

# Etanercept immunotherapy in women with a history of recurrent reproductive failure

Zastosowanie etanerceptu u kobiet z niepowodzeniami rozrodu

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## Abstract

**Objective:** The aim of the study was to evaluate the effect of etanercept immunotherapy on peripheral natural killer (NK) cell activity in women with a history of recurrent miscarriage (RM) or failed in vitro fertilization (IVF).

**Materials and methods:** Thirty nonpregnant women with reproductive failure and increased peripheral NK-cell number and/or activity before conception were studied. Women with reproductive failure received 4 doses (25 mg) of etanercept twice weekly before conception. Peripheral NK-cell activity before and after etanercept therapy in RM women was measured using flow cytometry. In addition, the peripheral blood NK-cell surface antigens- CD16- and CD56 and peripheral blood regulatory T cell (T reg) antigens- CD4- and CD25 were studied using flow cytometry, before treatment and 2 weeks after the last etanercept dose.

**Results:** NK-cell activity was significantly decreased after etanercept therapy in the study women ( $P < .05$ ). This effect was significantly higher in women with subsequent pregnancy success ( $P < .05$ ), but not in those with pregnancy failure ( $P > .05$ ). There were no significant differences in T reg level before and after etanercept therapy ( $P > .05$ ).

**Conclusion:** Etanercept therapy might be effective treatment for women with increased NK-cell activity. Regulation of immune system activity may underlie the possible effect of such therapy.

Key words: **Etanercept / in vitro fertilization / NK-cell activity / recurrent miscarriage /**

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## Streszczenie

**Cel pracy:** Celem pracy była ocena wpływu etanerceptu na aktywność komórek NK krwi obwodowej u kobiet z poronieniami nawykowymi (RM) lub wielokrotnymi niepowodzeniami zapłodnienia pozaustrojowego (IVF).

**Materiał i metody:** Badaniem objęto 30 kobiet z niepowodzeniami rozrodu i zwiększoną liczbą i/lub aktywnością komórek NK krwi obwodowej. Chore otrzymały 4 dawki (25mg) etanerceptu dwa razy w tygodniu przed planowaną ciążą. Aktywność komórek NK zbadano przed i po podaniu leku metodą cytometrii przepływowej. Dodatkowo oceniono ekspresję markerów powierzchniowych komórek NK CD16- i CD56 oraz limfocytów T regulatorowych (T reg) CD4- i CD25 metodą cytometrii przepływowej przed leczeniem oraz 2 tygodnie po ostatniej dawce leku.

**Wyniki:** Aktywność komórek NK była znacząco zmniejszona po podaniu etanerceptu w grupie badanych chorych ( $P < 0,05$ ). Efekt ten był znacząco wyższy u kobiet, których ciąży zakończyły się sukcesem położniczym ( $P < 0,05$ ), efektu tego nie zaobserwowano u kobiet z niepowodzeniem położniczym po podaniu etanerceptu ( $P > 0,05$ ). Nie stwierdzono różnic znamiennych statystycznie w ekspresji T reg w grupie badanej po podaniu etanerceptu ( $P > 0,05$ ).

**Wnioski:** Etanercept może być efektywną opcją terapeutyczną u kobiet ze zwiększoną ekspresją komórek NK. Regulacja aktywności układu immunologicznego może leżeć u podstaw skuteczności tej eksperymentalnej terapii.

Słowa kluczowe: **aktywność komórek NK / etanercept / poronienia nawykowe /  
/ zapłodnienie pozaustrojowe /**

## Introduction

Etanercept (trade name Enbrel) is a drug that treats autoimmune diseases by interfering with the tumor necrosis factor (TNF) by acting as a TNF inhibitor [1-4]. Etanercept is a TNF antagonist with anti-inflammatory effects. Its major mode of action is to suppress TNF- $\alpha$ , a Th-1 embryotoxic cytokine produced by activated natural killer cells (NK cells) [1-4]. Etanercept is a fusion protein produced through expression of recombinant DNA. That is, it is a product of a DNA construct engineered to link the human gene for soluble TNF receptor 2 to the gene for the Fc component of human immunoglobulin G1 (IgG1). Expression of the construct produces a continuous protein "fusing" TNF receptor 2 to IgG1. It is a large molecule, with a molecular weight of 150 kDa, that binds to TNF- $\alpha$  and decreases its role in disorders involving excess inflammation in humans and other animals, including autoimmune diseases such as ankylosing spondylitis, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, and, potentially, in a variety of other disorders mediated by TNF- $\alpha$  excess [1-4].

Recent studies suggest that etanercept, while blocking TNF- $\alpha$  release, may also deactivate NK cells [1-4]. If this indeed proves to be correct, etanercept might be effective in the treatment of in vitro fertilization (IVF) failure and recurrent miscarriage (RM) patients with NK-cell activation. Elevated numbers of peripheral blood NK cells and increased infiltration of endometrial NK cells have been reported as complications related to pregnancy [5, 6].

Cellular immune abnormalities with increased peripheral blood NK-cell numbers in women who experienced implantation failures after documented embryo transfer or recurrent pregnancy losses have been reported [5, 6]. NK cell activity may also predict pregnancy loss [6]. Although the immunophenotypes of the majority of peripheral blood NK cells are different from those of endometrial NK cells, peripheral blood NK cells seem to be closely related to decidual NK cells and may reflect the decidual NK-cell functional status [7]. It is now also accepted that uterine NK cells are at least partially derived from blood [7].

Additionally, some data suggest that women with RM have altered peripheral blood NK parameters and NK cells as a percentage of lymphocytes best discriminate RM and control women [8-10]. NK cell alteration may be associated with impaired pregnancy, and the modulation of the number of circulating NK cells is most likely to be a primary event in the pathogenesis of immunological infertility [11]. T regulatory (T reg) cells are a subset of T-cells with potent suppressive activity and pivotal roles in curtailing destructive immune responses and preventing autoimmune disease, and may be used as a new target for infertility treatment [12].

Our previous case report indicated that etanercept immunotherapy may allow a successful pregnancy outcome after multiple IVF failure [13]. The aim of this study was to determine etanercept immunotherapy influence on NK cells' activity in women with a history of at least three RM or failed IVF.

## Materials

### Patients

Thirty women with increased NK cell number and/or activity before were studied. Among women with reproductive failure, 17 had at least three RM and 13 at least three IVF failures (Table I). Women received 4 doses (25mg) of etanercept twice weekly before conception. Two women were excluded from the final analysis since they refused to have the peripheral blood assay re-checked after etanercept therapy. There were no significant differences among RM and IVF women with regard to their age or any of the studied parameters ( $P > 0,05$ ) except the number of reproductive failures, which was significantly higher in IVF women ( $P < 0,05$ ), (Table I). Consent for the study from the Bioethics Committee of the Military Institute of Medicine and from the patients was obtained. Natural killer cell activity was measured using flow cytometry. In addition, the peripheral blood NK cell surface antigens- CD16- and CD56 and T reg were studied using flow cytometry, before treatment and 2 weeks after the last etanercept dose.

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All tests were performed at least six months after the last miscarriage or IVF failure. The women were informed of the aim of the study and the best interests of participating patients always outweighed those of the trial. All measurements, interventions, and blood collections were performed after informed consent was obtained from each woman participating in the study in accordance with the bioethics committee-approved protocol. Consent for the study from the Bioethics Committee of the Military Institute of Medicine, Warsaw, and Institutional Review Board (IRB) approval were obtained. All data obtained from the subjects were confidential and accessible only to the investigative personnel. Recurrent miscarriage was defined as three or more consecutive pregnancy losses before 20<sup>th</sup> week of gestation with the same partner, according to the definition formulated by Committee for Diagnosis and Treatment of Recurrent Spontaneous Abortions [14].

All women were investigated in terms of any possible causes of abortion, and no apparent reason for their previous losses was found. Anatomic, genetic, microbiological, and hormonal causes of abortions or infertility were studied. A complete medical, surgical and social history was obtained in all cases. Also, all couples had peripheral blood chromosome assessment. Hysterosalpingography or hysteroscopy did not reveal any abnormalities of the patient's uterus. Levels of thyroid-stimulating hormone and prolactin were normal. We investigated the existence of inherited (deficiencies of protein C, protein S, antithrombin III, resistance to activated protein C including Leiden V mutation, prothrombin gene mutation, hyperhomocysteinemia) or acquired thrombophilia (anticardiolipin antibodies, lupus anticoagulant, beta-2-glycoprotein antibodies). We also studied the existence of autoimmunity, i.e. antinuclear antibodies and antithyroid antibodies. Nine study women have polycystic ovary syndrome (PCOS) and 6 women have subclinical thyroid deficiency.

After the diagnostic procedure, the patients were asked to participate in our program. During this time no other therapy was introduced. After finishing etanercept therapy, we introduce additional immunotherapy: heparin/aspirin, aspirin alone, steroids or combined therapy according to the presence of auto- or alloimmunity. The treatment protocol included aspirin 75-100 mg starting on day 1 of the cycle and prednisone 5mg starting on day 16 of the cycle. Once pregnancy occurred the dose of prednisone was increased to 10 mg every evening and stopped at 14 weeks and aspirin was discontinued at 36 weeks of gestation. In addition, enoxaparin (Clexane) 40 mg was administered subcutaneously during gestation. Metformin usually 1.5g daily was introduced at least 12 weeks before pregnancy and this therapy was continued during pregnancy at 1.0g daily in PCOS patients. We noted allergic skin reaction in the etanercept injection site in one study woman. All babies born after etanercept therapy were healthy except one with cardiac defect-atrial septal defect (ASDI) corrected at age 6 month. Now the boy is a two-year old, normal, healthy child. Mother of this boy has born healthy child in her next pregnancy under the same therapy.

## Methods

Peripheral blood NK-cell activity was measured using flow cytometry [10, 15]. The peripheral blood NK-cell surface antigens CD16 and CD56, and peripheral blood T reg surface antigens CD4 and CD25 were also studied using flow cytometry

(Simultest CD3/CD16+CD56, CD4/CD25, FACSCalibur, Cell Quest software, Becton-Dickinson, San Jose, CA, USA). Measurements were performed before etanercept therapy and two weeks after the last dose of the drug.

## Endpoints

Primary endpoints were peripheral blood NK-cell activity and T reg percentage (before and after etanercept therapy).

Secondary endpoints were pregnancy outcome in the study group.

## NK assay

### Separations of effector cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of patients by Ficoll gradient centrifugation. The mononuclear cells were adjusted to  $1 \times 10^7$  cells/2.6 mL in culture medium (RPMI 1640, 10% FBS). Target cells were human erythroleukemia K562 (ATTC, UK). The K562 human erythroleukemia cell line is used as a standard target for human NK-cell assays. The K562 cells were washed in PBS 120g and labeled with 1.2mL DIO (3,3-dioctadecyloxycarbocyanine perchlorate (Sigma) per 1mL PBS and incubated for 20 minutes in 5% CO<sub>2</sub> at 37°C. After two washes in PBS, the cell concentration was adjusted to  $1 \times 10^6$  cells/mL in medium (RPMI 1640, 10% FBS) and used for the cytotoxicity assay.

## Cytotoxicity assay

Groups of eight replicate wells were incubated for 4 hours in 5%CO<sub>2</sub> at 37°C: PBMC in medium, K562 in medium, mixed PBMC with target cells in ratios of 50:1 and 12:1. Twenty-five microliters of propidium iodide solution (0.1 µg/mL in water; Sigma) was added to each sample to stain dead cells (total volume in each sample 0.2 mL).

Live target cells (T) were identified by strong green fluorescence, whereas dead target cells (Td) showed strong green and red fluorescence. The percentage of dead target cells (%Td) was calculated by  $\%Td = (Td/T) \times 100$ . Specific lysis was calculated by  $\%Td$  cultured with effector cells -  $\%Td$  cultured without effector cells.

## Statistical analysis

All of the data were analyzed using Student's t test or the Mann-Whitney test for comparison of two groups or Kruskal-Wallis analysis of variance for comparison of a few groups;  $P < 0.05$  was considered to be statistically significant. Data were processed using Statistical Analysis System (SAS) version 8 (SAS Institute, Cary, NC).

## Results

We determined that 16 of 28 women (57.2%) had successful pregnancy outcomes and 6 of 28 (21.4%) had failures. Six of the 28 women (21.4%) women did not attempt to achieve their next pregnancy by the end of this study. There were no significant differences in any study parameters between RM women when compared with IVF ( $P > 0.05$ ) except the number of previous reproductive failures ( $P < 0.007$ ), (Table I).

Peripheral blood NK-cell activity was significantly decreased after etanercept therapy compared with NK-cell activity before therapy in the study group ( $P < 0.0008$ ), (Table II).

**Table I.** Characteristics of the study groups: in vitro fertilization (IVF) group (n=12) and recurrent miscarriage (RM) group (n=16). <sup>a</sup>P<0.05.

Variable	IVF n=12							RM n=16						
	Age (yrs)	Number of failures	CD16 (%)	CD56 (%)	CD3 (%)	NK activity (%)	T reg (%)	Age (yrs)	Number of failures	CD16 (%)	CD56 (%)	CD3 (%)	NK activity (%)	T reg (%)
Mean	39.30	7.00 <sup>a</sup>	16.00	13.50	69.10	22.30	8.00	36.00	3.90 <sup>a</sup>	13.40	14.90	72.10	19.80	7.00
SD	4.70	3.40	6.50	9.00	5.60	10.30	3.40	3.80	1.00	4.90	16.50	5.40	13.80	2.30
Median	38.00	6.00	16.50	12.00	69.00	24.00	8.00	37.00	4.00	13.00	11.50	72.00	21.50	7.50
Lower quartile	35.50	5.00	11.50	7.50	65.00	17.50	7.00	35.00	3.00	11.00	6.50	67.00	6.50	7.00
Upper quartile	44.00	9.00	20.00	15.50	74.50	28.00	10.00	38.00	4.00	15.00	15.50	77.00	27.00	8.00
Minimum	33.00	3.00	5.00	3.00	60.00	3.00	3.00	25.00	3.00	6.00	1.00	63.00	0.00	1.00
Maximum	46.00	15.00	28.00	33.00	77.00	41.00	12.00	41.00	6.00	27.00	71.00	80.00	43.00	9.00

**Table II.** Peripheral blood natural killer cell activity (%) in the study group (n=28) before and after etanercept therapy. <sup>a</sup>P<0.05.

Variable	Median	Lower quartile	Upper quartile	Minimum	Maximum
Before	22.00 <sup>a</sup>	9.00	28.00	0.00	43.00
After	11.00	6.00	15.00	2.00	39.50
Difference	-7.00	-19.00	-1.00	-37.00	13.50

**Table III.** Peripheral blood natural killer cell activity (%) in the group with subsequent successful pregnancy outcome (n=16) before and after etanercept therapy. <sup>a</sup>P<0.05.

Variable	Median	Lower quartile	Upper quartile	Minimum	Maximum
Before	21.00 <sup>a</sup>	7.50	32.00	3.00	43.00
After	12.00	6.00	15.00	2.00	39.50
Difference	-6.00	-20.00	-1.00	-37.00	7.00

There were no significant differences when comparing NK-cell activity in women with pregnancy success to those with pregnancy failure ( $P>0.05$ ). However, this effect was significantly higher in women with subsequent pregnancy success ( $P<.008$ ), but not in those with pregnancy failure ( $P>0.05$ ), (Table III). There were no significant differences in T reg level in the study group before and after etanercept therapy ( $P>0.05$ ), (Table I).

## Discussion

An anti-TNF- $\alpha$  therapy is considered a category B drug for pregnancy. Data in humans are limited with regard to safety for pregnancy [1-4, 16-18]. So far, there has been no evidence that TNF- $\alpha$  antagonists are associated with embryo toxicity, teratogenicity or increased pregnancy loss [1-4].

Some data suggest that, anti-TNF agents used during pregnancy in patients with rheumatic diseases may increase congenital anomalies comprising the association of vertebral abnormalities, anal atresia, cardiac defect, tracheoesophageal, renal, and limb abnormalities (VACTERL) [1-4]. However, the VACTERL claim was rubbished by Koren and Inoue [16]. The most complete database on etanercept in pregnancy is the OTIS registry, and when last reported, there was no statistically significant increase in anomalies in rheumatologic patients talking the drug in the first trimester, and no evidence for any particular type of abnormality [17]. However, current guidelines suggest that TNF- $\alpha$  blockers should be avoided at the time of conception in women with rheumatoid arthritis [18].

In our study, etanercept was safe both for the mothers and for the babies.

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Anti-TNF- $\alpha$  therapy can reset the immune system at a lower level of activity. Some data suggest that etanercept can exert adverse effects of inflammation mediated through elevated TNF- $\alpha$  activity and TNF- $\alpha$ -independent pathways, including the stress hormone response, factors connected to reproductive failure [19]. Etanercept may control proinflammatory cytokine expression [20]. According to recent data pregnancy loss was prevented by blocking TNF- $\alpha$  activity after treatment with etanercept in an animal model [21]. The association of success with correction of immune test abnormalities by anti-TNF- $\alpha$  blockers including etanercept or adalimumab has been reported, which was also shown by Winger [22-23]. According to a recent review by Clark, anti-TNF- $\alpha$  therapy appears promising for treatment of immune-mediated subfertility [24]. Some data suggest that etanercept promotes fertility since overproduction of TNF- $\alpha$  in the uterine lining by NK cells impairs implantation [25]. The effectiveness of such therapy may derive from regulation of immune, inflammatory, and/or procoagulant pathways that collaborate in the pathogenesis of pregnancy failure. According to a recent paper by Thaher et al., etanercept significantly decreased the activation marker CD 69 expression by CD56+NK-cells [26].

According to current knowledge NK-cell involvement in pathogenesis of pregnancy loss is controversial, but in our study etanercept therapy promoted successful pregnancy through diminished NK-cell activity [27]. As recurrent miscarriage and infertility encompassed a heterogeneous group of patients, it would be important to fully characterize such patients immunologically before proceeding with further studies. As yet, such parameters do not exist in clinical practice. In our study, there were no differences between IVF and RM women at least in the NK cell and T reg populations. An observational study could be done to identify the subgroups most likely to benefit from treatment, and then a proper multicenter randomized controlled trial in such patients would be appropriate.

## Conclusion

Etanercept therapy might be effective treatment for women with increased NK-cell activity. Regulation of immune system activity may underlie the possible effect of such therapy.

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