The serum level of C-reactive protein in patients undergoing GnRH agonist protocols for in vitro fertilization cycle

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Summary

Background: The synchronization of the uterus and mature eggs at the molecular level is the key factor in embryo transfer, and the regulation of synchronization depends on a variety of cytokines. C-reactive protein (CRP), as the first acute phase reaction protein, is involved in the entire process of embryo transfer. The study is designed to investigate the correlation among CRP, sex hormone, controlled ovarian hyperstimulation (COH) cycle, and pregnancy outcome. *Materials and Methods:* Ninety-two patients who accepted in vitro fertilization (IVF) treatment cycles because of tubal factor were included in the study. Seventy treated cases were included to complete final analysis with the full set of results. Respectively on the second day of the menstruation (Day-2) in gonadotropin-releasing hormone agonist (GnRH-a) short program treatment, on the morning in human chorionic gonadotropin (hCG) treatment (Day-hCG) and the embryo transplant day (Day-ET), plasma CRP level was tested by enzyme-linked immunosorbent assay (ELISA). The correlativity among CRP level, sex hormone, COH, and pregnancy outcome was analyzed by statistical methods. *Results:* In the short program GnRH-a of 70 cases, there was no relationship between serum CRP level and the infertility age, gonadotropin (Gn) dosage, number of oocytes retrieved, the number of normal fertilization, and sex hormone. In the short program of GnRH-a, the change of serum CRP levels in Day-2, Day-hCG, Day-ET: serum CRP in Day-2 < Day-hCG < Day-ET and the level of serum CRP gradually increased in Day-2, Day-hCG, and Day-ET in both the pregnant group and non-pregnant group. In non-pregnant group, the ratio of hCG / D2 and ET / hCG-day were significantly higher than the pregnant group. The area under receiver operating characteristic (ROC) curve was 0.806, indicating the accuracy of diagnostic tests is medium. the authors chose the point which presents the ratio of CRP in Day-ET to Day-hCG which was less than 1.752 as a predictor of treatment outcome, the sensitivity of the experiment was 77.8%, and the specificity 75%. *Conclusion:* CRP as a sensitive inflammatory marker, CRP ratio of Day-ET/ Day-hCG could be a predictor of treatment outcome by ROC curve analysis in COH program.

Key words: C-reactive protein; Controlled ovarian hyperstimulation; GnRH; Embryo transfer.

Introduction

Human assisted reproductive technology (ART) is a developed new technology in recent decades, having greater prospects, which is the most effective treatment to infertility. The most representative method in ART is fertilization in vitro and embryo transfer (IVF-ET), also called test-tube baby. The success of embryo transfer is a key link in IVF-ET. After quality four- to eight-cell embryo was transplanted into patients' endometrium, whether the endometrium accepts the embryo has become the key to the growth of embryos. In the process of IVF-ET, controlled ovarian hyperstimulation (COH) is a potentially inflammatory reaction. whereas, the body's levels of inflammatory mediators might be the most important factor of the ability of endometrium to accept embryos [1]. Therefore, to explore levels of inflammatory factors in IVF-ET process is important to the success of IVF.

In the study of the inflammatory response, C-reactive protein (C of reactive protein, CRP), as the first acute phase reaction protein (ARP), participates in non-specific immunity and combines with a variety of pathogens and other polysaccharide substances. The complexes could activate the complementary system, neutrophils, mononuclear phagocytes, and the production of cytokines such as IL-6, IL-1, and result in the inflammatory response [2]. IVF test confirmed that the IL-1 could ensure normal pregnancy through the inhibition of uterine stromal cell decidualization [3]; IL-6 stimulates the production of protease and inhibin involved in the formation of the placenta, which is beneficial to the trophoblast and endometrial differentiation [4]. Therefore, the level of CRP in the body is closely related to the success of ET. Researches indicate that CRP could decrease the stability of nitric oxide synthase (nitric oxide synthase, NOS) mRNA in endothelial cell, and lead to reduced production of nitric oxide, endothelial cell apoptosis, thus inhibiting angiogenesis, as well as disturbing the implantation of the embryo [5]. Studies [1] found that CRP increases in IVF patients since the first week of oocytes retrieval in the future, and the CRP ratio of pregnancies between transplantation and oocyte retrieval day was lower than that of non-pregnancies, which has similar trend of IL-1 in Karagouni *et al.* study [6], indicating that the increase of CRP may facilitate the production of cytokines in IVF patients, changing the preimplantation endometrial environment, thus affecting embryo implantation. At the same time, as an inflammatory marker, there is no circadian fluctuation of CRP com-

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pared with other cytokines such as IL-1. Additionally, its high sensitivity, simple, and rapid clinical testing make it more economical than the LIF and IL-1 and receive more and more attention from scholars in the field of auxiliary reproductive study.

At present, the COH program is different between reproductive centers; the main difference lies in the gonadotropin-releasing hormone (gonadotropic releasing hormone of GnRH) analogues and their administration time [7]. GnRH analogues, include gonadotropin-releasing hormone agonist (GnRH-a) and gonadotropin releasing hormone antagonist (GnRH-anta). GnRH-anta rapidly inhibits excitatory effects of endogenous GnRH on the pituitary through the competitive combination of GnRH-R. While GnRH-a inhibits the release of gonadotropin (Gn) through down regulation of GnRH receptors (GnRH-R) and desensitization of pituitary Gn cells. This inhibition is a slow process, which could reduce the body's inflammation process and is preferred by researchers. In the present research, the authors detected the quantity of CRP in plasma at different time point, including the next day of GnRH-a short protocol ovarian hyperstimulation (COH) cycle period (Day 2), the day of hCG (Day-hCG), and the day of embryo transplant (Day-ET). They then explored its relationship between hormone quantities of plasma, COH, and other variables.

Materials and Methods

Ninety-two patients who accepted IVF treatment cycles because of tubal factor were included in the study. There were 22 cases of incomplete CRP results or hemolysis. Therefore, only the data of 70 women who had a full set of results were included to complete the final analysis, except for the patients subjected to intracytoplasmic sperm injection (ICSI), natural cycles, and frozen ET. Patients aged 25 to 38 years , normal basal body temperature, body mass index (BMI) < 30, six sex index (FSH, LH, PRL, E, P, and T) test was normal. The infection tests were negative for anti-sperm antibody, anti-endometrium. Before accepting IVF progesterone, the subjects did not receive Gn and clomiphene citrate therapy, and did not have previous serious systemic disease and ovarian hyperstimulation syndrome (OHSS). The short program of use GnRH-α: two days before menstruation, the subjects were given GnRH-a 0.1 mg by subcutaneous injection once every two days. The drug use time was adjusted according to B ultrasound monitoring of follicle diameter and menstrual cycle of patients. rFSH 225 IU once a day was added on the third day of menstruation. At the eighth day of menstruation, the growth of follicle was monitored by B ultrasound. Diphereline and Gonal-F were stopped, given hCG 10000IU by intramuscular injection at 9:00 pm, and harvested the oocyte after 36 hours when the following situations appeared: there were two or three dominant follicles whose diameter was 18~20 mm in vaginal, and the plasma E2 peak value was more than 2,000 pmol/l. At 48-72 hours after oocyte harvesting, one to two embryos were transferred intrauterine. Daily supplement progesterone 60 mg after ovulation was given. At 14 days after ET, urinary hCG was positive, and at six to eight weeks, clinical pregnancy characteristics, such as intrauterine gestation and fetal heart beat, were observed.

Table 1. — *In the short program of GnRH-a, the CRP level had no relationship with the infertility age, Gn dosage, number of oocytes retrieved, and fertilization rate ($\bar{x} \pm s$).*

Index	Serum CRP (mg/ml)		
	Mean ± SD ($\bar{x} \pm s$)	Pearson coefficient of correlation	*p* value
Serum CRP (mg/ml)	174.453 ± 136.597	–	–
Infertility age (years)	29.889 ± 3.984	0.101	0.691
Gn dosage (IU)	2044.286 ± 515.890	0.009	0.972
Oocytes retrieved (n)	13.741 ± 8.934	0.235	0.349
Normal fertilization Number (2pn) (n)	7.185 ± 5.241	0.115	0.648

Table 2. — *In the short program of GnRH-a, there was no relationship between the ratio of serum CRP/BMI, and E2 in Day-2, Day-hCG, and Day-ET ($\bar{x} \pm s$).*

	Day-2	Day-hCG	Day-ET
E2 (pg/ml)	56.584 ± 34.057	375.625 ± 1685.865	2783.952 ± 1962.871
Serum CRP/BMI	5.677 ± 2.481	6.847 ± 1.987	12.162 ± 4.982
Pearson coefficient of correlation	0.495	0.897	0.891
p value	0.505	0.103	0.109

Sample collection

Serum: respectively on the second day of menstruation (Day-2), on the day use of hCG (Day-hCG) early in the morning, on the transplant day early in the morning (Day-ET), five ml venous blood was drawn from each patient and centrifuged at 5,000 r/min for ten minutes. The upper serum was taken, then stored at -20°C. Serum samples were collected with the consent of the Clinical Ethics Committee and the informed consent of patients.

Specimens detection

The CRP in serum samples was detected by enzyme-linked immune sorbent assay (ELISA) method. The operation was in strict accordance with the kit specifications.

Statistical methods

The subjects were divided into the pregnant group and the non-pregnant group. Through the paired t-test, ANOVA analysis, and the classification and the regression trees (CART), the rate difference of transplantation/oocyte retrieval was determined. SPSS11.5 and CART were applied to perform statistical analysis; $p \leq 0.05$ was considered a significant difference.

Results

In the short program of GnRH-a in the 70 cases, there was no relationship between serum CRP level and the infertility age, Gn dosage, number of oocytes retrieved, and the number of normal fertilization, as shown in Table 1.

In the short program of GnRH-a, the relationship between CRP and sex hormone: as we all know, CRP and body weight have a significant correlation, and for infertility patients, their weight and sex hormones have a certain relationship. However, under the premise of no difference in the underlying sex hormone levels and BMI ≤ 30, there

Table 3. — *The comparison of CRP levels in Day-2, Day-hCG, and Day-ET ($\bar{x} \pm s$).*

	Serum CRP (mg/ml)
Day-2	120.351 ± 107.726**
Day-hCG	145.174 ± 97.245*
Day-ET	257.834 ± 160.745

* $p < 0.05$ compared with Day-ET; ** $p < 0.01$ compared with Day-ET.

Table 4. — *The clinical outcomes and infertility age in the short program of GnRH-a ($\bar{x} \pm s$).*

Clinical outcomes	Case No. (%)	Infertility age
Pregnancy	23 (32.86)	29.778 ± 4.265
Non-pregnancy	47 (67.14)	29.944 ± 3.963
Total	70 (100)	29.889 ± 3.984

was no correlation between serum CRP/BMI and E2, as shown in Table 2.

In the short program of GnRH-a, the change of serum CRP levels in Day-2, Day-hCG, Day-ET: serum CRP in Day-2 < Day-hCG < Day-ET. While the level of serum CRP gradually increased in the COH process. Single factor analysis of variance: the F and P value of serum CRP levels of different groups is 5.838 and 0.005, indicating that serum CRP concentrations in the groups had statistically significant differences. Therefore pairwise comparisons by the analysis of variance are shown in Table 3.

In the short program of GnRH-a, the comparison between the pregnancy and non-pregnant group: this study was aimed to understand the relationship between CRP and embryo implantation. Therefore clinical pregnancy was taken as the demarcation of the pregnant group and non-pregnant groups.

In the short program of GnRH-a, the clinical outcomes and infertility age is shown in Table 4. Regardless of pregnancy or non-pregnant group, the level of serum CRP gradually increased in Day-2, Day-hCG, and in day-ET. Moreover, there was no significant difference between the pregnancy and non-pregnant groups at the same time point. (respectively, *p* values were 0.366, 0.840, and 0.595). Therefore, the single factor analysis of variance to the concentration of the serum CRP at three different time points was performed: the F value was 2.625 in non-pregnant group, and *p* value was 0.096.There was no significant difference in serum CRP concentration between groups; pairwise comparisons also showed that there was no difference in different groups. The F value was 3.374 in pregnant groups, and *p* value was 0.052.There was no significant difference in serum CRP concentration between groups, but only the CRP concentration in Day-ET was higher than that of Day-2 ($p < 0.05$). The data are shown in Table 5 and in Figure 1.

The level of CRP in pregnancy and non-pregnant groups was parallel (Figure 1, Table 5). However, the ratios of CRP

Table 5. — *The comparison of CRP levels in Day-2, Day-hCG, and day-ET between pregnant and non-pregnant groups ($\bar{x} \pm s$).*

	CRP (mg/ml) in pregnant group	CRP (mg/ml) in non-pregnant group
Day-2	87.426 ± 66.035	145.103 ± 92.745
Day-hCG	144.828 ± 106.526	157.392 ± 136.309
Day-ET	243.742 ± 182.167*	280.809 ± 155.963*

* $p < 0.05$ compared with Day-2.

Table 6. — *In the short program of GnRH-a, the comparison of ratios of CRP level in Day-2 to Day-hCG, and Day-ET to Day-hCG in pregnant and non-pregnant groups ($\bar{x} \pm s$).*

	Day-hCG/Day-2	Day-ET/Day-hCG
Pregnancy	1.704 ± 0.991	1.474 ± 0.748*
Non-pregnancy	2.503 ± 0.535	2.566 ± 1.252
p value	0.634	0.043 ($p < 0.05$)

* $p < 0.05$ compare with the ratio of CRP in Day-ET to Day-hCG in non-pregnant group.

level in Day-2 to Day-hCG, and Day-ET to Day-hCG in pregnant and non-pregnant group had significant differences ($p < 0.05$), as shown in Table 6.

As shown in Figure 2, the area of ROC curve was 0.806, indicating the accuracy of diagnostic tests is medium. Then the point which presents the ratio of CRP was chosen in Day-ET to Day-hCG which was less than 1.752, as a predictor of treatment outcomes from the upper left corner of the figure. The sensitivity of the experiment was 77.8%, which signifies that the ratio of Day-ET to Day-hCG in 18 from 23 pregnant cases was less than 1.752. The specificity was 75%, which signifies that the ratio of Day-ET to Day-hCG in 36 from 47 pregnant cases was less than 1.752.

Discussion

Firstly, the authors identified that the serum CRP level has no relation with infertility age, Gn dosage, number of oocytes, and the number of normal fertilization in the stop GnRH-a protocols of COH and IVF-ET process. Furthermore, there was no correlation between the CRP/weight index ratio and E2. Secondly, in the stop GnRH-a protocols, the serum CRP level gradually increased in the COH process in both pregnant and non-pregnant groups. The level of serum CRP in D2 day (CRPD2) was less than that in the hCG day (CRPhCG) and the serum CRP level in hCG day (CRPhCG) was less than that in the ET day (CRP ET). However, at the same time point, there were no differences between pregnant and non-pregnant group. Thirdly, the ratio of CRP hCG/CRPD2 with CRPET/CRPhCG was significantly different between pregnant and non-pregnant groups. The sensitivity and specificity was 77.8% and 75% when using the ratio for predicting pregnancy.

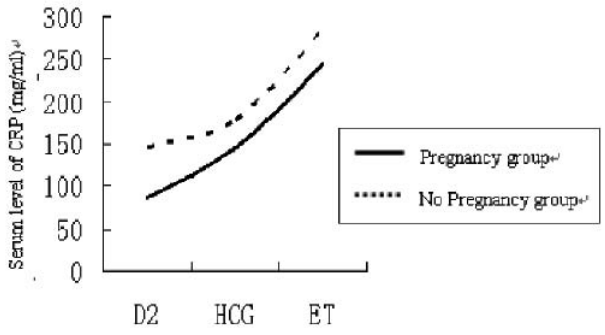

Figure 1. — The comparison of CRP level between pregnancy and non-pregnant groups $(\bar{x} \pm s)$.

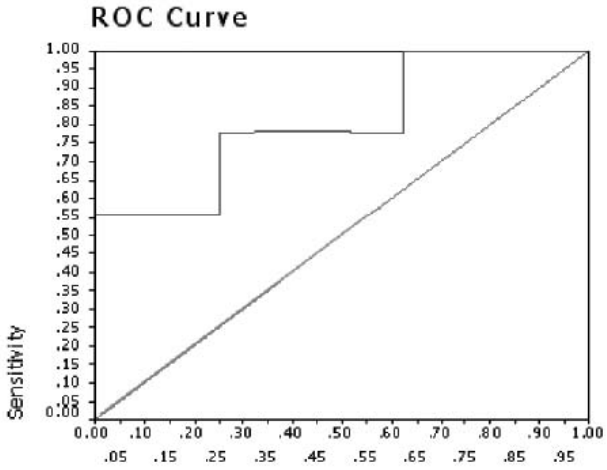

Figure 2. — The ROC curve in the short program of GnRH-a. The area under the curve is the area of ROC curve, diagonal line is the opportunity ROC curve.

CRP is a type of sensitive inflammation marker and was discovered in the serum of acute inflammation patient in 1930. There are no circadian fluctuations in plasma CRP concentration and the concentration is not affected by radiotherapy, chemotherapy, corticosteroids, and other treatment. The clinical detection can be conducted at any time of the day, therefore, CRP became a sensitive and reliable indicator [2, 8]. On one hand, CRP synthetized of by the liver participate in non-specific immune, combined with a variety of pathogens and other polysaccharide substances and with lecithin and nucleic acids in the presence of calcium. The combined complexes could activate the complement system as well as neutrophils, monocytes, and phagocytes with the production of cytokines as IL-1 and IL-6, triggering the opsonization and phagocytosis to invasion cells and showing inflammatory response. On the other hand, activated immune cells release a variety of cytokines as IL-6, can promote hepatocyte synthesis of CRP [2]. At present, the CRP could be detected in human serum, pleural effusion, and in follicular fluid. In the field of assisted reproduction, IL affect the reproductive activities in all aspects such as the regulation of egg development, maturity, ovulation, and other ovarian functions, as well as processes like fertilization, implantation, and pregnancy. In particular, IL-1 expression in embryonic and endometrial constitutes the material basis of the cross-talk between embryo and endometrium. IL-1 could induce and affect endometrial receptivity as well as the expression of LIF that mediates embryo implantation. Meanwhile, IL-6 increase instantaneously in the planting window and jointly promotes implantation with LIF [3, 4]. In addition, COH is an essential factor for the success of IVF-ET, with the most important complications as OHSS. Accurate OHSS is caused by large amount of cytokine increase or neutrophil activation in system infection [9-11]. So the authors found that the CRP is related with reproduction.

Current studies show that serum CRP level increases up from D2 day to hCG day and to ET day, which is consistent with the rising of E2 level in COH process. Additionally, the CRP level is stable at long-term without circadian disturbance. Therefore, even in the case of E2 declined due to loss of granule cells after egg retrieval, serum CRP is also increases. In an in vitro study of cytokines released from peripheral lymphocytes in super-ovulation patients, Orviet found that the level of interleukin cultured with whole blood significantly increased at E2 peaks in COH process [12]. Ricoux *et al.* also indicated that the increase of estradiol does not affect CRP level in the ovarian stimulation stage [13]. These researches support the results of this experiment and also proved the presence of infection in the COH and IVF-ET process. IL-1 can quickly produce CRP and preimplantation embryos in human can secrete IL-1. Therefore, IL-1 deletion will cause endometrial decidualization abnormalities and embryo resorption [13-15]. In this experiment, CRPhCG is higher than CRPD2 without significant difference and CRPET is significantly higher than CRPhCG. It can be inferred that the increase of CRP mainly commenced before implantation and after egg retrieval, which can be used as a signal of implantation. The initial adhesion process of the embryo and endometrium is regulated by their signal exchange and local factors promote embryo implantation through mechanisms of paracrine and autocrine.

The results also show that: the ratio of CRP (day of embryo transfer/day of hCG) is lower in pregnant cycle than that of non-pregnant cycle, which may indicate that the infection or potential infection level is lower in pregnant woman than that of non-pregnant women. In embryo implantation stage, in order of the embryo positioning and implantation, the endometrium will reduce the release of mediators of immunoregulation, as cytokines to stabilize lysosomal membrane to promote placental macrophages secreting prostaglandin E2, inhibit phospholipase C activity, block part of the prostaglandin synthesis, and inhibit

uterine contraction [16-18]. Furthermore, CRP could interact with both humoral infectious effect system and cellular infectious effect system [19-20]. Therefore, fundamentally speaking, there appears to be some immunosuppressive effect to the proper maturity of endometrium and embryo from CRP, which indicates that the infection degree of endometrium plays a role in the establishment of a suitable endometrial receptivity. For clinical, it is important to find an appropriate cutoff for CRP ratio to predict the success or failure of early embryo implantation. Inflammatory state and degree of IVF-ET patients could be understood in real time through the detection of indicators, the level of which could be used as guidance for individualized fetus protection and duration of anti-inflammatory treatment. In this experiment, through the ROC curve analysis of the COH program, the authors calculated the CRP ratio on transplant day and hCG day as < 1.752, which was used as a predictor of treatment outcome. However, the sensitivity and specificity of the predictor is not satisfactory and a more appropriate predictor could be established by increasing the number of detected cases, joint detection from multi-center, and expanding the sample size, conditions permitting.

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