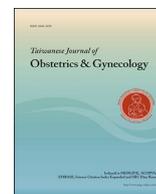




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## Original Article

## A randomized controlled trial of etanercept in the treatment of refractory recurrent spontaneous abortion with innate immune disorders

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## ABSTRACT

**Objective:** To evaluate the effectiveness of etanercept in the treatment of refractory recurrent spontaneous abortion with innate immune disorders.

**Materials and methods:** A randomized controlled trial in patients with refractory innate immune RSA was conducted in our hospital. 188 patients were selected, all with at least 4 consecutive miscarriages and caused by innate immunity disorders. Patients were randomly allocated into 2 groups. One group ( $n = 95$ ) used etanercept 25 mg per week starting from the first day after menstruation, while the other ( $n = 93$ ) with placebo. Delivery of a healthy baby without malformations was regarded as the primary outcome.

**Results:** In etanercept group, 85 (89.47%) patients delivered a healthy baby, while in placebo group, this number was only 67 (72.04%) [ $P = 0.01$ ,  $OR = 3.30$ ; 95%  $CI(1.49 \sim 7.32)$ ]. Significantly lower levels of TNF- $\alpha$  and NK cell activity were observed in gestation weeks 4–10 in etanercept group versus placebo group ( $P < 0.05$ ).

**Conclusion:** The results provide a proof of principle that etanercept can be an attractive therapeutic strategy for refractory innate immune RSA.

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Spontaneous abortion that occurs twice or more in a row is called recurrent spontaneous abortion (RSA) [1]. It is estimated that the prevalence of RSA in women of childbearing age is 5% [1]. At present, the recognized etiologies of RSA are chromosomal abnormalities, infectious diseases, endocrine factors, uterine abnormalities, autoimmune diseases, innate immune disorders and so on [2,3]. Among them, more than 50% of RSA is associated with innate immunity, mainly due to disorders of number or activity of NK cells [4]. During normal pregnancy, the number and activity of NK cells in peripheral blood decreased before and after implantation of fertilized eggs, while cytotoxic CD56<sup>+</sup>/CD16<sup>+</sup> NK cells in peripheral blood of RSA patients can attack the pregnant tissue, leading to RSA, repeated implantation failure (RIF) and increased risk of pregnancy complications (such as preeclampsia) [5,6]. Since

2010, our center used prednisone, heparin, aspirin, cyclosporine A (CSA), intravenous immunoglobulin (IVIG) to treat RSA patients with innate immune disorders. The live birth rate was 85%, but 15% of patients still failed. The main reason was considered to be that under the existing treatment strategy, TNF- $\alpha$  and the cytotoxicity of NK cells could not decrease to an ideal level.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is an inflammatory cytokine mainly produced by mononuclear macrophages, NK cells and T lymphocytes [7]. By binding to its receptors, it plays an important role in regulating the innate immune response, T cell-mediated tissue damage, and the occurrence and development of chronic inflammation [7]. Many studies have reported that high levels of TNF- $\alpha$  were closely related to miscarriage [7–9]. Serum TNF- $\alpha$  levels of threatened abortion patients were significantly higher than those of normal pregnant women at the same period [8]. After treatment, TNF- $\alpha$  levels returned to normal in patients who continued pregnancy, suggesting that TNF- $\alpha$  may play a role in RSA [8]. The specific mechanism of how TNF- $\alpha$  leads to RSA is still unknown. It is now generally accepted that increased TNF- $\alpha$  can

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activate Th1 cells, promoting the synthesis of prostaglandin E2 which can stimulate uterus smooth muscle, leading to trophocyte apoptosis, oxidative stress upregulation and blood coagulation system activation, resulting in placental vascular thrombosis, which eventually leads to adverse pregnancy outcome [9].

Etanercept (trade name: enbrel) is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kDa (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. It consists of 934 amino acids and has an apparent molecular weight of approximately 150 kDa. It can competitively bind to TNF- $\alpha$ , inhibiting binding of TNF- $\alpha$  to cell surface TNFRs, rendering TNF- $\alpha$  biologically inactive. This can inhibit excessive inflammatory reaction and treat autoimmune diseases such as ankylosing spondylitis, rheumatoid arthritis, psoriasis, psoriatic arthritis and so on [10,11].

Etanercept is now widely used in the treatment of RSA, but clinical evidence is scarce [12]. A famous retrospective cohort study in 2008 showed that compared with IVIG + aspirin + heparin group, the clinical pregnancy rate, embryo implantation rate and live birth rate of IVIG + TNF- $\alpha$  inhibitors + aspirin + heparin were significantly higher, which proved that TNF- $\alpha$  inhibitors can significantly improve the success rate of pregnancy [13]. A recent prospective observational multicenter cohort study showed that in patients with autoimmune disease who were treated with TNF- $\alpha$  inhibitors during early pregnancy, live birth rate and fetal weight were comparable with normal pregnant women [14]. In addition, in animal models, inhibition of TNF- $\alpha$  with etanercept significantly improved pregnancy outcomes as well [15].

To sum up, we treated patients who failed conventional treatment (heparin, aspirin, prednisone, CSA) with etanercept with the consent of the patients.

## Materials and methods

The refractory RSA patients with innate immune disorders (no previous successful pregnancy) referred to Weifang people's hospital between August 2014 and August 2017 were considered eligible for the study. All pregnancy events were defined and diagnosed according to the European Society for Human Reproduction and Embryology (ESHRE) Special Interest Group for Early Pregnancy. This study was evaluated and approved by the medical ethics committee of our hospital, and the clinical study was conducted according to Chinese law and the Declaration of Helsinki for Medical Research involving Human Subjects.

The patients had to fulfil the following inclusion criteria: woman's age  $\leq 39$  years, more than four previous miscarriages, serum TNF- $\alpha > 5.14$  pg/ml or increased NK cell activity (either ratios of 50:1 or ratios of 25:1 is greater than 15%) during pregnancy [5], failure of conventional treatment for RSA (heparin, aspirin, prednisone, CSA) and they had to be negative for all of the known causes of RSA (abnormal karyotype, uterine defects, infections, endocrine problems, coagulation defects or thrombophilia and autoimmune defects). All the patients underwent several examinations and only the couples in whom no abnormalities were found were included. The karyotype of both parents was normal, semen analysis of the male partners was also normal, hysteroscopy and/or pelvic ultrasound examination did not show any uterine abnormalities, and hormonal blood tests (FSH, LH, prolactin, 17 $\beta$ -estradiol, estrone, progesterone, D4androstenedione, dehydroepiandrosterone, testosterone and 17 $\alpha$  OH progesterone), thyroid tests (TSH, free T3 and T4, antiperoxisome and antitireoglobulin antibodies), glycemic tolerance test and insulin were in the range of normal values. Indirect Coombs test, antiphospholipid autoantibody (lupus anticoagulant, anticardiolipin antibodies) and other autoantibodies (antigastric wall cell, anti-mitochondrion, anti-smooth muscle cell,

anti-nucleus) were negative (a transient low positivity for one of these autoantibodies was considered as negative). The blood test for the screening of toxoplasma, rubella, cytomegalovirus, herpes virus I and II and infections other than for chlamydia and mycoplasma were negative. No abnormalities were found for anti-thrombin III, coagulative protein C and S, and blood coagulation factors II, V, VII, VIII and XII and homocysteine; furthermore, all patients were negative for Factor V Leiden prothrombin gene mutation. In addition, the patients to be included in the study had to show a normal karyotyping of embryonic tissues in their last miscarriage (during conventional treatment), and they were 46/XX in 90 miscarriages and 46/XY in 98 miscarriages. Both partners of all the couples included in the study had to be fertile, without any fertility impairment, and none of them had undergone fertility treatments, such as in vitro fertilization (IVF).

A total of 193 patients were considered eligible for the study and 188 of them agreed to participate. All the patients were informed about the possible risks of this treatment for the mother and the fetus, other than the lack of information about the developmental toxicity of etanercept. The patients were randomized by means of a computer-generated randomization number sequence. They were randomly assigned to the two arms of the study, one to basic treatment (heparin, aspirin, prednisone, cyclosporine) + etanercept and the other to basic treatment + placebo administration; the patients were blind to which treatment they were assigned to. The patients underwent randomization only once and were not allowed to re-enter in the study. The etanercept group of 95 women underwent a daily s.c. administration of etanercept (enbrel), at a dosage of 25 mg from the end of menstruation until the occurrence of menstruation or to the end of the 10th week of gestation. The placebo group, consisting of 93 subjects, was treated daily with s.c. saline solution at the same dosage and same time. Serum TNF- $\alpha$  concentration was measured before every administration in both groups. NK cell activity was assessed weekly between 4 and 10 weeks of gestation in both groups. All the patients conceived within 3 months from randomization and inclusion in the study. All the patients were followed up during the pregnancy in our clinic, and every 2 weeks, they underwent transvaginal ultrasound scans from the 4th through 12th gestational week to observe heart beat and embryo growth, or to diagnose miscarriage (according to ESHRE published criteria). Biochemical miscarriages were not included in the study. All the babies born underwent a paediatric examination to exclude congenital malformations. The gestational age of miscarriage was calculated by ultrasound scan using the crown-rump length and the gestational sac dimension. The primary outcome was live birth of a healthy baby without major or minor congenital malformations. The secondary outcomes were side effects of the treatment, possible pregnancy complications (pre-eclampsia, pre-term delivery, gestational diabetes, pregnancy hypertension, bleeding and thrombosis) and newborn weight.

NK cells activity assay was described previously [16]. Groups of eight replicate wells were incubated for 4 h in 5%CO<sub>2</sub> at 37 °C: Peripheral blood mononuclear cells (PBMC) in medium, K562 (ATTC, UK) in medium, mixed PBMC with target cells in ratios of 50:1 and 25:1. 25  $\mu$ l of propidium iodide solution (0.1  $\mu$ g/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.

The statistical analysis was performed using the SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). For continuous variables, statistical significance was assessed by the use of the two-tailed

Student's t-test for unpaired data with the Bonferroni correction for multiple comparisons. Fisher's exact test and  $\chi^2$  were used when appropriate for discontinuous variables.  $P < 0.05$  was defined as statistically significant.

**Results**

*The demographic data of the patients*

There were no differences between the two groups for the age when pregnancy started, number of previous miscarriages, body mass index (BMI) when pregnancy started and gestational week of miscarriage (Table 1). None of the patients dropped out of the study. The patients were strictly followed up during pregnancy and no violation of the study was recorded.

*Primary outcome and secondary outcomes*

85 live births (89.47%) were found in the 95 patients in etanercept group, while only 67 live births (72.04%) were found in the

93 patients in placebo group; this difference was significant [ $P = 0.01$ , odds ratio (OR) = 3.30; 95% confidence interval (CI) (1.49–7.32)]. The number of patients needed to treat (NNT) for one additional live birth was 5.74. Compared with placebo group, the incidence of pregnancy complications in etanercept group was lower. There were no significant differences in gestational age of miscarriage, neonatal weight and therapeutic side effects between two groups: 3 cases of skin rash and 1 case of gestational hypertension were found in etanercept group. In placebo group, 1 patient developed skin rash and 7 patients developed gestational hypertension. 2 patients in the etanercept group were born with congenital malformations, one was atrial septal defect, which was cured by interventional treatment; The other was inguinal hernia, which was cured by surgical treatment. One patient in placebo group was born with congenital malformations (cleft lip), which was cured by surgical treatment. Data were shown in Table 2. The embryonic tissue was available for karyotype in 26 of 36 miscarriages, including 6 karyotype abnormalities, 3 in etanercept group and 3 in placebo group. In the 20 cases with normal karyotype, 9 were 46XX and 11 were 46XY.

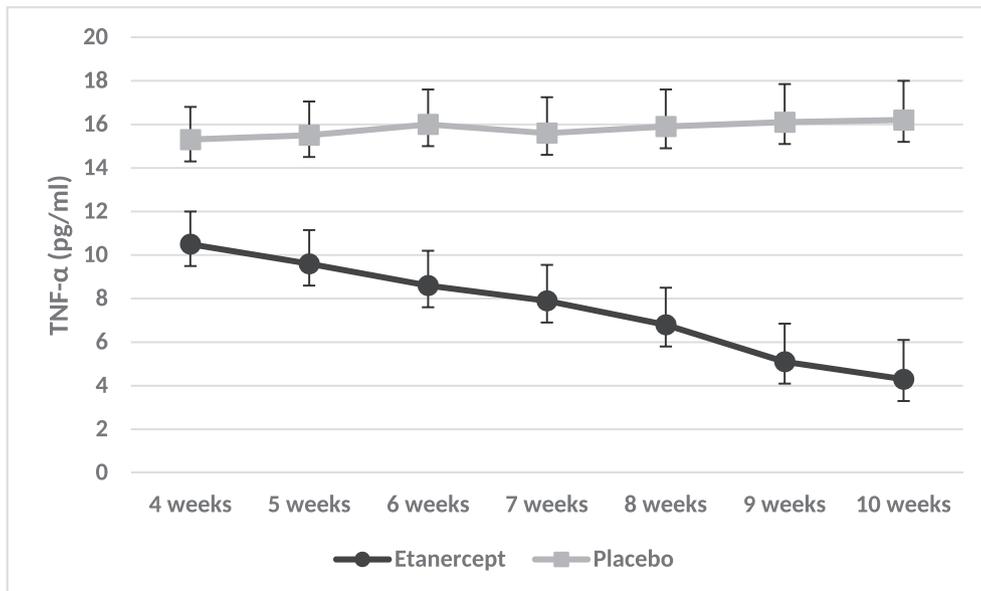
**Table 1**  
The demographic data of two groups.

	Number of patients	Age (years)	BMI	Smokers <sup>a</sup>	Number of previous miscarriages	Gestational week of miscarriage
Etanercept	95	34.5 ± 2.7	27.8 ± 1.9	0	5.4 ± 0.3	6.3 ± 1.2
Placebo	93	34.0 ± 2.8	27.5 ± 2.0	1	5.5 ± 0.4	6.2 ± 1.1

<sup>a</sup> More than 10 cigarettes per day.

**Table 2**  
Comparison of results between two groups.

	Number of live births (%)	Number of miscarriages (%)	Gestational week of miscarriage (mean ± SD)	Apgar score of newborns		Newborn weight (g, mean ± SD)	Side effects		
				1min	5min		Skin rash	Gestational hypertension	Congenital malformation
Etanercept	85 (89.47)	10 (10.53)	7.0 ± 1.2	9.6 ± 0.5	9.9 ± 0.2	3060 ± 230	3	1	2
Placebo	67 (72.04)	26 (27.96)	7.2 ± 1.1	9.5 ± 0.5	9.8 ± 0.3	3120 ± 220	1	7	1
P-value	<0.01	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05	>0.05



**Fig. 1.** Serum TNF- $\alpha$  level of two groups.

### Comparison of serum TNF- $\alpha$ concentration

As shown in Fig. 1, serum TNF- $\alpha$  level of etanercept group was lower than that in placebo group in gestation weeks 4–10 ( $P < 0.05$ ).

### Comparison of NK activity

As shown in Fig. 2, the peripheral blood NK cell activity of etanercept group was significantly lower than that of placebo group in gestation weeks 4–10 ( $P < 0.05$ ).

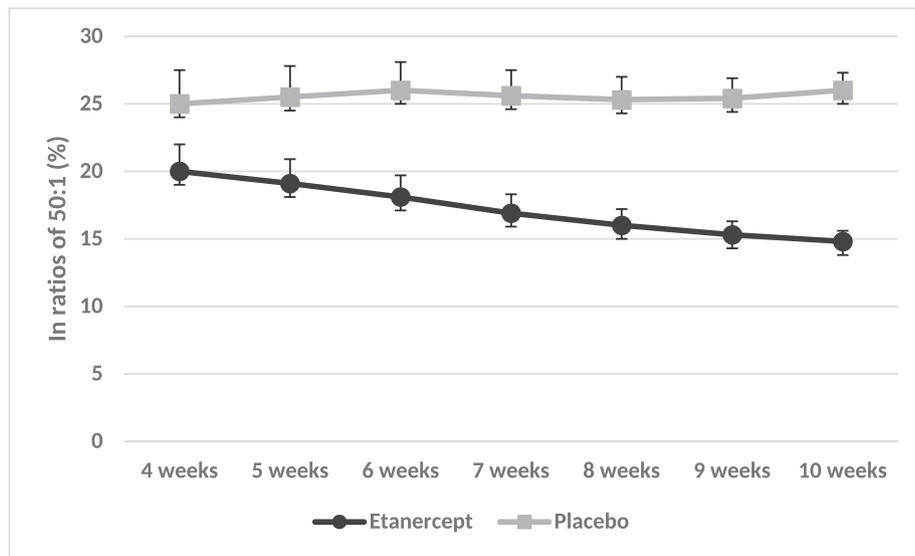
### Discussion

There are three reasons for the varying degrees of difference in the results of the studies on RSA treatment. First, all the studies have a bias for the spontaneous resolution of the problem; Second, the limited sample; Third, the inclusion criteria of these studies (especially the age of patients and the number of previous

miscarriages) were quite different, and the risk of miscarriage increased with maternal age and the number of previous losses [17]. An ideal trial to test the effectiveness of a treatment for RSA should be conducted with a randomized block design rather than a completely randomized design, taking into account the maternal age and number of previous miscarriages [17]. Although this study was very strict in terms of the age and the number of previous miscarriages of patients, this bias was still shown to some extent due to the limited sample size.

In this study, only patients aged  $\leq 39$  years old without fertility problems in either partner were included to eliminate confounding factors. In addition, all patients had failed a previous cycle of treatment for RSA and the embryo karyotypes were normal. All these standards make this trial closer to the ideal trial proposed by Christiansen et al. [17]. Because the large sample size in multi-center trial would inevitably result in less selective criteria for patient inclusion and an increase in confounding factors, this study appropriately reduced the sample size. In this study, the incidence

A



B

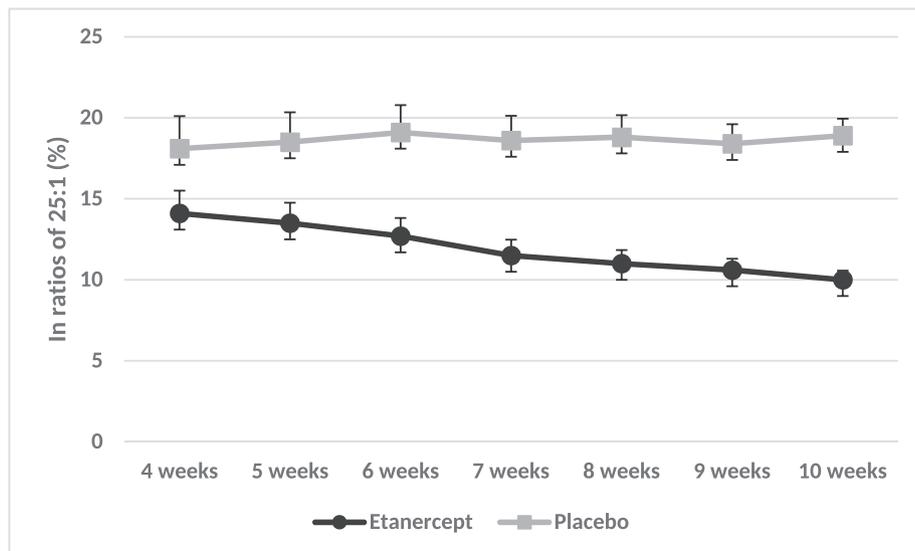


Fig. 2. (A) NK cell activity in ratios of 50:1 in two groups. (B) NK cell activity in ratios of 25:1 in two groups.

of pregnancy complications was very low, which may be due to the physicians' strict supervision, the safe lifestyle during pregnancy and the relatively young age of patients.

In this study, the effect of etanercept on refractory innate immune RSA was evident, with a significant increase in success rate and a remarkable reduction of miscarriages. This is consistent with the retrospective cohort study in 2008 [13], and we excluded the effect of IVIG. The NNT for one additional live birth was 5.74. This result is quite different from the traditional treatment. It has been reported that the NNT of paternal leukocyte transfusion and IVIG were 10 and 6, respectively [18]. It may be because the sample size of this study is small or the inclusion criteria is stricter than other studies, therefore the study results cannot be generalized for all RSA patients. Etanercept was basically safe and had no major adverse reactions except for a mild local skin rash and curable congenital malformations in this study, which is consistent with the prospective observational multicenter cohort study in 2015 [14].

Etanercept has a high molecular weight and requires active transport through human placenta, which is similar to the active transport of IgG and generally occurs only after 20 weeks of gestation. Therefore, etanercept cannot penetrate placenta during early pregnancy, and there is no evidence that etanercept increases the risk of fetal malformation. In this study, there were no differences in newborn weight, Apgar score and congenital malformation between two groups, suggesting that etanercept had no adverse effect on neonates. Although there have been several reports of fetal abnormalities in patients with TNF- $\alpha$  inhibitor, these abnormalities were diverse and no obvious pattern has been observed, which most likely was due to the underlying disease and its activity rather than drug toxicity during pregnancy [19]. A recent prospective observational multicenter cohort study showed that TNF- $\alpha$  inhibitors might carry a risk of adverse pregnancy outcome of moderate clinical relevance [14]. Considering the impact of insufficiently controlled autoimmune disease on the mother and the unborn child, TNF- $\alpha$  inhibitors may nevertheless be a treatment option in women with severe disease refractory to established immunomodulatory drugs [14].

NK cells have cytotoxic effects that can directly kill tumor cells, virus-infected cells and other target cells without antigen sensitization. Studies have shown that NK cell activity was not increased in women with successful pregnancy, but increased in 50% of patients with infertility, biochemical pregnancy and pregnancy loss [20]. Studies have shown that etanercept can not only inhibit TNF- $\alpha$ , but also inactivate NK cells [10,21]. In this study, advanced methods were used to detect the activity of NK cells, and it was found that etanercept could significantly reduce NK cell activity and improve the pregnancy outcome in RSA patients.

Many studies have shown that TNF- $\alpha$  concentration and NK cell activity were increased in RSA patients after pregnancy [5,8]. In this study, the live birth rate is not low in placebo group, maybe because conventional treatment blocked these increase of TNF- $\alpha$  concentration and NK cell activity.

To sum up, our results provide a proof of principle that etanercept can be an attractive therapeutic strategy for refractory innate immune RSA. However, it is far from proven that etanercept is effective and safe in the treatment of all immune RSA patients, and there were not enough pregnant women receiving etanercept to rule out any possible teratogenic effects. As a result, the use of etanercept should remain cautious. So far, few researches have been done on the effect of etanercept on human reproductive

function, and the mechanism of how etanercept interacts with NK cells and immune system remains to be elucidated.

## Conflicts of interest

The authors declare that there is no conflict of interest.

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