The impact of HSF on endometrium

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SUMMARY

Objective: We conducted the research in order to explore the impact of hydrosalpinx fluid (HSF) on endometrium.

Method: HSF group: 261 patients with HSF scheduled to undergo laparoscopic surgery 3 to 7 days after menstruation in our center. Hysteroscopy would also be performed in order to observe the endometrial morphology during the surgery. Sixty (60) patients would be randomly selected for endometrial biopsy in order to detect the inflammatory cytokines TNF-α and IL-2 mRNA. Non-HSF group: 210 patients with no evidence of HSF due to chronic salpingitis or pelvic adhesion. IVF-ET treatment was performed after eliminating the factor of male infertility and hysteroscopy was conducted before the treatment. Fifty (50) patients underwent endometrial biopsy in order to detect TNF-α and IL-2 mRNA.

Results: Hysteroscopy was performed in 261 patients with HSF and 210 patients without HSF. The incidence rate of endometritis manifestation among these two groups of patients was 37.2% (97/261) and 20.5% (43/210), respectively. The incidence rate of endometritis in the patients with HSF is significantly higher than in the patients without HSF (p<0.05). Sixty (60) patients from the HSF group and 50 patients from the non-HSF group were regrouped according to inflammatory and normal manifestation after the endometrial biopsy. There were 49 patients in the inflammatory manifestation group and 61 patients in the normal manifestation group. RT-PCR technology was adopted to detect the expression of inflammatory cytokines TNF-α and IL-2 mRNA in endometrial tissue. The level of TNF-α mRNA expression in endometrial tissues with inflammatory manifestation was higher than in normal endometrium (76.75±11.95 vs. 23.45±9.75, p<0.01). There are significant differences between them. The level of IL-2 mRNA expression in endometrial tissues with inflammatory manifestation was higher than that found in normal endometrium (80.56±13.35 vs. 35.12±8.35, p<0.01). There are significant differences between them.

Conclusion: Chronic endometritis is related to HSF and may therefore affect endometrial receptivity.

Keywords: HSF, endometrium, immunohistochemistry, polymerase chain reaction.

INTRODUCTION

Endometrial receptivity is an important factor to determine the success of pregnancy and hydrosalpinx fluid (HSF) may have a bad effect on endometrial receptivity through various mechanisms. Using immunohistochemical examination on patients with HSF after endometrial biopsy, Mayer et al.1 found that endometrial integrin αvβ3 of the patients with HSF during implantation window period significantly decreased compared with the non-HSF control group, but the above endometrial integrin had clearly improved in expression after salpinx surgery. As integrin αvβ3 is an important index for endometrial receptivity, it can be concluded that HSF influences the endometrial receptivity. In fact, many studies in recent years showed that indexes of endometrial receptivity for HSF patients including endometrial inflammatory re-
sponse, integrin, matrix metalloproteinases, endometrial blood flow, leukemia inhibitory factor are moderately affected. Our study explores the connection between HSF patients and chronic endometritis (CE) and verifies whether HSF would influence endometrial receptivity by inducing CE. We observed the general performance of endometrium in patients with HSF, first through hysteroscopy and then collecting endometrial samples from different patients for the detection of TNF-α and IL-2 mRNA in order to assess whether HSF has negative effects on the endometrium.

**METHOD**

**Study object**

Four hundred seventy-one (471) patients undergoing infertility treatment in the Reproductive Medicine Centre due to salpingitis from July 2010 to June 2014 were chosen. Their average age was 31.5 years and infertility lasted from 2 to 9 years.

**Inclusion criteria**

- (1) The female partner of infertile couples needing treatment due to salpingitis.
- (2) Age range from 25 to 35 years old.
- (3) Normal menstrual cycle lasting from 25 to 35 days with a variation of 3 days.
- (4) Weight range from 45 to 70 kg and body mass index between 18 and 25 kg/m².

**Exclusion criteria**

- (1) Infertility due to unknown reasons other than salpingitis.
- (2) Routine examination of male semen abnormal more than twice.
- (3) Female partner with one of the following endocrine abnormalities: polycystic ovary or polycystic ovary syndrome, hyperprolactinemia.
- (4) Female partner presenting abnormal cervical cytological examination: HPV infection or atypical cell hyperplasia or more severe lesions indicated by TCT.
- (5) Female partner’s baseline endocrine levels (menstruation D2 ~ D5) FSH and/or LH > 10 IU/mL.
- (6) Acute inflammation of the genital tract.
- (7) Organic lesions present including submucosal uterine fibroids, endometrial polyps, uterine adhesions.

**Diagnostic criteria**

HSF group: HSF was indicated by hysterosalpingography (HSG). Non-HSF group: no HSF found after HSG that would be caused by chronic tubal inflammation or pelvic adhesion.

**Experiment group**

HSF group: 261 patients with HSF scheduled to undergo laparoscopic surgery 3 to 7 days after menstruation in our center. Hysteroscopy would also be performed in order to observe the endometrial morphology during the surgery. Sixty (60) patients would be randomly selected for endometrial biopsy in order to detect the inflammatory cytokines TNF-α and IL-2 mRNA.

Non-HSF group: 210 patients with no evidence of HSF due to chronic salpingitis or pelvic adhesion. IVF-ET treatment was performed after eliminating the factor of male infertility and hysteroscopy was conducted before the treatment. Fifty (50) patients underwent endometrial biopsy in order to detect TNF-α and IL-2 mRNA.

**Experiment methods**

**Methods and procedures for endometrial examination with hysteroscopy**

- (1) Evaluation time: the best period is the early to the middle stage of endometrial proliferation, i.e., 3 to 7 days after menstruation.
- (2) Anesthesia: Hysteroscopy can be performed in outpatient setting without anesthesia and few patients need intravenous or general anesthesia. In the case of inpatients who prepare to undergo combined laparoscopic surgery, general anesthesia was used.
- (3) The objective lens of hysteroscope is placed into the cervical canal slowly under direct vision after routine disinfection and speculum placement. At the same time, physiological saline is applied for cervical canal expansion and uterine distention, the distending pressure is 100-120 mmHg.
- (4) Endometrial morphology observation: Normal uterine cavity was covered by newly formed smooth endometrium, yellowish and reddish, with few blood capillary and open glandular ducts (Figure 1). Endometritis patients often show endometrial hyperemia, edema and exudation, even necrosis. In this study, the biopsy would be performed only for patients with normal endometrial appearance and inflammatory manifestations like endometrial hyperemia, edema etc. Other lesions such as endometrial polyp, submucosal myoma of uterus etc. were excluded from our study. Diagnostic criteria for CE under hysteroscope can refer to the description of Zolghadri et al. endometrial hyperemia presents a crimson red or fire red color and the subepithelial vascular network is significantly dense and thickened (Figure 2).
- (5) Sampling: endometrial tissue was taken as specimen by a slim catheter (Wallace) after hysteroscopy. The sam-
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FIGURE 1. Normal uterine cavity: smooth endometrium, in pale red color without abnormal blood vessel.

FIGURE 2. Uterine cavity with inflammatory manifestation: endometrium presents purple red or red colors after hyperemia and the subepithelial vascular network is significantly dense and thickened.

ple was then rinsed by physiological saline in order to reduce blood contamination as much as possible, inserted into a 1.5 mL Ep tube with high pressure sterilization and DEPC water treatment and then stored in a freezer at -70°C ultra-low temperature in order to extract RNA from the tissue during RT-PCR detection.

Detection of the expression of TNF-α and IL-2 mRNA in endometrium with RT-PCR

Relevant reagents: Total RNA extraction kit with Trizol reagent produced by Invitrogen Company (USA). RNA reverse transcription kit, 10 mmol/L dNTPs, 5 U/uL Taq DNA polymerase and agarose were all products of Promega Company. Synthesis of PCR primers was entrusted to Sangon Biotech (Shanghai) Co., Ltd. and TNF-α primer sequences were: upstream primer 5'-G A G T G A C A A G C C T G T A G C C C C-3', downstream primer 5'-G C A A T G A T C C A A A G T A G A C C-3' with the amplified product length of 363 bp (Figure 3). And the primer sequences of Internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were: upstream primer 5'-C C A C C A C C A C T C T C A C C A C C T T C T T G -3' with the amplified product length of 572 bp. IL-2 primer sequences were: upstream 5'-C T G G A G C A T T A C T G C T G G A T -3', downstream 5'-G C C T T C T T G G G C A T G T A A A A C C-3' with the amplified product length of 110 bp (Figure 4).

The specific procedure in the RT-PCR experiment was as follows:

- (1) Treatment of endometrium: about 100 mg fresh frozen endometrial tissue sample was cut into small pieces, cleaned with D-Hanks liquid and centrifuged for 12 minutes in a low temperature centrifuge at a temperature of 4°C and 10,000 rpm. The supernatant was removed and the tissue sediments were washed for 2 to 3 times with D-Hanks liquid.
- (2) Total RNA extraction: total RNA was extracted according to procedures in the manual of total RNA extraction kit with Trizol reagent. The optical density values of A_{260} and A_{280} were determined using a DU800 spectrophotometer from Beckman Coulter with a ratio of ranging from 1.8 to 2.0. The concentration of total RNA was then calculated. At the same time, total RNA electrophoresis yielded three strips of 28S, 18S and 5S or two strips of 28S and 18S, showing that RNA quality met the requirements (Figure 5).
- (3) cDNA synthesis: reverse transcription kit from Invitrogen Company was adopted to synthesize cDNA according to the manual. The cDNA obtained from reverse transcription was used as a template for PCR reaction.
- (4) PCR reaction: cDNA of 2 μL, primer of 200 nmol/L, dNTP of 150 μmol/L, Taq DNA polymerase 1 U and corresponding buffer solution were included in the 25 μL reaction system. TNF-α, IL-2 and internal reference GAPDH in type 9600 DNA cycler (Perkin Elmer Cetus) were amplified according to the reaction conditions. The amplification conditions of the three genes were as follows: pre-denaturation for 3 minutes at 94°C in the beginning; repeated cycling 35 times for 30 seconds at 94°C, for 30 seconds at 55°C, for 1 minute at 72°C; 7 additional minutes at 72°C in the end.
Identification of amplification products: Absorbance scanning for TNF-α, IL-2 and GAPDH was performed by using GD 2000 gel scanning and analyzing system D with pUC Mix treated as molecular weight standard after taking PCR amplification product of 10 μL, 2% of agarose gel electrophoresis and ethidium bromide staining (including the ethidium bromide with a final concentration of 0.5 μg/mL). And then the ratio of gene expression of TNF-α and IL-2 to that of GAPDH should be calculated respectively.

Statistical method
SPSS 11.0 statistical software was used for data analysis. The obtained data was expressed as $\bar{x} \pm s$ and tested with $t$-test. And enumeration data was detected by $\chi^2$ check. Statistical significance was defined as $p<0.05$.

Results
Hysteroscopy result
In this study, hysteroscopy was performed in 261 patients with HSF and 210 patients without HSF. The incidence rate of endometritis in these two groups of patients were 37.2% (97/261) and 20.5% (43/210), respectively. As shown in Table 1, the incidence rate of endometritis in the patients with HSF is significantly higher than that of patients without HSF.
Sixty (60) patients from HSF group and 50 patients from non-HSF group were regrouped according to the inflammatory or normal status after endometrial biopsy. There were 49 patients in the inflammatory manifestation group and 61 patients in normal group. RT-PCR technology was adopted to detect the expression of inflammatory cytokines TNF-α and IL-2 mRNA in endometrial tissue.

Result of RT-PCR detection of TNF-α and IL-2 mRNA in endometrium
- (1) The level of TNF-α mRNA expression in endometrial tissue with inflammatory manifestation was higher than that seen in normal endometrium (p<0.01). There was significant differences between them (Table 2).

| Group                        | Total number of cases (n) | Number of cases with inflammatory manifestation (n) | Inflammatory manifestation rate (%) |
|------------------------------|----------------------------|-----------------------------------------------------|-------------------------------------|
| HSF group                    | 261                        | 97                                                  | 37.2 (97/261)                       |
| Non-HSF group                | 210                        | 43                                                  | 20.5 (43/210)*                      |

p<0.05* compared with HSF group.

- (2) The level of IL-2 mRNA expression in endometrial tissues with inflammatory manifestation was higher than in normal endometrium (p<0.01). There was significant difference between them (Table 3).

| Group                        | Number of cases | Relative content of TNF-α (%) |
|------------------------------|-----------------|------------------------------|
| Inflammatory manifestation group | 49              | 76.75±11.95                  |
| Normal manifestation group    | 61              | 23.45±9.75                   |

p<0.01

Discussion
Embryo quality and endometrial receptivity are two key factors that influence the clinical outcome of pregnancy. The embryo implantation process undergoes many special changes, involving a series of signal transduction processes both cellular and molecular. Hormones produced by the ovaries can initiate autocrine or paracrine activities of various downstream cytokines, trigger expression of integrin and other adhesion molecules and then mediate the mutual recognition between embryo and endometrium in order to prepare for implantation. For example, estrogen and progesterone can regulate the expression of HOXA10 in endometrium, which affects the expression of integrin β3 in endometrium, being closely related to the change seen in endometrial hyperplasia and pregnancy. Almost all of the studies for endometrial receptivity show that HSF hinders embryo implantation by reducing endometrial receptivity. Since HSF is a manifestation of sequelae of pelvic inflammatory disease, it is speculated that HSF may be related to CE. However, the related research is still rarely reported up to now. The value of hysteroscopy in evaluating intrauterine environment has already reached a consensus that the correct treatment of lesions after hysteroscopy does not only improve the pregnancy rate, but also has a predictive value on the pregnancy outcome of IVF treatment after hysteroscopy. But, in hysteroscopy, people usually pay close attention to the treatment of endometrial polyps, endometrial hyperplasia, submucosal uterine myoma, uterine adhesion and other uterine organic lesions, and often ignore the adverse effects of minor lesions (like CE) in intrauterine environment. CE may lead to infertility and abortion. It mainly presents as endometrial matrical edema, local punctate or diffuse hyperemia, under hysteroscope. Under a microscope, in turn, delayed maturation of endometrium and stroma (influenced by inflammation) can be seen, while capillary and venous sinus have enlarged and remained in that state, with presence of many different plasma cells and lymphocytes infiltrated in the endometrial stroma. The presence of plasma cells is one of the mainstays of CE diagnosis. Recently, a retrospective analysis reported the sensitivity and specificity of hysteroscopy in the diagnosis of CE compared with a pathological analysis: the incidence rate of the abnormalities such as endometrial polyp, hyperemia etc. is 66.3% with hysteroscope, while the incidence rate of CE reported after pathological analysis is 43.6%. Taking pathological diagnosis as standard for calculation, the sensitivity and specificity of hysteroscopy in the diagnosis of CE is 35.23% and 67.54%, respectively. It has been found that, compared with histological examination, hysteroscopy can reflect more effectively inflammatory conditions of the uterine cav-
It is not clear but better ways to diagnose CE are still needed. Whether CE affects embryo implantation is still controversial; the high incidence rate of CE in patients with embryo implantation failure is an indisputable fact.10,11

Inflammatory cytokines are important mediators for the occurrence of CE and both TNF-α and IL-2 play an important role in the process of inflammatory response.12 TNF-α is mainly expressed by mononuclear macrophages, CD4+Th1 cells and natural killer (NK) cells etc. in the immune system. Besides the expression in immune cells, TNF-α can also be expressed in reproductive tissues such as ovary, salpinx, uterus, placenta etc. And it also takes part in the process of gametogenesis, embryonic development, follicle growth and steroid hormone synthesis etc., through autocrine and paracrine action. There must be a right amount of TNF-α in pregnant women to maintain pregnancy, but TNF-α at high concentrations may lead to a series of inflammatory lesions, stimulate the production of multiple inflammatory factors such as IL-1, IL-6, NO etc. and result in the occurrence of inflammation and damage in tissues by activating inflammatory cells and upregulating adhesion molecules, NO and oxygen free radicals. After detecting the levels of IL-6 (interleukin-6), IL-1β and TNF-α in menstrual blood of the patients diagnosed as CE by hysteroscopy and histology in follicular phase of the previous menstrual cycle, Tortorella et al.13 believed that proinflammatory cytokines IL-6 and TNF-α can be used as a biomarker for CE. TNF-α also has a direct toxic effect on endometrium and harms the decidua vessel, which brings about contraction of vascular smooth muscle, embolism of the embryonic blood-supply system and tissue necrosis.14 IL-2 is the glycoprotein produced and secreted by activated T cells with a molecular weight of about 15kD. At the same time, it is necessary for T cell proliferation, as well as a key mediator of the immune response. It plays a key role in the cellular immune response and possesses a mechanism of promoting the biological activity of T cells and B cells.15 IL-2 may also be expressed by endometrial glandular cells but excessive expression of IL-2 may affect embryo implantation.16 IL-2 level reflects the body’s cellular immune state to some degree and IL-2 has a function of anti-tumor, anti-microbial infection, also inducing graft rejection, autoimmunity and immune regulation. Therefore, detection of IL-2 levels is a sensitive index to assess the immune activation status of the body. TNF-α and IL-2 are all cytokines secreted by Th1 cells and play a key role in the inflammatory response. Piccinni et al.17 found that Th1 cytokines can downregulate the expression of LIF, and IL-2 is an indispensable cytokine in embryo implantation. HSF, in turn, may influence the endometrial receptivity by decreasing the expression of LIF in the endometrium during the implantation window period.16 After hysteroscopy, we found that the incidence rate of the inflammatory manifestation of endometria in patients with HSF and the expression of TNF-α and IL-2 in endometrial issues with inflammatory manifestation increased significantly, indicating that inflammatory cytokines such as TNF-α and IL-2 in the endometrium of patients with HSF were involved in the inflammatory manifestation of endometrium. In the process of embryo implantation, the matrix was in a state of immune tolerance with Th2 type immune response in a dominant position. The high expression of TNF-α and IL-2 in endometrium of patients with HSF may also indicate that the local immune balance of the endometrium biases towards the Th1 type immune response, which affects embryo implantation.

Due to limitation of experimental conditions, we only tested the typical TNF-α and IL-2 instead of detecting a large number of inflammatory cytokines. Detection of endometrial receptivity only involves inflammatory response, which may result in probable error for the conclusion. Further studies on the impact of HSF on endometrial receptivity are still needed.

**Conclusion**

CE is related to HSF, and endometrial receptivity may be influenced by HSF.

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