

Controlled Ovarian Hyperstimulation Changes the Prevalence of Serum Autoantibodies in *In Vitro* Fertilization Patients

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Keywords

Common autoantibodies, infertility, supraphysiological sex hormone levels

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Submitted 7 July, 2006;
accepted 21 September, 2006.

Citation

Haller K, Sarapik A, Talja I, Salumets A, Uibo R. Controlled ovarian hyperstimulation changes the prevalence of serum autoantibodies in *in vitro* fertilization patients. *Am J Reprod Immunol* 2006; 56:364–370

doi:10.1111/j.1600-0897.2006.00438.x

Introduction

A high prevalence of autoimmune mechanisms responsible for reproductive failure has been demonstrated by others¹ and conformed by us.^{2,3} However, the impact of a particular autoantibody on the path-

Problem

Autoimmune mechanisms are involved in etiology of female infertility, the medical problem frequently treated by *in vitro* fertilization (IVF). Controlled ovarian hyperstimulation (COH) with supraphysiological levels of sex hormones is achieved by IVF.

Methods of study

Anti-human-ovary and eight common autoantibodies [nuclear (ANA-H, ANA-R on human HEP-2 cell line and rodent antigen, respectively), smooth muscle (SMA), parietal cell, thyroid microsomal, mitochondrial, β 2-glycoprotein-I, cardiolipin antibodies] found in IVF patients ($n = 129$) were analyzed with regard to the number of previous IVF procedures and the age of the patient. The changes in autoimmune reactions caused by the COH were determined.

Results

Endometriosis and polycystic ovary syndrome were associated with a higher number of common serum autoantibodies compared with the tubal factor infertility (Proportion test, $P < 0.05$). ANA-R was associated with unexplained infertility [adjusted odds ratio (aOR) 8.79, $P = 0.038$]. SMA correlated with endometriosis (aOR 37.29, $P = 0.008$), male factor infertility (aOR 20.45, $P = 0.018$) and with the previous IVF procedures (aOR 2.87, $P = 0.013$). There was an overall decrease in the number of detectable autoantibodies after COH (Proportion test, $P < 0.05$).

Conclusion

COH may have a suppressive effect on the humoral immunity by the time of embryo transfer but more conclusive studies are needed.

ogenesis of infertility is not uniformly understood. Some of the autoantibodies have been associated with certain diseases as patients with endometriosis often possess anti-nuclear (ANA) and anti-smooth muscle autoantibodies (SMA). Ovarian autoantibodies have been associated with premature ovarian

failure, antiphospholipid and thyroid autoantibodies have been associated with recurrent pregnancy loss.^{1,4,5} Some studies suggest lesser importance of specific autoantibodies and stress the key role of overall activation of the immune system in reduced fecundity.⁶

Steroid sex hormones have substantial impact on both development and modulation of the immune system and general susceptibility to autoimmunity. Steroid hormones exert dose and time dependent actions both locally in the tissues in which they are produced and centrally after entering into the circulation.⁷ These hormones modulate immune system after extended administration but also during the menstrual cycle.^{8,9} Additionally, sex hormones are implicated in the immune response with dual effects: estrogens as enhancers of the humoral immunity and androgens and progesterone as immunosuppressors.^{7,8} At physiological level, estradiol and especially its metabolite 16 α -hydroxyestrone, may favor autoimmunity by increasing B cell differentiation and antibody production as well as by activating T cells.⁷ The effect of progesterone on immunoglobulin (Ig) secretion is believed to be mediated by hormonal effects on immunocompetent cells.⁹ Testosterone shifts suppressor/cytotoxic CD4⁻CD8⁺ cells to predominate over the helper CD4⁺CD8⁻ lymphocytes among mature thymocytes.⁸ The altered estradiol/testosterone ratio and peripheral conversion of sex hormones are believed to be pathogenic factors for impaired immunosuppression in several autoimmune diseases.⁷

In vitro fertilization-embryo transfer (IVF-ET) has become a promising treatment for infertility of many causes. During IVF-ET procedure, multiple follicles are enabled to grow and mature by stimulating the ovaries with the administration of exogenous follicle-stimulating hormone (FSH). This procedure is routinely known as controlled ovarian hyperstimulation (COH). COH is associated with rapid increase of the production of gonadal sex hormones, with the level of estradiol exceeding at least five to 10 times the estradiol level at the luteal stage of spontaneous menstrual cycle.¹⁰ In this context, the COH determines the hormonal and immunological status at which the embryo transfer takes place.

The prospective of the present study was to (i) describe the autoimmune reactions in IVF patients with different causes of infertility and to analyze the results with regard to the number of previous IVF procedures and the age of the patient, and (ii) detect the changes in autoimmune reactions caused by COH.

Materials and methods

Patients

The Ethics Committee of the University of Tartu approved the study and informed consent was obtained from all participants after the nature of the study was explained to them. A total of consecutive 129 infertile patients (mean age 33.0 years, SD = 5.5) undergoing IVF-ET at Nova Vita Clinic between July 2004 and October 2005 were enrolled in the study. The indications for IVF within the study group were as follows: 43.4% ($n = 56$) tubal factor infertility, 22.5% ($n = 29$) male factor infertility, 16.3% ($n = 21$) polycystic ovary syndrome (PCOS), 9.3% ($n = 12$) endometriosis and 8.5% ($n = 11$) unexplained infertility. Patients with other causes of infertility as endometrial hyperplasia, myoma uteri, ovulatory dysfunction, autoimmune diseases (Hashimoto's thyroiditis) or chronic infections were excluded from this study. Among the patients participated, 85 women (65.9%) were about to start their first IVF procedure, 26 (20.2%) had had one, 10 (7.7%) had had two, and eight (6.2%) had had three or more previous IVF procedures. All patients had been suffering from infertility for at least 1 year before entering the study. The pregnancy rate (positive chorionic gonadotropin test) and clinical pregnancy rate per embryo transfer were 40.5% (51/126) and 30.2% (38/126), respectively.

Endometriosis and PCOS were diagnosed as reported previously.³ Tubal factor infertility due to the fallopian tube occlusion was diagnosed either by hysterosalpingography or diagnostic laparoscopy.¹¹ The main cause for tubal occlusion was an episode of infection (pelvic inflammatory disease). Male factor infertility was diagnosed when the woman lacked known reasons for infertility, while her partner experienced decreased semen quality.¹² Unexplained infertility was assumed if the woman lacked any of the abovementioned reasons for infertility and her partner had normal semen quality, but the couple had suffered from infertility for more than a year.

The blood samples were drawn twice from each patient: (1) during the 3–5 days of the patient's spontaneous menstrual cycle before administering gonadotropin-releasing hormone (GnRH) agonists/antagonists and starting the COH; and (2) on the day of oocyte retrieval immediately after the COH. The ovarian hormonal stimulation was conducted

according to either the conventional long protocol using GnRH agonists [$n = 7$ (5.4%)], or antagonists [$n = 122$ (94.6%)] protocols and administering recombinant FSH. The levels of serum estradiol, progesterone, and testosterone were measured using chemiluminescence immunoassay (Immulite 2000[®] station, Diagnostic Products Corporation, Los Angeles, CA, USA).

Immunological Methods

Stored sera were assayed for the presence of anti-human ovary (AOA) and common autoantibodies: nuclear (ANA-H, ANA-R on human HEp-2 cell line and rodent antigen, respectively), SMA, parietal cell (PCA), thyroid microsomal (TMA) and mitochondrial (AMA) antibodies using the indirect immunofluorescence method as described previously.² The antibody levels were expressed as negative or as positive at lower (1:10) or higher (1:100) titers.

Indirect in-house ELISA was used to detect antibodies against β 2-glycoprotein I² (B2-GPI) and cardiolipin¹³ (ACA). The results were expressed in enzyme-immunological units (EIU). The cut-off values for weak and strong positive result were 10 and 30 EIU for B2-GPI, and 30 and 60 EIU for ACA. All immunological tests are clinically available and have been periodically subjected to external quality assessment by UK NEQAS (Sheffield, UK).

Statistical Analysis

To evaluate the statistical difference between autoantibody levels before and after COH the R 2.3.1 A Language and Environment (Free Software Foundation, Boston, MA, USA) was used for proportion test with continuity correction, Wilcoxon test, paired *t*-test and logistic regression analysis. Patients with tubal factor infertility participated in the study as a reference group. A *P*-value of < 0.05 was considered statistically significant.

Results

The Patients' Hormonal Profile Before and After COH

The following steroid hormone profiles of patients were recorded during the follicular phase of the spontaneous menstrual cycle (SPC): estradiol (mean \pm SD)

161.1 \pm 117.0 pmol/L, progesterone 1.6 \pm 1.4 nmol/L, and testosterone 1.5 \pm 0.7 nmol/L. The levels of the hormones increased significantly (paired *t*-test, $P < 0.0001$) by the day of the oocyte pick-up (OPU): estradiol 5768.0 \pm 7867.0 pmol/L, progesterone 32.3 \pm 19.1 nmol/L, and testosterone 3.3 \pm 1.9 nmol/L. The levels of estradiol, progesterone and testosterone at SPC were similar in all patient groups (data not shown). The level of estradiol at OPU was significantly higher in PCOS patients compared with the tubal factor infertility group [medians 6533 (from 885 to 45520) pmol/L and 2818 (from 712 to 31130) pmol/L, respectively] (Wilcoxon test, $P = 0.017$). Patients with endometriosis, male factor infertility and unexplained infertility demonstrated similar OPU-estradiol levels as the tubal factor infertility group (data not shown). The levels of progesterone and testosterone at OPU were similar in all patient groups (data not shown).

The Prevalence of Autoantibodies in Different Patient Groups at Early Follicular Phase of Spontaneous Menstrual Cycle

All patients who represented autoantibodies followed the GnRH antagonist protocol (94.6%), while the patients in the GnRH agonist protocol group (5.4%) were negative for all measured autoantibodies. We did not detect any patients positive for AOA or AMA antibodies. In the group of endometriosis and PCOS, there were more patients positive for at least one autoantibody at the lower titer (endometriosis only) and at the higher titer than in the group of the tubal factor infertility (Table I).

To evaluate the putative associations between the certain autoantibodies at SPC and the cause of infertility the logistic regression analysis was used. The logistic regression models for each antibody studied were adjusted by the age and the number of previous IVF procedures. The number of previous IVF cycles was only associated with the higher frequency of 1:10 SMA (adjusted OR 2.87, $P = 0.013$). Higher prevalence of 1:10 SMA was also associated with endometriosis and male factor infertility (adjusted OR 37.29, $P = 0.008$ and 20.45, $P = 0.018$) and showed a tendency to be more frequent in PCOS (adjusted OR 11.19, $P = 0.067$). Additionally, our model revealed higher frequency of ANA-R 1:10 antibodies in unexplained infertility group (adjusted OR 8.79, $P = 0.038$) compared with the tubal factor infertility.

Table I The prevalence of autoantibodies (Ab) in serum obtained during the 3–5 days of the patients' spontaneous menstrual cycles

Infertility group (n)	Ab at lower titer*, n (%; 95% CI)	Proportion test (P-value)	Ab at higher titer**, n (%; 95% CI)	Proportion test (P-value)
Tubal factor (56)	17 (30.4; 19.2–44.3)	Reference	6 (10.7; 4.4–22.6)	Reference
Male factor (29)	11 (37.9; 21.3–57.6)	N.S.	1 (3.4; 0.2–19.6)	N.S.
PCOS (21)	9 (42.9; 22.6–65.6)	N.S.	5 (23.8; 9.11–47.5)	< 0.05
Endometriosis (12)	7 (58.3; 28.6–83.5)	< 0.05	4 (33.3; 11.3–64.6)	< 0.05
Unexplained infertility (11)	4 (36.4; 12.4–68.4)	N.S.	2 (18.2; 3.2–52.2)	N.S.

*Antibody-positivity at the lower titer was assessed by counting the number of positive tests at the following titers: 1:10 for ANA-R, ANA-H, SMA, TMA and PCA or ACA and B2GP I present at least at the weak positive value.

**Antibody-positivity at the higher titer was assessed by counting the number of positive tests at the following titers: 1:100 for ANA-R, SMA, TMA, 1:10 for PCA and 1:40 for ANA-H or highly positive results for ACA and B2GP I autoantibodies.
N.S., statistically not significant ($P > 0.05$).

The Dynamics of Autoantibody Levels During the COH

The increased ANA-R, ANA-H, SMA, TMA and PCA antibody levels after the COH were measured as changes in titers from negative to positive at the lower titer, or from positive at the lower titer to positive at the higher titer. The decrease in antibody levels was assessed by the opposite changes in titers. The increase of ACA and B2GP I levels were assessed in two ways: (i) by changes from negative to weak positive reading, and from weak positive to highly positive reading (comparisons were done using proportion test); and (ii) as a continuous values (comparisons were done using paired *t*-test). The decrease of ACA and B2GP I levels were detected opposite way to the increase. The levels of SMA, PCA, TMA, ACA and B2-GP I antibodies did not change during the COH. However, the ANA-H level decreased significantly (proportion test between the patients with decreased and increased antibody levels, $P < 0.05$). In comparison, the level of B2-GP I antibody raised during the COH (mean levels \pm SD before and after COH were 2.0 ± 8.4 and 2.3 ± 6.9 EIU, $P = 0.031$, paired *t*-test), but this change was insufficient to increase the number of tests above the cut-off (proportion test, $P > 0.05$). The same changes in ANA-H and B2-GP I antibody levels were detected for patients studied with only the GnRH antagonist protocol (data not shown).

The Changes in Autoantibodies During the COH

After the COH, we counted patients falling into the following groups: (i) patients with more autoantibodies detected at OPU than at SPC either at lower

or higher titer, representing the phenomenon of antibody-increase after the COH; (ii) patients with unchanged number of detectable autoantibodies after the COH either at lower or at higher titer; or (iii) patients with fewer autoantibodies detected at OPU than at SPC either at lower or at higher titer, representing the phenomenon of antibody-decrease after the COH. The number of patients with increased antibody titer was compared with the number of patients with decreased antibody titer using the proportion test. Our results showed an overall reduction in autoantibodies during the COH, as there were significantly more patients who showed a decrease in detectable autoantibodies after the COH compared with the patients with increased number of autoantibodies (Table II). Same results were obtained for patients following only the GnRH antagonist protocol (data not shown).

Table II The comparison of the frequency of patients showing the increase or the decrease in number of detected autoantibodies (Ab) during the COH

	Patients with increased number of Ab (95% CI) ^a	Patients with decreased number of Ab (95% CI) ^b
Ab at lower titer	9.0% (5.0–15.6)	18.8% (12.7–26.7)*
Ab at higher titer	3.8% (1.4–9.0)	9.8% (5.5–16.5)*

The number of patients was counted for both situations following the COH: ^amore autoantibodies were detected at OPU than at SPC either at lower or higher titer, or ^bfewer autoantibodies were detected at OPU than at SPC either at lower or higher titer.

*Statistically significant difference (proportion test, $P < 0.05$)

Discussion

A large variety of non-organ- and organ-specific autoantibodies are associated with female infertility.^{1,14} Therefore, it has been challenging to look for a particular antibody most strongly associated with certain cause of infertility.^{1,4,5} Concordant with previous studies, we found that a low titer of ANA (detected by rodent antigen substrate) was strongly associated with the unexplained infertility. The association was independent of the age of the patient and the number of previous IVF procedures. Because ANA has been associated with early implantation failure,¹ these antibodies may promote infertility in otherwise clinically healthy woman. We also showed an association between the presence of lower titer SMA and endometriosis and male factor infertility. In addition, SMA was the only antibody in our study which correlated positively with the previous use of IVF procedures in an age-independent fashion. The association between the production of SMA and infertility due to the male factor could not be easily explained. In the group of patients with male factor infertility, unexplained or undetected subfertility of the woman cannot always be ruled out. In addition, there is clear evidence that the production of SMA can be caused by persistent viral infection and these antibodies alter the fallopian tube function.¹ Although we were not aware of any viral infections among our IVF patients one cannot exclude some similarities between the immune system dysregulation in chronic inflammation and endometriosis or repeated IVF procedures. An increase of autoantibodies after repeated IVF attempts has previously been reported.¹⁴⁻¹⁶

Rather than the presence of one particular autoantibody, the detection of activation of immune system, in general, can help to predict the patient's fecundity and the outcome of IVF-ET treatment.^{6,17} We analyzed the association between the cause of infertility and the autoimmune reaction, with regard to the number of autoantibodies detected. Our results showed that infertile patients with endometriosis and PCOS were associated with significantly higher number of common serum autoantibodies compared with the women with tubal factor infertility. The higher prevalence of autoantibodies in these patient groups compared with the fertile controls has been demonstrated by many authors.^{2,18-20}

It was somewhat surprising to us, that the number of autoantibodies detected after the COH showed an overall decrease. Of all the autoantibodies studied,

the level of ANA-H decreased, while only the level of B2-GPI increased slightly. Regardless of the high prevalence of autoantibodies in the group of IVF patients¹ and the hazardous effect of anti-ovarian,¹⁴ ANA and antiphospholipid^{1,16} antibodies on embryo as well as on early pregnancy, only a few studies have been conducted so far to explore the effect of COH on the levels of common autoantibodies. Fisch et al.²¹ showed no significant changes in the levels of ANA or ACA during the hormonal stimulation. Similarly, Monnier-Barbarino et al.²² concluded the absence of influence of ovarian stimulation (performed by classical long GnRH agonist protocol) on anti-ovarian autoimmunity. We were not able to compare two protocols used in ovarian stimulation – GnRH antagonists and agonists, because of only seven (5.4%) patients followed the agonist protocol none of whom represented the autoantibodies studied. Thus, our results are restricted to GnRH antagonist protocol only.

The activating effect of estrogens on the humoral immune system has been reported by studying women during menstrual cycles, by evaluating the patients suffering from rheumatoid arthritis or systemic lupus erythematosus, and by using animal models.⁷⁻⁹ The level of serum estradiol is typically within the physiological range in abovementioned autoimmune diseases⁷ and serum levels used in animal studies were five to 14 times lower than the levels found during pregnancy.²³⁻²⁵ However, it has been demonstrated for chronic inflammatory diseases that depending on the concentration, estradiol can exert the opposite effect on immune system.⁷ In our study, the production of estradiol increased rapidly during COH and the levels reached the values of 10 times higher than those at luteal stage of spontaneous menstrual cycle. In addition to the supraphysiological levels of estradiol, the production of immunosuppressive testosterone and progesterone^{7,8} also increased. Therefore, the specific changes in the levels of all three sex hormone associated with COH may explain the decrease in number of autoantibodies detected after the COH.

Although the effect of sex hormones on immune reactions can take place within a short period of time^{9,24} the produced autoantibodies could stay in the circulation for a longer period. The time period for COH in our study was 9–12 days. IgG has a half-life of 7–23 days, depending on the specific subclass.²⁶ However, in case of autoimmunity, the considerable increase in the catabolic rate of IgG

antibodies has been recorded, shortening their expected half-life to less than a third of the normal value.^{27–29} Our data along with the data from literature suggest that COH may have an effect on the humoral immunity by the time of embryo transfer but profound studies are still required to further explore this effect. A question of how these changes in autoimmune reactions caused by COH could be related to the outcome of IVF treatment is the priority of our future studies.

Conclusions

Our results indicate that different forms of infertility are associated with various autoimmune disturbances along with the slight deviations in the profile of autoantibodies. We showed that the ovarian hormonal stimulation and repeatedly performed IVF procedures themselves could conversely modulate the autoimmune reactions. These data suggest that COH may have a suppressive effect on the humoral immunity by the time of embryo transfer but further studies are required.

Acknowledgments

This study was supported by the Estonian Science Foundation (Grants no. 6498 and 6514) and the Estonian Ministry of Education and Science (Core grants no. 0182582Cs03 and 0182586s03). We thank Anu Kaldmaa, Küllike Koppel and Ele Prans from the Department of Immunology of the University of Tartu, Estonia for their skillful laboratory assistance. Heti Pisarev, MSc from Department of Epidemiology and Biostatistics of the University of Tartu, Estonia is greatly appreciated for her advice with statistical analysis. We thank Dr Elle Talving and Dr Peeter Karits from Nova Vita Clinic, Estonia for collecting the patient data. This study was presented at the 16th European Congress of Immunology – ECI, September 6–9, 2006, Paris, France.

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