolium reduction reaction after the cultivation of isolated NK cells with sperm cells.

Our method allows for the simple and non-radioactive determination of substance-induced NK cell activation. This method is certainly more concerned screening than measuring serum or plasma cytokines or looking at intracellular NK cells cytokines. Clearly, much additional research is required to translate the value of this test in guiding treatment strategies.

Material and Methods

Twenty women with primary idiopathic infertility and forty-two healthy women of childbearing age with the median age 34.5, ranging from 29 to 38 years, were enrolled the study. Before sampling, all subjects provided written Informed Consent and the study was approved by the institutional Ethics Committee.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised human blood by density-gradient centrifugation (Ficoll *Histoprep*, BAG, Germany). After washing and counting in the *Bürker chamber*, the concentration of isolated PBMC was adjusted to 10^6 cells per one ml by adding RPMI-1640 medium (P-Lab, Czech Republic).

The second isolation step consisted of an isolation of untouched human NK cells from PBMC suspension by depleting T cells, B cells, monocytes, dendritic and other cells so that isolated NK cells will be bead- and antibody-free and suitable for subsequent application. For this purpose, the NK Cell Negative Isolation Kit (DynaL Biotech, ASA, Norway) was used.

The Dynal NK Cell Negative Isolation Kit contains Depletion Dynabeads and an Antibody Mix. Depletion Dynabeads are supplied in phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide. These are uniform, superparamagnetic polystyrene beads (4.5 μm diameter) coated with a monoclonal human anti-mouse IgG4 antibody. The Antibody Mix contains mouse IgG antibodies for CD3, CD14, CD36, CDw123, HLA class II DR/DP and CD235a (Glycophorin A) and is supplied in PBS and 0.02% sodium azide (NaN_3). The principle of isolation is the following: after adding a mixture of monoclonal antibodies (Antibody Mix) against the non-NK cells to the starting sample of PBMC suspension, depletion Dynabeads are added to bind to the unwanted non-NK cells during a short incubation. Consequently, bead-bound cells are removed by a magnet (DynaL MPC-15, Dynal Biotech, ASA, Norway) and discarded. As a result, untouched NK cells remained in the tube for subsequent applications. Finally, the concentration of obtained NK cells was adjusted to 10^6 cells per one ml after the counting in the *Bürker chamber*. Yield and purity of obtained suspension were checked by a flow cytometry and were about 80%

Table 1 – Volumes for NK cells isolation

Chemicals Volumes	
Heparinised Human Blood	3 ml
Ficoll Histoprep (BAG, Germany)	4–5 ml
RPMI-1640 medium (P-Lab, Czech Republic)	5 ml
Antibody Mix (DynaL Biotech, ASA, Norway)	20 μl
Depletion Dynabeads (DynaL Biotech, ASA, Norway)	100 μl
Bovine Serum Albumin (BSA)	25 μl
Phosphate Buffered Saline (PBS)	3.5 ml

of NK cells in resulting suspension. These impurities consist mainly of non-separated beads with no interference with the assay.

Volumes for NK cells isolation are shown in Table 1.

Semen samples from healthy fertile normospermic donors were used. Specimens were produced by masturbation into a plastic container and prepared within one hour after the ejaculation. Pooled semen specimens were counted according to the procedure indicated in the WHO laboratory manual [9], washed twice and diluted with RPMI-1640 medium to the final concentration of 10^6 sperm cells per one ml. Frozen/dead sperm cells ($-80\text{ }^\circ\text{C}$) were used to avoid interference with metabolic activity of sperm cells with NK cell activity. To confirm non-viability of frozen sperms, propidium iodide cell viability flow-cytometric assay was used.

NK cells cultivation was performed in 96-well flat-bottomed microtitre plates. $10\text{ }\mu\text{l}$ of NK cells suspension and $60\text{ }\mu\text{l}$ of sperm cells suspension were seeded and grown in each well for twenty-four hours in RPMI-1640 in a total volume of $200\text{ }\mu\text{l}$ at $37\text{ }^\circ\text{C}$ and 5% CO_2 . This ratio was obtained with repeated advances – see Figure 1. As a negative control, NK cells without sperms were added to the well.

The metabolic activity of NK cells was measured by means of a tetrazolium reduction assay (EZ4U, Biomedica GmbH, Austria). The method described by Klöcking et al. [10] is based on the finding that living cells are capable of reducing slight or uncoloured tetrazolium salts into intensely coloured formazan derivatives as shown in Figure 2.

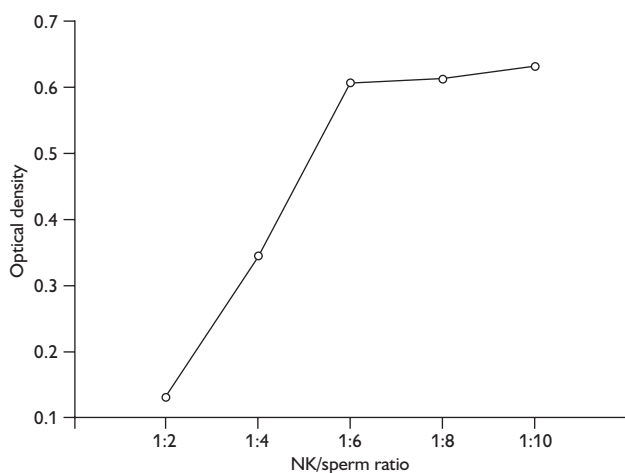


Figure 1 – Optical density of various proportions between sperms and NK cells.

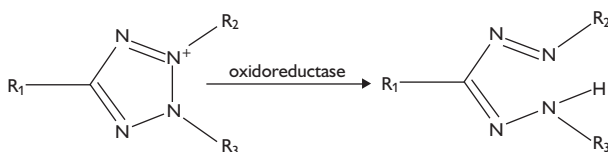


Figure 2 – Chemical formula of tetrazolium reduction.

The tetrazolium compound is reduced by as yet not completely understood reactions in the mitochondria, yielding a coloured, soluble formazan dye. The water-soluble tetrazolium compound penetrates the cell membrane by an unknown mechanism. An intracellular reduction system, most likely located in the mitochondria, converts the yellow tetrazolium salt into an intensely coloured formazan. This water soluble formazan is secreted into the culture medium and can be measured.

After twenty-four hours incubation time, 20 µl of EZ4U reagent was added to each well, and the plates were incubated for another four hours. Optical densities (OD) were measured at 450 nm in a Dynex MRXII Revelation double-beam spectrophotometer. Each experiment was performed in duplicate. The stimulation index was counted for each sample as a rate of OD of the stimulated sample to the OD of unstimulated control. The stimulation index is in direct proportion to the metabolic activity of investigated NK cells after sperm cells stimulation.

Results

The results of NK cells metabolic activity are summarized in Figure 3.

Discussion

Maternal immunological mechanisms have been postulated to explain idiopathic infertility [11, 12, 13]. A successful pregnancy depends on protection or down-regulation of potentially harmful maternal immune responses in the uterus. Although fecundation and implantation are typically localized processes, it is interesting to evaluate whether any systemic immunological changes can have an

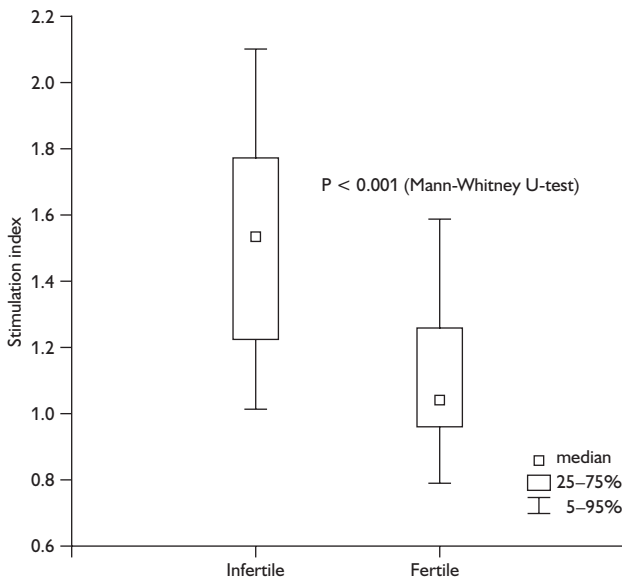


Figure 3 – NK cell's metabolic activity representation via stimulation indexes.

effect on these processes. In addition, it is difficult to examine the local environment at the implantation site.

Recently, the discovery of lymphocyte and cytokine functions opened a new approach to the diagnostics of infertility [14, 15, 16]. The basic principle of these new diagnostics methods is the *in vitro* monitoring of the cell activation in target cell lysis assay. The classical cytotoxicity assay is based on the release of radioactive chromium [17, 18]. In addition to its radioactivity, this method has some disadvantages, one of which is its high cost. Attempts have been made to measure cell activity by various colorimetric, fluorescent and enzymatic assays [19, 20, 21]. In our work, we adapted a reliable, inexpensive and easy to prepare method in conjunction with the EZ4U system.

The first step, target cells isolation, was solved using Dynabeads® technology. This gentle method is critical in obtaining high yields of viable and functional cells. With this tube-based magnetic separation method, cells are not exposed to the stress of going through a column.

The second detection step of our assay is based on tetrazolium salts reduction. Due to soluble end products, this test is easy and fast to perform. An important benefit is the ease of adapting incubation times due to non-toxic substrates. Compared to other tests of metabolic assay, our assay is easy (no washing and solubilisation steps are required), fast (it consists of simple one-step incubation and the response time is fast), and sensitive (very low cell numbers can be detected).

In summary, we propose that it is possible to use our technique to measure NK metabolic activity. The ease of its execution, its safety and its suitability for analyzing a relatively small number of cells makes this method a promising contender for amending routine techniques. Despite the relatively small number of subjects studied, we have found significant differences in results between infertile and fertile women. However, additional analyses are necessary to determine the sensitivity and specificity of the introduced test within immunological diagnostics of infertility.

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