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Reproductive Outcomes and General Health in Women with High Natural Killer Cell Levels

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Abstract

Objective: The role of natural killer (NK) cells in reproductive failure has been investigated extensively, but the topic remains controversial and poorly understood. NK cells are known to play an important protective role in cancer and infection, but the implications of high NK cell levels have not been studied with respect to these diseases. This study aimed to identify any differences in reproductive outcomes as well as general health and disease between women with high and normal NK cell parameters.

Materials and Methods: 258 female patients at a fertility clinic who received peripheral blood NK cell testing were divided into groups based on their NK cell levels. Their medical history was analysed with regard to fertility, neoplasia, autoimmune disease, atopic disease, gynaecological disorders and mental illness.

Results: Women with high NK cell levels had a significantly higher number of embryo transfer procedures ($p < 0.05$) and lower number of live births ($p < 0.05$) than women with normal levels. They also trended to having longer periods of infertility and more miscarriages. Higher NK levels were associated with an increased incidence of autoimmune thyroid disease, endometriosis, asthma and positive autoantibody assays, and a reduced incidence of cervical intraepithelial neoplasia, depression and allergy.

Conclusions: This study shows that women with elevated NK cell levels have significantly worse reproductive outcomes than those with normal levels. They also appear to have an altered risk of various other diseases. The trends we observed should be targeted in larger, longer-term studies.

Keywords: Natural killer (NK) cells; Female infertility; Reproductive failure; In vitro fertilisation (IVF); Reproductive immunology

Introduction

Few topics in recent reproductive medicine have been the subject of as much controversy, media attention and passionate debate as natural killer (NK) cells and their role in reproductive failure [1,2]. Reproductive failure, a broad set of conditions encompassing infertility, recurrent miscarriage (RM) and recurrent implantation failure (RIF), is common but often poorly understood. In a large proportion of patients-including 50% of women with RM and 15-30% of infertile couples-no explanation is found for their reproductive failure from traditional fertility investigations [3,4], so it is not surprising that practitioners and patients alike are investigating other potential explanations.

The assessment of NK cells in the peripheral blood in the investigation of subfertile women was popularised by claims in the mid-1990s that NK cell testing could be used to predict miscarriage and infertility [5-7]. Over the intervening 20 years, many investigators have sought to prove or disprove this hypothesis and have produced a multitude of conflicting evidence and passionate debate [1,2,8-11].

It is not yet proven that NK cell levels can predict reproductive outcomes, and the wider implications of high NK cell levels beyond the reproductive system are unknown. There is no known disease associated with elevated NK cell levels, nor has a clinical phenotype been defined among patients with this finding. NK cells are known to play a protective role against cancer and infection and are involved in both promoting and preventing autoimmune disease [12-14], but high levels have almost never been studied in relation to these pathological processes.

One of the only studies to examine the correlation between NK cell levels and cancer showed that individuals with high NK cytotoxicity had a significantly reduced risk of cancer, while those with low NK cytotoxicity had a significantly increased risk, compared to those who fell within the normal range. In fact, the relative risk of cancer was halved in women with increased NK cytotoxic activity [15]. Although it was published fifteen years ago, this study has never been repeated.

This study aimed to identify any differences in general health and disease between women with high and normal NK cell parameters and to determine whether there was a difference in reproductive outcomes between these groups.

Materials and Methods

Study population

Blood NK testing was developed at St George Hospital and IVFAustralia as previously described for women with recurrent miscarriage [16] and repeated IVF failure [17]. A protocol was created using forward and side scatter, and labelling with CD45, CD3, CD56, CD16, CD69, CD19 and CD14. Those studies used normal fertile women as a control group to create reference ranges which have since been used to identify those with high or borderline levels. Empirical immune therapy given to those women has demonstrated better than expected outcomes for subsequent pregnancies or IVF treatments in our observational studies [18,19] and others [20,21]. For clarity in this study, we chose strict criteria to define those with 'normal' and 'high' NK results, using both the percentage of NK cells as a proportion of lymphocytes, and the concentration of activated NK cells as defined by the CD69 marker (as previously described [16,17]). The resulting 3 groups were as follows:

- Normal NK cell levels ("normal group") - NK cells as a percentage of total lymphocytes < 12% and CD69 concentration $< 12 \times 10^6$/l
- High NK cell levels ("high group") - NK cell percentage ≥ 18% and CD69 concentration $\geq 12 \times 10^6$/l
- Borderline NK cell levels ("borderline group") -all others.

437 women attending IVF Australia clinics for the investigation and management of fertility problems who underwent peripheral blood NK cell testing between October 2012 and April 2015 were identified. Of these, 258 patients' medical files were assessed to collect retrospective data on their medical histories and the results of other investigations. The other 179 cases were excluded, most often because the patients' medical files were not accessible (n = 174). Another five cases were excluded from the study as they represented repeat tests. All of the data collected reflected the patients' medical histories prior to the date of their NK cell testing.

Statistical Analysis

The results were analysed using GraphPad Prism software. Mann-Whitney and Fisher's exact tests were used to compare continuous and categorical variables respectively.

Results

Of the 258 participants, 43 (16.7%) were deemed to have high NK cell levels, 125 (48.5%) borderline levels, and 90 (34.9%) normal levels (Table 1).

Reproductive Outcomes

Women in the high group were aged 23-50 (mean = 37.4) and experienced on average 3.63 years of infertility (range = 0-1), 3.24 embryo transfer procedures (range = 1-9), 1.95 miscarriages (range = 0-8) and 0.20 live births (range = 0-2).

Those in the borderline group were aged 25-49 (mean = 37.4) and experienced on average 2.81 years of infertility (range = 0-15), 2.92 embryo transfer procedures (range = 0-18), 1.73 miscarriages (range = 0-10) and 0.37 live births (range = 0-3).

Women in the normal group were aged 26-48 (mean = 36.9) and experienced on average 2.72 years of infertility (range = 0-12), 2.45 embryo transfer procedures (range = 0-15), 1.73 miscarriages (range = 0-10) and 0.55 live births (range = 0-5).

Women in the high group had undergone significantly more embryo transfer procedures ($p = 0.04$) and had fewer live births ($p = 0.01$) than those in the normal group. They also experienced a greater average length of infertility and number of miscarriages, but these differences were not statistically significant (Table 1).

Other Disease

Benign neoplasms, and particularly uterine fibroids, were more prevalent in the high and borderline groups than the normal group, although this difference was not statistically significant. Very few women across all three groups had had malignancies. Rates of cervical intraepithelial neoplasia (CIN) were marginally lower in the high group than the normal group (9.3% vs. 13.3%; relative risk [RR] = 0.75) and endometriosis slightly higher (27.9% vs. 20.0%; RR = 1.33).

Rates of atopic disease were similar between the normal and high groups (28.9% vs. 25.6%) but highest in the borderline group (35.2%). The prevalence of autoimmune disease-broadly defined to include alopecia, coeliac disease, Graves' disease, Hashimoto's thyroiditis, inflammatory bowel disease, multiple sclerosis, psoriasis, rheumatoid arthritis and systemic lupus erythematosus-was similar between the normal and high groups (13.3% vs. 14.0%) but the women in the high group had almost double the risk of autoimmune thyroid disease (Graves' disease and Hashimoto's thyroiditis; RR = 1.92).

Depression was reported twice as often in the normal group compared to the high and borderline groups (12.2% vs. 4.7% and 4.8%). This trend approached but did not reach statistical significance ($p = 0.09$) (Table 2).

Other Investigations

Autoantibodies were more likely to be present in the high and borderline groups than in the normal group (30.6% and 27.5% vs. 16.9%). In the high group, 23.1% of patients tested positive for thyroid antibodies (anti-thyroid peroxidase or anti-thyroglobulin) compared to 11.5% in the normal group (RR = 1.71). Although only a small number of participants received antinuclear antibody (ANA) testing, a trend was still observable in that 80% of those tested in the high group were positive compared to 50% in the normal and borderline groups.

The average peripheral blood concentration of anti-Müllerian hormone (AMH) was marginally higher in the high group and the concentration of vitamin D marginally lower, although neither of these differences was statistically significant (Table 3).

Discussion

The relationship between NK cell levels and reproductive outcomes has been studied often, with researchers reporting vastly different results. This study demonstrates that women with high NK cell levels have significantly fewer live births and undergo significantly more embryo transfer procedures than their immunologically normal counterparts. This supports others who have reported low live birth rates [5,23] and poor chances of IVF success [24,25] in subfertile women with abnormal NK cell levels or activity.

It is not known why some women have higher blood NK cell activity than others, or how long they have such activity for. We have previously shown than in 42 women who had repeat testing with a mean of 7.5 months apart, and a range of up to 42 months, there was remarkable stability for the test result [17]. It is certainly possible that measurable NK activity is altered by acute events such as exercise or virus infection at the time of the test, and this needs to be considered in all blood NK cell studies. Hence the need for careful interpretation, more studies, and continued examination of the concepts proposed in this paper from as many different angles as possible.

In this population, 16.7% of women were identified as having high NK cell levels. Using an NK cell percentage of 18% as a cut-off, previous groups have found that 12.5-14.7% of women with RM and 11% of women with RIF had high NK cell levels [5,16,17]. That our estimate is slightly higher is perhaps due to the fact that previous estimates were based on more narrowly focused populations while ours drew from a less discriminate sample. Our definition of high NK cell levels, however, was stricter than in previous reports since we included CD69 concentration as an additional cut-off, meaning that the proportion of patients deemed to have high levels would probably have been larger if NK cell percentage was the sole criterion.

Table 1: Basic demographics and reproductive outcomes.

Group	Normal	Borderline	High	Total	
Number of participants (%)	90 (34.9)	125 (48.4)	43 (16.7)	258 (100.0)	
Age, mean ± SD, years	36.9 ± 4.5	37.4 ± 4.6	37.4 ± 5.5	37.2 ± 4.7	
NK cell percentage, mean ± SD, %	8.1 ± 2.1	13.7 ± 3.6	23.1 ± 4.6	13.3 ± 6.1	*
NK CD69 concentration, mean ± SD, × 10^6/l	6.8 ± 2.8	16.2 ± 15.8	25.0 ± 12.4	14.4 ± 13.7	*
Length of infertility, mean ± SD, years	2.72 ± 2.2	2.81 ± 2.2	3.63 ± 3.7	2.92 ± 2.5	
Number of embryo transfer procedures, mean ± SD	2.45 ± 3.1	2.92 ± 3.2	3.24 ± 2.4	2.80 ± 3.0	^
Number of miscarriages, mean ± SD	1.73 ± 1.9	1.73 ± 1.9	1.95 ± 1.8	1.77 ± 1.9	
Number of live births, mean ± SD	0.55 ± 0.8	0.37 ± 0.6	0.20 ± 0.5	0.40 ± 0.7	^

* $p < 0.001$ between normal and high groups (Mann-Whitney test)
^ $p < 0.05$ between normal and high groups (Mann-Whitney test)
NK, natural killer; SD, standard deviation.

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Table 2: Non-reproductive diseases.

Group	Normal	Borderline	High
Atopic disease, n (%)	26 (28.9)	44 (35.2)	11 (25.6)
Allergy	25 (27.8)	33 (26.4)	8 (18.6)
Asthma	6 (6.7)	16 (12.8)	4 (9.3)
Autoimmune disease, n (%)	12 (13.3)	23 (18.4)	6 (14.0)
Alopecia	2 (2.2)	0 (0.0)	0 (0.0)
Coeliac disease	1 (1.1)	7 (5.6)	0 (0.0)
Graves' disease	0 (0.0)	2 (1.6)	2 (4.7)
Hashimoto's thyroiditis	2 (2.2)	7 (5.6)	1 (2.3)
Inflammatory bowel disease	3 (3.3)	1 (0.8)	0 (0.0)
Multiple sclerosis	0 (0.0)	1 (0.8)	1 (2.3)
Psoriasis	3 (3.3)	1 (0.8)	0 (0.0)
Rheumatoid arthritis	3 (3.3)	4 (3.2)	2 (4.7)
Systemic lupus erythematosus	0 (0.0)	1 (0.8)	0 (0.0)
Benign neoplasia, n (%)	14 (15.6)	26 (20.8)	9 (20.9)
Acoustic neuroma	1 (1.1)	0 (0.0)	0 (0.0)
Fibroadenoma	1 (1.1)	5 (4.0)	0 (0.0)
Hepatic adenoma	0 (0.0)	1 (0.8)	0 (0.0)
Lipoma	1 (1.1)	1 (0.8)	1 (2.3)
Osteochondroma	0 (0.0)	0 (0.0)	1 (2.3)
Uterine fibroid	10 (11.1)	19 (15.2)	6 (14.0)
Cervical intraepithelial neoplasia, n (%)	12 (13.3)	18 (14.4)	4 (9.3)
Endometriosis, n (%)	18 (20.0)	23 (18.4)	12 (27.9)
Malignant neoplasia, n (%)	3 (3.3)	2 (1.6)	2 (4.7)
Breast cancer	1 (1.1)	0 (0.0)	0 (0.0)
Skin cancer	1 (1.1)	1 (0.8)	0 (0.0)
Thyroid cancer	1 (1.1)	1 (0.8)	2 (4.7)
Mental illness, n (%)	13 (14.4)	11 (8.8)	5 (11.6)
Anxiety	4 (4.4)	8 (6.4)	2 (4.7)
Bipolar disorder	0 (0.0)	0 (0.0)	1 (2.3)
Depression	11 (12.2)	6 (4.8)	2 (4.7)
Panic disorder	0 (0.0)	1 (0.8)	0 (0.0)
Polycystic ovaries, n (%)	9 (10.0)	14 (11.2)	3 (7.0)
Polycystic ovary syndrome *	3 (3.3)	6 (4.8)	1 (2.3)

* Rotterdam criteria

Table 3: Autoantibody assay results and other investigations.

Group	Total	Normal NK	Borderline NK	High NK
Autoantibodies (any) n positive / n tested (%)	54/222 (24.3%)	13/77 (16.9)	30/109 (27.5)	11/36 (30.6)
Antinuclear antibodies n positive / n tested (%)	26/46 (56.5%)	6/12 (50.0)	12/24 (50.0)	8/10 (80.0)
Cardiolipin antibodies n positive / n tested (%)	11/193 (5.7%)	3/69 (4.3)	7/93 (7.5)	1/31 (3.2)
Lupus anticoagulant n positive / n tested (%)	2/186 (1.1%)	0/68 (0.0)	2/90 (2.2)	0/28 (0.0)
Thyroid antibodies n positive / n tested (%)	27/169 (16.0%)	7/61 (11.5)	14/82 (17.0)	6/26 (23.1)
AMH, mean ± SD, pmol/l	N=258 (100%)	19.5 ± 18.4	18.0 ± 18.5	22.3 ± 24.9
Vitamin D, mean ± SD, ng/ml	N=258 (100%)	71.1 ± 24.6	65.7 ± 20.5	65.1 ± 19.4

AMH: Anti-Müllerian Hormone; SD: Standard Deviation.
Antibody assays were performed by Douglas-Hanly-Moir Pathology, Ryde, Sydney, Australia. Normal ranges for thyroid antibodies were less than 20iu/ml (thyroglobulin) and less than 10iu/ml (peroxidase), for cardiolipin antibodies less than 20iu/ml, and for antinuclear antibodies less than 1:80.

The relatively small size of our sample meant that very few relationships between variables were statistically significant, especially when analysing diseases such as cancer that were only present in small numbers. Despite this, a number of trends could still be observed when comparing the prevalence of diseases and findings in the high and borderline groups with the normal group. These trends suggest that women with higher NK cell levels tend to be at an increased risk of autoimmune thyroid disease, positive autoantibody tests, endometriosis, asthma and benign neoplasia, and at a reduced risk of depression, CIN and allergy (Table 4).

The relationship between NK cells and autoimmune thyroid disease is unclear, as NK cell parameters have variously been reported to be increased, decreased or normal in Graves' and Hashimoto's patients. While some groups have reported depressed NK cytotoxicity in these patients, our results are consistent with the findings of Hidaka et al. [26] that NK cell activity is enhanced in autoimmune thyroid disease [26].

Our observation that higher NK cell levels tended to be associated with thyroid antibodies echoes a previous report from Kim et al. [27] Previous studies have shown a correlation between NK cell levels or CD69 concentration and cardiolipin antibodies [5,16,28], although such a trend was not seen in our sample.

The role of NK cells in endometriosis has not been thoroughly studied, but it has been proposed that alterations in NK cell function, particularly in cytotoxicity, may impair the ability to eliminate ectopic

Table 4: Summary of trends.

Disease or finding	Trend in high/ borderline groups	$p < 0.05$
Allergy	Reduced	
Asthma	Increased	
Autoantibodies	Increased	
Autoimmune thyroid disease	Increased	
Benign neoplasia	Increased	
Cervical intraepithelial neoplasia	Reduced	
Depression	Reduced	
Endometriosis	Increased	
Length of infertility	Greater	
Number of embryo transfer procedures	Greater	*
Number of live births	Fewer	*
Number of miscarriages	Greater	

For more detail see tables I and II

endometrial cells [29]. Our observation that endometriosis was more prevalent in the high group supports the hypothesis that aberrant behaviour by NK cells may represent a risk factor for endometriosis.

Suppressed NK cell activity has frequently been associated with depression [30-32], and this is supported by the increased prevalence of depression in the normal group compared to the borderline and high groups.

The absence of a discernible trend in cancer across the three groups is unsurprising given that in our sample of relatively young participants, very few had had malignancies (n = 7). With a larger sample size, or a prospective design to follow up patients as they age, we perhaps would have observed a reduced risk in cancer among the women in the high group, consistent with what is known about the onco-protective role of NK cells. We would expect to see results consistent with the findings of a landmark study by Imai et al. that demonstrated a significant reduction in the relative risk of cancer in individuals with high NK cytotoxic activity over a follow-up period of eleven years [15]. Despite these incredible findings, no similar studies have ever been published. We saw a similar trend to that reported by Imai et al. [15] in the prevalence of CIN, which was slightly lower in the high group.

It has been previously reported that NK cell parameters were significantly increased in RM patients with vitamin D deficiency (defined as a concentration <30 ng/ml), suggesting that vitamin D, an important immunomodulator, may contribute to NK cell aberrations [33]. Very few women in our sample were deficient in vitamin D, but we nevertheless observed that vitamin D levels were marginally lower in the high group, lending some credence to this theory.

Numerous groups have shown that immunotherapy is effective in reducing NK cell levels and improving reproductive outcomes in women with high levels [5,21,34-37]. Yet we observed that elevated NK cell activity appears to lower the risk of some diseases and, indeed, many immunotherapeutic approaches to cancer management are targeted at expanding or stimulating the NK cell population [12,38].

Conclusion

This study is the first of its kind to attempt to define a broad clinical phenotype among women with elevated NK cell levels. It demonstrates that women with high NK cell levels typically undergo more embryo transfer procedures and have fewer live births. Furthermore, a number of trends were observed in the general health of these women which, with larger prospective studies, can improve our understanding of what it means to have increased NK cell levels. To date, most investigations into abnormal NK cell levels have focused on reproduction, and we believe this study demonstrates the merits of casting a wider net beyond the reproductive system when considering the implications of high NK cell levels.

This study is too small to draw any definite conclusions regarding how NK cell levels relate to aspects general health. Yet it demonstrated, firstly, significant relationships and trends between increased NK cell levels and measures of infertility. Secondly, a number of trends were seen in the cohort with high NK cell levels: they tended to have higher rates of autoimmune thyroid disease, endometriosis and asthma, and lower rates of CIN, depression and allergy. These diseases and outcomes should be targeted in a larger, longer-term follow-up study, with particular control for the many confounders in these associations.

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