

# Expression level of TNF- $\alpha$ in decidual tissue and peripheral blood of patients with recurrent spontaneous abortion

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## Abstract

**Objective:** This study aimed to determine the express level of tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) in the decidual tissue and peripheral blood of patients with recurrent spontaneous abortion (RSA).

**Material and methods:** Eighty RSA patients and 100 control women were recruited in this study. Enzyme-linked immunosorbent assay (ELISA) was applied to determine the expression level of TNF- $\alpha$  in peripheral blood and decidual tissues from both groups. Additionally, the expression level of TNF- $\alpha$  was compared between RSA patients with different numbers of abortions, as well as primary and secondary RSA patients.

**Results:** The expression level of TNF- $\alpha$  in peripheral blood and decidual tissues of RSA patients was significantly higher compared to the controls ( $p < 0.001$ ). Patients who had undergone RSA twice expressed TNF- $\alpha$  in peripheral blood and decidual tissues at a similar level to patients who had experienced RSA three times ( $p > 0.05$ ), but significantly lower than patients who had experienced RSA more than three times ( $p < 0.001$ ). The expression level of TNF- $\alpha$  in peripheral blood and decidual tissues was significantly higher in the secondary RSA patients, when compared with primary RSA patients ( $p < 0.001$ ).

**Conclusions:** Taken together, the relatively high expression level of TNF- $\alpha$  in decidual tissue and peripheral blood may be one of the causes of RSA and therefore could be used as a clinical indicator.

**Key words:** RSA, TNF- $\alpha$ , ELISA, peripheral blood, decidual tissue.

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## Introduction

Recurrent spontaneous abortion (RSA) is used to define individuals who have experienced two or more consecutive spontaneous abortions with the same sexual partner, and RSA has an approximate incidence rate of 1-3% in women of childbearing age [1]. Aside from physical pains, RSA can also cause significant psychological harm to patients. In addition, this disease can bring financial loss to both the affected family and society as a whole [2, 3]. Therefore, understanding the aetiology and pathogenesis of RSA, as well as how to provide preventative measures and clinical treatments, are active areas of research.

There are numerous causes of RSA, including chromosomal, genital abnormalities, endocrine dyscrasia, immune dysfunction, infection, and psychological and environmental factors. Nevertheless, approximately 50% of RSA cases are unexplained and found to be related with certain mater-

nal immune responses against the foetus [4-7]. Among the immunological factors, the abnormal expression of tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) expression in pregnant women is considered a major factor in driving RSA [8, 9].

However, comprehensive investigations of the association between RSA and TNF- $\alpha$  expression level are very limited in China. This study was therefore conducted to investigate the level of TNF- $\alpha$  in decidual tissue and peripheral blood of RSA patients, and to further analyse their correlation, with the aim to improve treatment and prevention for RSA patients.

## Material and methods

**Ethics Statement:** The study was approved by the Ethics Committee of Taishan Medical University (Permit No. ECTSMU2015-008).

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## Study population

### *Inclusion and exclusion criteria of cases*

Individuals with regular menstruation and normal endocrine function were included, whereas those with diabetes and autoimmune thyroid disease were excluded. Patients with abnormal genital structures were excluded after B-ultrasound, hysterosalpingography, and hysteroscopy examinations. Couples who both had normal karyotype analysis results, and consistent ABO and Rh blood types, were included. Individuals were selected who had negative immunology tests for anti-sperm, anti-cardiolipin, and anti-nuclear antibody, as well as negative tests for cytomegalovirus and herpes simplex virus. Those with infections such as ureaplasma and urealyticum were excluded. Inclusion criteria also included no history of contact with toxic mercury or other lead-based chemicals and radiation, as well as unhealthy habits such as smoking, drinking, or heroin use, and no excessive fatigue or strenuous exercise.

A total of 180 abortion cases were recorded at the Maternal and Child Care Service Centre in Laiwu city of Shandong Province, China between January 2015 and December 2015. The patients were aged between 21 and 39 years. All of the abortions occurred within 12 weeks of pregnancy. Among them, 80 RSA patients were assigned to the case group, while the other 100 cases – patients with a normal early pregnancy but who voluntarily decided to terminate the pregnancy – were used as controls.

### *RSA patients*

Out of the 80 cases of early pregnancy RSA patients, there were 32, 25, and 23 cases with two, three, or more than three RSAs, respectively. Thirty-seven of the cases showed the presence of an embryo and primitive heart tube pulsing in ultrasound examinations, followed by the loss of heart rate and termination of embryonic development. Forty-three cases showed the presence of an intrauterine gestation sac with the absence of embryo and primitive heart tube pulsing, resulting in termination of embryonic development. Eighteen cases had a history of live birth and were considered secondary RSA, while 62 cases had no live birth and were considered primary RSA.

### *Control group*

One hundred cases of individuals with a normal early pregnancy, but who chose to discontinue the pregnancy, were selected as controls. All selected individuals were healthy and without other diseases, had at least one or more live birth experience, and had no history of adverse pregnancy including spontaneous miscarriage, stillbirth, or deformed foetus. In addition, pregnancies included in this study had no adverse symptoms such as abdominal pain or vaginal bleeding, and they were checked to ensure that the intrauterine gestation sac was of a size consistent with normal gestational age

and showing clear presence of an embryo and a primitive heart tube pulsing by ultrasound examination.

## Methods

TNF- $\alpha$  expression level in peripheral blood and decidua

**TNF- $\alpha$  expression level in peripheral blood:** Prior to the artificial abortion, 4 ml of blood was collected from all selected patients in both case and control groups using heparin-containing collection tubes. The blood samples were centrifuged at 2000 rpm for 10 min at room temperature, and serum was collected in 1.5-ml Eppendorf tubes. ELISA kits purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) were then used to measure the serum level of TNF- $\alpha$ , following the manufacturer's instructions.

**TNF- $\alpha$  expression level in decidual tissues:** Fresh decidual tissues extracted from all selected patients, in case and control groups, were washed extensively with normal saline to remove residual blood. Remaining saline and blood were dried off using filter paper, and the tissue samples were then weighed and stored in 1.5-ml Eppendorf tubes. For every milligram of decidual tissue 1 ml of 50 mmol/l phosphate buffered saline was added and homogenised in an ice bath using a tissue grinder. Homogenates were centrifuged at 10000 $\times$ g at 4 $^{\circ}$  for 20 min and the supernatants were collected into 1.5-ml Eppendorf tubes. ELISA kits purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) were then used to measure the level of TNF- $\alpha$  in the extracted tissue solution, according to the manufacturer's instructions.

### *Statistical analysis*

SPSS version 19 Software (SPSS, Inc., Chicago, IL, USA) was used to analyse the results. Comparison between two groups was analysed by Student *t*-test, and one-way ANOVA was used to test three or more groups.  $P < 0.05$  was considered statistically significant.

## Results

### **Comparison of background information between both groups**

There was no significant difference in age, body mass index (BMI), gestational age (determined by last menstruation), ultrasonic gestational age, and gravida births between patients from the two groups ( $P > 0.05$ ) (Table 1).

### **Comparison of TNF- $\alpha$ expression level in peripheral blood and decidual tissues between both groups**

The expression level of TNF- $\alpha$  in peripheral blood of RSA patients ( $463.21 \pm 102.15$  ng/l) was significantly high-

**Table 1.** Basic information about subjects in case and control groups (mean ± standard deviation)

Basic information	Case group	Control group	P value
No. of subjects	80	100	
Age (year)	29.03 ±4.40	28.50 ±5.16	> 0.05
Body mass index (BMI) (kg/m <sup>2</sup> )	20.31 ±1.27	20.42 ±1.12	> 0.05
Gestational age (weeks)	9.12 ±1.02	9.08 ±1.17	> 0.05
Ultrasonic gestational age (weeks)	8.02 ±1.13	8.16 ±1.15	> 0.05
Pregnant frequency	3.12 ±1.25	2.98 ±1.43	> 0.05
Childbirth frequency	0.69 ±0.35	0.81 ±0.31	> 0.05

**Table 2.** Comparison of the expression level of TNF-α in peripheral blood and decidual tissue of case and control groups (ng/l, mean ± standard deviation)

	Case group	Control group	P value
No. of subjects	80	100	
Peripheral blood (TNF-α)	463.21 ±102.15	193.51 ±121.76	< 0.001
Decidual tissue (TNF-α)	1921.26 ±527.34	793.82 ±296.43	< 0.001

**Table 3.** Comparison of the expression level of TNF-α in peripheral blood of RSA patients with different number of abortions (ng/l, mean ± standard deviation)

No. of abortions	No. of cases	Peripheral blood (TNF-α)
2	32	409.67 ±102.92
3	25	428.31 ±89.46
≥ 3	23	652.59 ±47.35

**Table 4.** Comparison of the expression level of TNF-α in decidual tissue of RSA patients with different number of abortions (ng/l, mean ± standard deviation)

No. of abortions	No. of cases	Decidual tissue (TNF-α)
2	32	1692.16 ±412.34
3	25	1732.11 ±572.92
≥ 3	23	2721.13 ±290.41

**Table 5.** Comparison of TNF-α expression level in peripheral blood and decidual tissue between patients with primary and secondary RSA (ng/l, mean ± standard deviation)

Groups	No. of cases	Peripheral blood (TNF-α)	Decidual tissue (TNF-α)
Primary	62	399.96 ±102.32	1721.17 ±398.37
Secondary	18	557.62 ±102.55	2271.6 ±457.62
P value	–	<0.001	<0.001

er than that of the control group (193.51 ±121.76 ng/l). The expression level of TNF-α in decidual tissue samples of RSA patients (1921.26 ±527.34 ng/l) was also significantly higher than that of the control group (793.82 ±296.43 ng/l) (Table 2).

**Comparison of TNF-α level in peripheral blood of RSA patients with different numbers of historical abortions**

No significant difference was detected in the expression level of TNF-α in the peripheral blood of patients who had two RSA experiences (409.67 ±102.92 ng/l) compared with patients who had experienced three RSAs (428.31 ±89.46 ng/l). However, a significant difference was observed when the TNF-α level of patients having had two RSAs was compared to those who had experienced three or more RSAs (652.59 ±47.35 ng/l). Similarly, a significant difference was observed between the level of TNF-α in patients who had experienced three RSAs and patients who had experienced three or more RSAs (Table 3).

**Comparison of TNF-α level in decidual tissue of RSA patients with different numbers of historical abortions**

No significant difference was detected in the level of TNF-α in decidual tissues of patients who had experienced two RSAs (1692.16 ±412.34 ng/l) compared to patients who had experienced three RSAs (1732.11 ±572.92 ng/l). However, a significant difference was observed when the TNF-α level of patients having had two RSAs was compared to those who had experienced three or more RSAs (2721.13 ±290.41 ng/l). A significant difference was also observed between the level of TNF-α in patients who had experienced three RSAs and patients who had experienced three or more RSAs (Table 4).

**Comparison of TNF-α level in peripheral blood and decidual tissue between patients with primary and secondary RSA**

The level of TNF-α expression level in both peripheral blood and decidual tissue of patients in the primary RSA group (399.96 ±102.32 ng/l; 1721.17 ±398.37 ng/l) was significantly lower than that in the secondary RSA group (557.62 ±102.55 ng/l; 2271.6 ±457.62 ng/l).

**Discussion**

Tumor necrosis factor acts primarily as an inflammatory cytokine in the immune system, and has a broad range of biological activities, including anti-tumour functions, immunoregulation, inflammatory effects, cytotoxicity, and apoptosis. It is also involved in cell proliferation, differen-

tiation, angiogenesis, and tissue reconstruction [10]. It has been shown that the TNF- $\alpha$  levels in serum and placental tissues of RSA patients (with unknown causes) was significantly higher than the levels in normal pregnant patients at the same gestational stage [11-14]. This indicates that aberrantly expressed TNF- $\alpha$  may play an important role in driving the occurrence of RSA.

This study was conducted to compare the expression of TNF- $\alpha$  in serum and decidual tissue, between patients with RSA and normal pregnancy. The results indicated that the levels of TNF- $\alpha$  in both serum and decidual tissue were significantly higher in the case group compared to the controls, thus linking high expression of TNF- $\alpha$  to RSA. Interestingly, the level of TNF- $\alpha$  detected in the peripheral blood directly reflected the levels measured in the local decidual tissue. Hence, the level of serum TNF- $\alpha$  can be used to guide the clinical treatment of RSA and also to measure the efficacy of immunotherapy [15-17].

A comparison was also made between the expression of TNF- $\alpha$  in RSA patients who had experienced a different number of abortions. No significant difference in the level of TNF- $\alpha$  was found between RSA patients who had experienced two and three abortions, potentially due to the small sample size or the differences in individual factors. Thus, an increased sample size is necessary for future studies. The increase in TNF- $\alpha$  expression was significant in RSA patients who had experienced three or more abortions. An increase in TNF- $\alpha$  expression level was found to correlate with an increase in the number of RSA, indicating that TNF- $\alpha$  may be an important reference for the outcome of pregnancy, and it could potentially serve as a clinical indicator for predicting the occurrence of RSA [18]. The reason for an increase in the expression level of TNF- $\alpha$  as the number of abortions increases could be linked to other possible causes of the abortion, such as maternal infection, inflammation, viral infection, and clotting abnormalities, all of which also increase levels of inflammatory cytokines like TNF- $\alpha$  [19, 20].

A comparison of TNF- $\alpha$  expression between patients with primary and secondary RSA demonstrated that the level of TNF- $\alpha$  in peripheral blood and decidual tissue of secondary RSA patients was significantly higher than that of primary RSA patients. Secondary RSA patients were defined as those having had at least one previous live birth and as a result had some residual foetal or placental antigens, which in turn might act to increase the number and activity level of T helper cells. As a result, higher susceptibility to immune dysfunction, or a reduction in histocompatibility antigens between the foetus and trophoblasts, may occur [21]. The dysregulation of immune function increased the production of TNF- $\alpha$ , resulting in foetal growth retardation, premature birth, or the recurrence of RSA [22]. Alternatively, the imbalance of cytokines at the maternal-foetal interface could be due to a weakened Treg-mediated immunosuppressive effect against foetal

and trophoblast antigens. This leads to over-expression of inflammatory cytokines and induces an increase in TNF- $\alpha$  expression, ultimately increasing the incidence of RSA [17, 23-25].

Since secondary RSA patients have had at least one previous live birth, this demonstrates prior success in embryonic implantation. Thus, the appearance of RSA in a subsequent pregnancy would suggest that other factors can influence the intrauterine environment, such as inflammatory cytokines [19]. However, the mechanisms involved are still unclear, and therefore further studies are required to define this association.

## Conclusions

The abnormal elevation of TNF- $\alpha$  expression level in peripheral blood and decidual tissue of RSA patients may be one of the contributing factors to RSA. The expression level of TNF- $\alpha$  in peripheral blood and decidual tissue is proportional, and the level of TNF- $\alpha$  detected in peripheral blood was found to reflect that in the local tissues. Moreover, TNF- $\alpha$  expression level in peripheral blood and decidual tissue in secondary RSA patients was significantly higher than that in primary RSA patients, indicating that TNF- $\alpha$  has a greater influence on secondary RSA.

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