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Inflammatory cytokines IL-6, IL-10, IL-13, TNF-α and peritoneal fluid flora were associated with infertility in patients with endometriosis

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Abstract. – OBJECTIVE: To investigate the correlations of inflammatory factors, interleukin-6 (IL-6), IL-10, IL-13 and tumor necrosis factor-α (TNF-α), and composition of bacterial flora in the peritoneal fluid with infertility in endometriosis patients.

PATIENTS AND METHODS: A total of 55 patients diagnosed with endometriosis and infertility in the Gynecology Clinic of our hospital from June 2014 to July 2017 were selected as observation group, and another 30 non-endometriosis and non-infertility patients were enrolled as control group. The peritoneal fluid was extracted from patients in both groups, and the total white cell count and the percentage of leukocyte subset were determined. The total genome deoxyribonucleic acid (DNA) of microorganisms in peritoneal fluid was extracted, and the composition of microorganisms was analyzed using the Ion Torrent PGM platform (BGI). The levels of IL-6, IL-10, IL-13, and TNF-α in peritoneal fluid were detected via enzyme-linked immunosorbent assay (ELISA). Moreover, the correlations of inflammatory factors in the peritoneal fluid with endometriosis complicated with infertility were analyzed via Logistic regression analysis.

RESULTS: The total white cell count, monocytes, neutrophils, eosinophils and basophils in endometriosis patients complicated with infertility were significantly higher than those in control group ($p<0.05$). Results of ELISA showed that the levels of IL-6, IL-10, IL-13, and TNF-α in peritoneal fluid of endometriosis patients complicated with infertility were significantly higher than those in control group ($p<0.05$). In peritoneal fluid of patients in both groups, Proteobacteria and Firmicutes were mainly dominated, followed by Actinobacillus, Bacteroidetes, Fusobacteria and Tenericutes, and there was no significant difference in the Eumycota between the two groups ($p>0.05$). Logistic regression analysis results showed that there were significant correlations of inflammatory factors (IL-6, IL-10, IL-13, and TNF-α) with endometriosis complicated with infertility.

CONCLUSIONS: There are many kinds of Eumycota in the peritoneal fluid of endometriosis patients complicated with infertility, but they are not the main pathogenic factors. Inflammatory factors (IL-6, IL-10, IL-13, and TNF-α) can be used as important reference indexes for the diagnosis of endometriosis complicated with infertility.

Key Words
Inflammatory factors, Peritoneal fluid, Composition of bacterial flora, Endometriosis patients, Infertility.

Introduction

Endometriosis is one of the common chronic diseases in women, which is characterized by the ectopia of endometrial cells and stroma outside the uterine cavity. Its clinical symptoms are often manifested as chronic pelvic pain and infertility[1]. Endometriosis causes severe pelvic pain and infertility in 15-25% women at child-bearing age, and its benign lesions can result in peritoneal inflammation, fibrosis, adhesions and ovarian cyst, and even show characteristics of malignant tumors in a small number of patients, such as neovascularization, local infiltration, and distant metastasis[2,3]. Due to the unknown etiology, inconsistent clinical manifestations and difficult diagnosis of endometriosis, the treatment is not standardized. Studies have found that the pathogenesis of the disease is very complicated, and it is related to the mechanical, hormonal, immune, environmental and genetic factors, but the specific pathogenesis is inconclusive[4]. New evidence suggests that bacteria-mediated inflammatory responses are involved in the occurrence and progression of endometrio-

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sis. Recent studies have shown that bacterial infection increases the occurrence of endometriosis in macaques[5]. It is also reported *via* the analysis of Escherichia coli in menstrual blood of patients with endometriosis that its colony formation is significantly higher than that in non-endometriosis women. Bacterial endotoxin can activate pelvic macrophages to release the secondary inflammatory mediators, which is also an important reason for infertility in patients at child-bearing age[6]. However, the specific bacterial colonies and inflammatory factors involved in the infertility of patients with endometriosis are not very clear.

Therefore, in this study, 55 endometriosis patients complicated by infertility and 30 non-endometriosis patients complicated by infertility were selected. The levels of inflammatory factors [interleukin-6 (IL-6), IL-10, IL-13, and tumor necrosis factor-α (TNF-α)] and colony formation in peritoneal fluid of subjects were detected, respectively, and their correlations with endometriosis complicated by infertility were analyzed.

Patients and Methods

Clinical Data

A total of 55 patients diagnosed with endometriosis and infertility *via* laparoscopy from June 2014 to July 2017 were enrolled in this study, and 30 non-endometriosis patients complicated by infertility were included into the control group. None of the patients received hormonal therapy at 2 months before the operation, and no patients were pregnant or lactated in the past 6 months. This study was approved by the Ethical Review Committee of our hospital, and all patients enrolled signed the informed consent.

Collection of Peritoneal Fluid

Under the direct vision of laparoscope, the trocar was inserted to quickly extract 10 mL peritoneal fluid from the vesicouterine pouch and rectovaginal pouch; then the fluid was placed in a 15 mL sterile centrifuge tube and centrifuged at 3000 rpm at 4°C for 10 min. The supernatant and sediment were taken, placed into 1.5 mL centrifuge tubes and stored in a refrigerator at -80°C.

Total DNA Extraction, Amplification, and Detection of Microbial Genome

The total genomic DNA was extracted from the bacterial microbes in peritoneal fluid samples *via* silica membrane-centrifugal column specific adsorption principle using the DNA Mini Kit (Art. No.: EE101-01, TransGen Biotech, Beijing, China). Specific operations were in strict accordance with the instructions. Polymerase chain reaction (PCR) amplification was performed for the total DNA extracted, and the product was subject to the 0.6% agarose gel electrophoresis to detect the integrity of DNA fragments. Purification and recovery were performed using the Quick PCR Purification Kit (Art. No.: EE121-01, TransGen Biotech, Beijing, China). The total genomic DNA was sent to the Ion Torrent PGM platform of BGI for backward sequencing (V5→V4).

Detection of IL-6, IL-10, IL-13, and TNF-α Levels in Peritoneal Fluid Via Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of inflammatory factors in peritoneal fluid of patients in two groups were detected *via* horseradish peroxidase-labeled ELISA. Briefly, IL-6 (RD, Art. No.: D6050, New York, USA), IL-10 (RD, Art. No.: DY417), IL-13 (RD, Art. No.: M1300CB) and TNF-α (RD, Art. No.: 210-TA) antibodies were coated in each well of a 96-well ELISA plate. An appropriate amount of serum was added for incubation. Tetramethylbenzidine was used as the substrate to determine the amount of enzyme and enzyme-binding TNF-α. The optical density (OD) values were detected using a microplate reader at a dual wavelength of 450 nm and 600 nm, and the sample concentration was calculated.

Statistical Analysis

GraphPad Prism software (Version 5.01, GraphPad Software, Santiago, Chile) statistical software was used for analysis. Measurement data were presented as $\bar{x}\pm s$, and Student's *t*-test was used to analyze the differences between observation group and control group. Logistic regression analysis was performed to detect the correlations of inflammatory factors and bacterial colonies in the peritoneal fluid with endometriosis complicated by infertility. $p<0.05$ suggested that the difference was statistically significant.

Results

General Conditions of Patients in Experimental Group and Control Group

55 women with endometriosis complicated by infertility met the requirements and were included

Table I. Baseline conditions of patients in experimental group and control group.

Group	Control group (n=30)	Endometriosis + infertility group (n=55)	*t*	*p*
Age (years old)	37.7±7.4	37.2±8.2	0.81	>0.05
BMI	22.9±2.1	22.5±2.3	0.51	>0.05
Menstrual cycle (d)	27.5±5.3	27.1±6.4	0.69	>0.05
Menstrual period (d)	4.5±1.5	5.7±1.2	0.63	>0.05
Times of pregnancy (time)	1.7±0.6	1.4±0.7	0.92	>0.05

into this study. 28 cases (50.91%) were diagnosed with stage I-II endometriosis, and 27 cases (49.09%) were diagnosed with stage III-IV endometriosis. The statistical data of general conditions of patients in each group are shown in Table I. There were no significant differences in the age, body mass index (BMI), menstrual cycle, menstrual period and times of pregnancy between two groups (p>0.054).

White Blood Cell Subset Frequencies in Peritoneal Fluid

The total white blood cell count in peritoneal fluid of patients in endometriosis + infertility group was significantly higher than that in control group (p<0.05). In addition, monocytes, neutrophils, eosinophils, and basophils in endometriosis + infertility group were significantly increased (p<0.05). However, there was no significant difference in the proportion of lymphocytes in the peritoneal fluid between two groups (p>0.05) (Table II).

Inflammatory Factor Levels in Peritoneal Fluid of Patients in Each Group

The levels of inflammatory factors in peritoneal fluid of patients were detected *via* ELISA. IL-6, IL-10, IL-13, and TNF-α levels in peritoneal fluid of patients in endometriosis + infertility group were significantly higher than those in control group (p<0.05) (Table III).

Bacterial Colonies in Peritoneal Fluid

As can be seen from Figure 1, a variety of bacteria were detected in peritoneal fluid of pa-

Table II. Comparisons of total white blood cell count and percentages of white blood cell subsets in peritoneal fluid.

Group	Control group (n=30)	Endometriosis + infertility group (n=55)	*t*	*p*
White blood cell count ($\times 10^3$/mL)	317	574	4.26	<0.05
Lymphocyte (%)	33.2	36.83	0.94	>0.05
Monocyte (%)	39.2	26.73	3.82	<0.05
Neutrophil (%)	8.13	24.35	6.31	<0.05
Eosinophil (%)	5.52	3.14	1.87	<0.05
Basophil (%)	0.91	1.31	1.52	<0.05

Table III. Inflammatory factor levels in peritoneal fluid of patients in each group.

Group	Control group (n=30)	Endometriosis + infertility group (n=55)	*t*	*p*
IL-16 (pg/mL)	18.14±9.03	48.15±9.83	5.21	<0.05
IL-10 (pg/mL)	33.74±8.32	46.86±10.42	3.45	<0.05
IL-13 (pg/mL)	26.34±8.25	48.36±7.43	4.28	<0.05
TNF-α (pg/mL)	37.06±12.73	87.29±14.26	7.33	<0.05

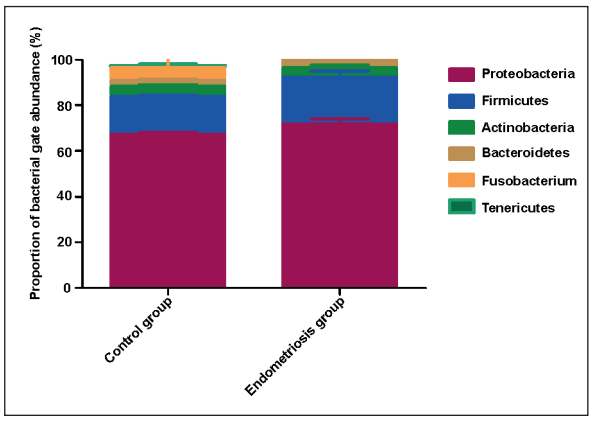

Figure 1. Bacterial colonies in peritoneal fluid of patients in two groups.

tients in both groups, mainly *Proteobacteria* and *Firmicutes*, followed by *Actinobacteria*, *Bacteroidetes*, *Fusobacterium*, and *Tenericutes*. Patients in two groups had no significant differences in levels of bacteria (p>0.05).

Correlations of Inflammatory Factors in the Peritoneal Fluid with Endometriosis Complicated by Infertility

There was no significant relationship between bacteria and endometriosis complicated by infertility, so Logistic analyses were performed with the endometriosis complicated by infertility as the dependent variable, and inflammatory factors (IL-6, IL-10, IL-13, and TNF-α) in peritoneal fluid as independent variables, and the results showed that inflammatory factors (IL-6, IL-10, IL-13 and TNF-α) had significant correlations with endometriosis complicated by infertility (Table IV).

Values of Inflammatory Factors in the Diagnosis of Disease

Receiver operating characteristic (ROC) curves were drawn according to the levels of IL-6, IL-10, IL-13 and TNF-α in peritoneal fluid of patients. The results showed that the areas under the curve (AUCs) of IL-6, IL-10, IL-13, and TNF-α were 0.893, 0.685, 0.661 and 0.893, respectively, with IL-6 ≥ 23.64 pg/mL, IL-10 ≥ 35.83 pg/mL, IL-13 ≥ 29.31 pg/mL and TNF-α ≥ 43.17 pg/mL as critical values (Table V & Figure 2).

Discussion

In the vagina of healthy women, there are many Gram-positive and Gram-negative aerobic and facultative anaerobic microorganisms, which are normal vaginal flora[7]. Bacteria are generally thought to play roles in diseases, such as sexually transmitted diseases and urinary infections. However, recent studies have found that the microbial growth in endometrial samples is closely related to the occurrence of endometriosis[8]. Plous et al[9] proposed the bacterial contamination hypothesis as a new concept of etiology of endometriosis. Moini et al[10] found that under certain conditions, certain Gram-negative bacteria can break through the cervix and migrate from the vagina to the uterine wall, resulting in pollution of menstrual blood, accumulation of toxins, peritoneal fluid, and pelvic

Table IV. Correlations of inflammatory factors in peritoneal fluid with endometriosis complicated by infertility.

Index	WALD value	*p*	Odds ratio (OR)	95% confidence interval (CI)
IL-6	1.432	<0.05	4.260	3.017-7.835
IL-10	1.224	<0.05	5.736	4.271-9.337
IL-13	1.023	<0.05	4.261	2.882-7.261
TNF-α	1.792	<0.05	8.346	7.354-10.386

Table V. Values of inflammatory factors in the diagnosis of disease.

Item	AUC	Standard error	*p*	95% confidence interval (CI)
TNF-α	0.903	0.065	<0.05	0.723-0.974
IL-6	0.873	0.082	<0.05	0.694-0.852
IL-10	0.705	0.057	<0.05	0.653-0.819
IL-13	0.661	0.059	<0.05	0.602-0.827

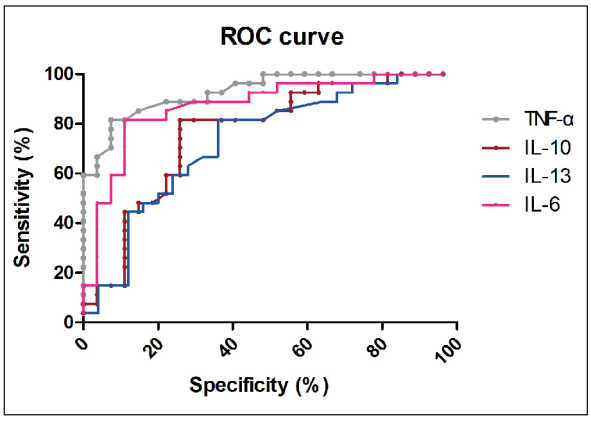

Figure 2. Values of inflammatory factors (IL-6, IL-10, IL-13, and TNF-α) in peritoneal fluid in the diagnosis of disease.

inflammatory disease. However, there have been no relevant reports on bacteria in peritoneal fluid and endometriosis yet so far.

It is now recognized that endometriosis is a kind of common chronic inflammatory disease, as well as one of the important causes of female infertility[11]. Specifically, after the sperm comes into the uterine cavity, bacterial toxins, leukocyte phagocytosis, and other inflammatory factors lead to death or reduce the motility of sperm, so that the number of sperm into the fallopian tubes is decreased. Secondly, fertilized eggs are not easy to be implanted in endometria with inflammation, or the endometrial antibody results in implantation dysfunction. And abortion occurs easily due to unstable implantation of fertilized egg, leading to infertility[12,13]. In this experiment, the analysis *via* Ion Torrent PGM platform found that a variety of bacteria were detected in peritoneal fluid of patients in both groups, mainly *Proteobacteria* and *Firmicutes*, followed by *Actinobacteria, Bacteroidetes, Fusobacterium,* and *Tenericutes*. Patients in two groups had no significant differences in levels of bacteria.

Furthermore, the patient's peritoneal fluid was extracted, and an increase in the white blood cell count in peritoneal fluid of women with endometriosis was observed. This result supports the inflammatory hypothesis that the number of immune cells entering the peritoneal cavity is increased in endometriosis, especially in the early stage[14]. In addition, it was found[15] that there is a high proportion of neutrophil in the abdominal cavity of patients with endometriosis. Neutrophil in the abdominal cavity can secrete VEGF, an effective pro-angiogenic factor, increasing its level in the peritoneal fluid in endometriosis. Therefore, neutrophils may support the growth of endometriotic lesions by secreting VEGF. Most importantly, monocytes in peritoneal fluid of patients with endometriosis were significantly reduced in this study. Monocytes can migrate into tissues and differentiate into macrophages. Moreover, Fakih et al[16] found that macrophages induce the secretion of a variety of inflammatory cytokines, thus playing a positive role in the occurrence, maintenance, and progression of endometriosis. Studies have found that the menstrual flow causes an inflammatory response and releases different chemotactic proteins in the pelvic cavity, attracting peripheral blood mononuclear cells into the peritoneum, and these cells become mature into macrophages in a time-dependent manner. Mature macrophages can lurk in peritoneal fluid or intact tissues, which can also respond to external or endogenous stimuli to produce inflammatory mediators[17]. As a part of the innate immune system, these activated macrophages release cytokines or growth factors that are suitable for the growth of endometriosis[18]. Others[19] have found the elevated level of chemotactic factor of eosinophil in peritoneal fluid in patients with endometriosis.

The intraperitoneal immune system is a complex network that contains different cell types and their products secreted, which may interact through autocrine/paracrine system. Functional deviation in local immune cells includes B lymphocytes, T lymphocytes, monocytes, macrophages, and NK cells in the peritoneal fluid, which may play key roles in the pathophysiological process of endometriosis by secreting different cytokines[20]. Therefore, in this report[20], the expression levels of inflammatory factors were analyzed *via* ELISA. It was found that IL-6, IL-10, IL-13, and TNF-α in the peritoneal fluid of patients with endometriosis were highly expressed. IL-6 is a pleiotropic cytokine produced by many types of cells, including lymphocytes and monocytes. Studies[21] have found that IL-6 is an activator of macrophages, which can promote the proliferation of endometrial cells. Activated macrophages further induce the secretion of IL-10, IL-13, and TNF-α. TNF-α can mediate immune and inflammatory responses, resulting in local pelvic adhesions, fibrosis and immunological abnormality, which are conducive to the formation of ectopic lesions. In addition, the elevated TNF-α concentration in the peritoneal fluid can directly reduce the sperm motility, thus affecting the entire process of fertilization and implantation, showing embryo toxicity, and resulting in increased infertility in patients wi-

th endometriosis. In addition, the elevated TNF-α concentration in peritoneal fluid can directly reduce the sperm motility, thus affecting the entire process of fertilization and implantation, showing embryo toxicity, and resulting in increased infertility in patients with endometriosis[22].

Conclusions

We showed that there were a variety of bacteria in peritoneal fluid of patients with endometriosis; however, bacteria were not the main factors affecting endometriosis and infertility in this experiment, while the inflammatory response may be the main factor leading to the ectopic growth of endometrial tissues and infertility.

Conflict of Interest

The authors declared no conflict of interest.

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