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Where and when should natural killer cells be tested in women with repeated implantation failure?

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ABSTRACT

The aim of this study was to identify the candidates for natural killer (NK) testing and to define the best methodology. For this purpose a prospective study was performed on 73 women with repeated implantation failure (RIF). RIF was considered to exist in patients not achieving clinical pregnancy after three transfers with at least one good-quality embryo. Idiopathic RIF was considered to exist in patients in whom thrombophilia, hysteroscopy and endometrial culture were normal, and no chromosomal factor was suspected. Thirty-two of the 73 patients were considered to have idiopathic RIF, and 17 fertile women with children were taken as controls. Immunohistochemical staining for endometrial CD56+ and blood CD56+ or CD16+ NK cells measured using flow cytometry were compared during the mid-luteal phase in both patients and controls. Seventeen out of the 32 patients with idiopathic RIF and only one of the controls had >250 CD56 cells per high power field 400× in endometrial biopsy ($p < 0.001$). The percentage of blood NK cells out of the total lymphocyte population was higher in women with idiopathic RIF (13.4 ± 1.2%; range, 2.63–29.01) than in controls (8.4 ± 0.7%; range, 5.72–13.28; $p = 0.026$). There was a positive correlation between blood and endometrial CD56 cells ($\rho = 0.707$; $p < 0.001$). No significant differences were found between patients with other types of RIF and controls. This study suggested that testing for NK cells might be useful in women with idiopathic RIF during the mid-luteal phase.

1. Introduction

Despite the new technologies, human implantation remains a barrier in assisted reproduction (Edwards, 2007). At the time of implantation, there seems to be a dialog between the embryo and the endometrium (Roberts et al., 2008). In this dialog, the main role is played by the embryo (Dior et al., 2014). Embryos resulting from sperm and egg donation or those chromosomally normal after preimplantation genetic diagnosis (PGD) are usually considered to be of very good quality (Capalbo et al., 2014). However, sometimes, even after transferring very good quality embryos, pregnancy is not achieved.

The first problem faced when treating repeated implantation failure (RIF) is that there is no well-established

Abbreviations: RIF, repeated implantation failure; NK, natural killer; h.p.f., high power fields of 400× magnification.

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definition (Tan et al., 2005; Coughlan et al., 2014). It is also unclear which tests should be included to detect the reasons for these failures.

As aneuploidy is one of the major factors affecting embryo implantation (Rodrigo et al., 2014), parental karyotyping, sperm fluorescence in situ hybridization (FISH), DNA fragmentation (Enciso et al., 2013), and even preimplantational genetic diagnosis could be recommended for women with RIF (Pagidas et al., 2008; Franssen et al., 2011).

Given that anatomical malformations of the uterus may interfere with normal implantation (Simon and Laufer, 2012), and that a chronic endometritis rate of up to 66.3% upon hysteroscopy has been described (Yang et al., 2014), hysteroscopy and endometrial biopsy may be recommended.

Hypothyroidism (Fumarola et al., 2013) has also been associated with reproductive failure and should therefore be included in the investigation. However, some women with repeated implantation failure do not have any of the above-mentioned factors, still do not achieve pregnancy, and would like to identify the reason. Despite there being many publications describing an increased amount of natural killer cells in both blood and endometrium in women with repeated implantation failure and recurrent miscarriage (Tuckerman et al., 2010; Coulam and Goodman, 2000), natural killer (NK) cell testing is not a recommended clinical practice (Tang et al., 2011; Seshadri and Sunkara, 2013), and not all studies agree that NK levels may be associated with implantation problems (Thum et al., 2005). Unless the diagnostic methodology is clear and standardized, it would be difficult to find a solution because of the lack of clarity regarding who should be included in therapeutic trials. A recent meta-analysis reports elevated peripheral NK cells in infertile women, but not a significant increase in uterine NK cells (Seshadri and Sunkara, 2013). A problem of most investigations is the low number of cases and controls. Controls are often infertile patients. In many publications, repeated implantation failure and recurrent miscarriage tend to overlap (Tang et al., 2011), although they have different physiopathology and immunology. T cells, for instance, decrease significantly in the first trimester in the decidua and are unlikely to play an important role in the maintenance of human pregnancy; however, T cells could be important for implantation where their relative numbers are greater (Johnson et al., 1999), and NK cells increase immediately after implantation and severely decrease by gestational day 6 (Takashima et al., 2013).

As there are great differences in cut-off values, the normal range of uterine NK cells for human embryo implantation is controversial.

The most effective methodology for testing NK cells that can be standardized in routine clinical practice is not clear either. It is not clear if NK cell levels should be measured in blood or in endometrium, and very little is known about the correlation between blood and endometrial levels of NK cells (Laird et al., 2011). Flow cytometry can be used to test both blood and uterine NK cells, but to analyze uterine NK cells, they have to be mechanically or enzymatically disintegrated (Manaster et al., 2008). Therefore, this method does not easily allow NK cells to be counted in a defined tissue volume and is hard to standardize for application in different centers.

In contrast, immunohistochemistry has been successfully used by Tuckerman et al. (2010) and is usually reported to be the percentage of stained cells from the total number of stromal cells. However, it would be interesting to compare NK cell testing as a percentage, total count per field, and relation to the epithelial glands. A previous meta-analysis has demonstrated that the significance of NK cells is different when they are expressed as numbers or as percentages (Seshadri and Sunkara, 2013). However, it is difficult to identify different phenotypes such as NK and natural killer T (NKT) cells. In particular, bright and dim CD56 cells cannot be distinguished by means of immunohistochemistry, and blood NK cells cannot be studied either. It seems that the total numbers of CD56 cells measured using flow cytometry and immunohistochemistry correlate well (Laird et al., 2011). Therefore, we aimed to study uterine NK cells using immunohistochemistry and blood NK cells using flow cytometry focusing on the total amount of CD56 cells rather than different phenotypes, as this is what can be compared in blood and endometrium.

The objective of this study is to define the candidates for this test and to find the best methodology. We also aimed to establish a reasonable cut-off value for normal uterine NK cells and check if blood NK cells correlated with uterine NK cells.

2. Materials and methods

This prospective study on 90 women had the approval of the local ethics committee and required patients to provide informed consent. Data were obtained from 73 women with RIF and 17 controls (Fig. 1). RIF was defined as no clinical pregnancy after three transfers with at least one good-quality embryo (grade A or B). Most transfers took place on the third day. In case the patient's own oocytes were used, only women under 40 years of age were included.

All patients with RIF went through our repeated implantation failure protocol, which includes the following: karyotyping of both the man and the woman; hysteroscopy; endometrial culture and microbiological analysis of endometrial biopsy; sperm FISH for chromosomes 21, 18, 13, X, and Y; and sperm DNA fragmentation.

We diagnosed a subgroup of these women with idiopathic RIF on the basis of these tests being normal. Exclusion criteria for this subgroup were as follows: uncompleted protocol, suspected chromosomal factor, and any immunological disorder, except for autoimmune hypothyroidism if TSH was under 2.5 with medication. We suspected a chromosomal factor in women over 39 years of age, if there were any abnormal embryos after PGD, and if sperm FISH or DNA fragmentation was altered. Women with diagnosed autoimmune disorders, such as rheumatoid arthritis and lupus erythematous, were excluded. The women excluded from the idiopathic RIF group were also analyzed and considered as cases of other types of RIF.

Fertile women under 35 years of age with no history of infertility and their own children were taken as controls.