CEOG Clinical and Experimental Obstetrics & Gynecology

Follicular phase serum and follicular fluid glycodelin measurements in gonadotropin-releasing hormone (GnRH)-antagonist assisted reproduction cycles: A prospective cohort study

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Summary

Purpose: To establish the serum pattern for glycodelin and to investigate the possible correlations of serum and follicular fluid (FF) glycodelin with clinical pregnancy in gonadotropin-releasing hormone (GnRH)-antagonist controlled cycles. *Materials and Methods:* A prospective observational study conducted with 80 infertile couples who received a GnRH-antagonist controlled cycle. Glycodelin levels were measured in FF, day 2-3, and ovarian pick-up (OPU)-day serum samples. *Results:* There were no significant differences in serum glycodelin concentrations in either the early follicular phase or the preovulatory phase, and in FF glycodelin concentrations between clinically pregnant and non-pregnant patients. OPU-day serum glycodelin was found to be significantly higher than early follicular serum glycodelin level in all patients whether pregnancy occurred or not. *Conclusion:* Although day 2-3 and OPU-day measurements of serum glycodelin levels were not significant in predicting clinical pregnancy, the pattern of serum glycodelin seems different in GnRH-antagonist controlled cycles than natural and GnRH-agonist controlled cycles.

Key words: Assisted reproductive technology; Glycodelin; Gonadotropin releasing hormone antagonist.

Introduction

Approximately 80-85% of assisted reproductive technology (ART) cycles reach the embryo transfer phase, as reported in the 2009 ART report. It was also reported that the clinical pregnancy rate was approximately 36% per cycle, with more than one embryo transfer administered in approximately 86% of cycles [1]. In general, failure of implantation appears to be one of the most important contributors to decreased ART success [2].

Implantation consists of the apposition, adhesion, and invasion phases; all of these steps are regulated by molecules derived from embryonic and maternal sources. Several molecules, such as integrin, L-selectin ligand, and leukemia inhibiting factor, have been demonstrated to play a role in the initiation and continuation of implantation. Glycodelin is one of the most important of these molecules [3]. Glycodelin is secreted from endometrial glands and, to a lesser degree, the salpinx, hematopoietic cells and ovaries [4]. In normal menstrual cycles, glycodelin concentrations are low during ovulation, and the highest levels are observed at the end of the luteal phase. The peak glycodelin serum level occurs six to eight days after the progesterone peak [5], and in cycles that result in conception, plasma glycodelin rises rapidly after implantation [6].

It is believed that glycodelin has a particularly important impact on the implantation phase [7] and may play a role in endometrial receptivity [8]. However, in a study by Bentin-Ley *et al.*, endometrial flushing fluid glycodelin concentrations measured on the first day after the luteinizing hormone (LH) peak in normal menstrual cycles, could not predict pregnancy in the subsequent in-vitro fertilization (IVF) cycle [9]. In contrast, patients with lower serum glycodelin levels in the normal menstrual cycle had higher pregnancy rates in the subsequent ovarian stimulation-IVF cycle [10]. Although pregnancy prediction was not examined, serum glycodelin levels in long gonadotropin-releasing hormone (GnRH)-agonist IVF cycles were significantly lower up to the seventh day of the spontaneous cycle. After the seventh day, the serum glycodelin in both stimulated and non-stimulated cycles reached equally low values [5]. However, circulating concentrations of glycodelin reach a nadir in the peri-ovulatory phase in both stimulated and non-stimulated cycles; therefore, the pattern of serum glycodelin changes during the follicular phase in natural and long GnRH-agonist

Revised manuscript accepted for publication January 20, 2014

IVF cycles appears to be similar, although the concentration is lower in GnRH-agonist cycles.

Based on these data, it is likely that serum glycodelin plays a role in the development of pregnancy in natural and GnRH-agonist IVF cycles. In contrast, some evidence suggests that GnRH-antagonists may have detrimental effects on endometrial receptivity and embryonic implantation, which may contribute to the decreased pregnancy rate in GnRH-antagonist cycles [11]. Controversies regarding the endometrial receptivity differences between GnRH-agonist protocols, GnRH-antagonist protocols, and natural cycles still remain [3]. However, there have been no studies investigating the glycodelin pattern in GnRH-antagonist ART cycles; therefore, glycodelin pattern changes in GnRH-antagonist cycles may be one of the causes of the differences between GnRH-agonist and antagonist cycles.

Glycodelin is also synthesized by the granulosa and theca cells, released in follicular fluid (FF) and taken up by cumulus cells [12]. In natural conception cycles, glycodelin regulates the functional competence of spermatozoa [13]. To date, the effect of follicular fluid glycodelin levels on the success of ART cycles has not been studied.

The aim of the present study was to establish the serum concentration pattern for glycodelin in GnRH-antagonist ART-intracytoplasmic sperm injection (ICSI) cycles and to investigate the possible correlations between serum and follicular glycodelin and clinical pregnancy in these treatment cycles. To the authors' knowledge, no studies have investigated the role of follicular phase serum glycodelin levels and follicular fluid-glycodelin levels in GnRH-antagonist cycles.

Materials and Methods

This prospective cohort study was performed between June 2011 and May 2012 at the Eskisehir Osmangazi University, Medical Faculty-Center for Reproductive Health. The study was approved by the Ethical Review Board of the hospital. Informed consent was obtained from all patients prior to participation in the study. To ensure that only couples with male-factor infertility were included in the study, females were examined to rule out female-factor infertility. To identify pathologies that may have caused subfertility, patients were evaluated with a pelvic examination and a patient history. In addition, no additional endocrine disorders or endometriosis were detected. To exclude anovulation, all women were determined to have a progesterone level of > 3 ng/ml on day 21 of their menstrual cycle. Day 3 basal ultrasonography and hormone profiles of all women demonstrated the following: follicle stimulating hormone (FSH) < 10 mIU/ml, estradiol <40 pg/ml, and an antral follicle count (AFC) > 6. However, although we ruled out probable endometriosis with the absence of specific signs/symptoms for endometriosis and with the presence of a normal gynecological pelvic examination, the authors did not perform routine laparoscopy (the gold standard in the diagnosis of endometriosis) in the diagnostic work-up of the study patients. They also did not detect any findings on ultrasonography that may be associated with endometriosis or other gynecological pathologies. Furthermore, only women who never smoked were included because any amount of smoking could affect the results. Women who smoked or had findings compatible with endometriosis, anovulation, polycystic ovarian syndrome, chronic pelvic inflammation, or diminished ovarian reserve were excluded from the analysis.

A total of 80 couples admitted for ART-ICSI treatment who met the World Health Organization (WHO) criteria [14] for primary infertility due to severe oligoasthenoteratospermia [severe oligospermia (< 5 million sperm/ml), asthenospermia (< 5% progressive motility), or teratospermia (< 4% normal forms by strict criteria)] were selected for the study.

A total of 80 females received controlled ovarian hyperstimulation (COH) with a fixed-dose GnRH-antagonist protocol. As soon as menstruation began, ovarian stimulation was initiated with 150-250 U/day of recombinant FSH (rec-FSH). On the sixth day of stimulation, 0.25 mg of cetrorelix was started and was continued until the administration of human chorionic gonadotropin (hCG). Recombinant hCG was administered when ≥ three follicles grew to ≥ 17 mm, and oocyte pick-up (OPU) was performed under sedation in the 36th hour after hCG was administered. On the oocyte-retrieval day, sperm were collected in a sterile plastic container from males who had refrained from ejaculation for three to five days prior to the procedure. Following sperm preparation using the density-gradient method, the ICSI procedure was performed with sperm that were selected by embryologists two to three hours after OPU. At 16-18 hours, the fertilization-two pronucleus check was conducted on embryos that were developed in cleavage stage medium, and embryos were observed until the third day after OPU. On the third day, embryologists performed controls and selected one Grade I embryo, classified according to the European Society for Human Reproduction and Embryology (ESHRE) consensus, for transfer [15]. All transfers were performed under ultrasonography using a soft transfer catheter.

Serum hCG levels were measured in all patients on the 14th day after embryo transfer. Patients were considered pregnancy-positive if their hCG level was >50 IU/L. In patients with a positive initial hCG measurement, a second assessment was performed at 48 hours to ensure a two-fold increase in hCG. Transvaginal ultrasonography was performed three weeks later in patients who exhibited an increase in hCG, and if fetal structures and fetal cardiac activity were observed, the patient was considered to be clinically pregnant.

On day 2-3, blood serum samples were obtained. In addition, FF and blood serum samples were simultaneously obtained from 80 women during the OPU procedure. FF samples from the leading follicles were collected in each woman and pooled. Only the visually blood-free FF samples were included in the analysis. FF and serum samples were immediately centrifuged at 1,000 g for 15 minutes, and the supernatants were collected and stored in tubes at -20 °C until they were needed for the assays. Glycodelin was measured with a glycodelin enzyme-linked immunosorbent assay (ELISA) kit. The intra-assay CV was <8%, the inter-assay CV was <10%, and the minimum detectable level of glycodelin was typically less than 7.8 pg/ml.

This study was statistically evaluated using SPSS IBM 20. For all variables, the Shapiro Wilk test was used for normality. For normally distributed variables, the paired sample t-test and independent sample t-tests were used; the mean ± the standard deviation values were reported. The Wilcoxon signed ranks test and the Mann Whitney test were used to evaluate non-normally distributed variables, and the median (25%-75%) percentiles were reported.

Table 1. — *Characteristics of the pregnant and non-pregnant patients.*

	Clinically pregnant (n= 20)	Clinically non-pregnant (n= 60)	*p* value
Age (years)	31.85±3.77	31.0±5.01	0.489
BMI (kg/m^2)	25.45±4.74	25.50±4.07	0.958
FSH (mIU/ml)	5.91±2.83	5.76±2.41	0.819
LH (mIU/ml)	4.42±3.10	4.41±2.18	0.977
E_2 (pg/ml)	35.03±15.13	46.21±43.91	0.270
Duration of Infertility (years)	9.02±5.67	7.64±5.66	0.347
AFC total	12.6±6.95	12.7±6.86	0.955
Unexplained infertile couples (n)-(%)	11-27.5	29-72.5	
Male factor infertile couples (n)-(%)	9-22.5	31-77.5	

Data expressed as mean± standard deviation (SD)
Abbreviations: BMI: Body Mass Index; FSH: follicle stimulating hormone; LH: luteinizing hormone; AFC: antral follicle count.
p-value < 0.05 was considered statistically significant.

Results

The clinical characteristics of 80 couples with male-factor infertility are summarized in Table 1. No significant differences were noted in the women's age, BMI, duration of infertility, day 3 FSH-LH-estradiol levels or total AFC between couples who had a clinical pregnancy and those who did not have a clinical pregnancy. The clinical pregnancy rate was 25%.

The median value of cycle day 2-3 serum glycodelin for all patients was 1.61 (1.13-2.13) ng/ml. The median value was 1.96 (1.40 - 2.66) ng/ml for the group in which pregnancy occurred and 1.53 (1.09 - 2.0) ng/ml for the group in which pregnancy did not occur. Although the glycodelin level was higher for the group in which pregnancy occurred, the difference was not significant ($p > 0.05$, Table 2).

The median value of the OPU-day serum glycodelin for all patients was 4.49 (2.47 - 10.02) ng/ml. The median value was 5.24 (2.92 - 11.95) ng/ml for the group in which

Table 2. — *Cycle characteristics, ART outcomes, and glycodelin concentrations of patients.*

	All cases (n=80)	Clinically pregnant (n=20)	Clinically non-pregnant (n=60)	*p* value
Stimulation duration (days)	8.57 (7.0-10.0)	9.0 (7.25-10.0)	8.0 (7.0-9.0)	0.233
Gonadotropins used (IU)	2541.56 (1800-3131.25)	2362.50 (1781.25-3775.0)	2312.50 (1800-3018.75)	0.498
Total oocytes retrieved (n)	10.0 (5.0-15.75)	11.0 (6.25-17.0)	9.50 (5.0-15.0)	0.449
Serum glycodelin on day 2-3 (ng/ml)	1.61 (1.13-2.13)	1.96 (1.40-2.66)	1.53 (1.09-2.0)	0.060
Serum glycodelin on OPU-day (ng/ml)	4.49 (2.47-10.02)	5.24 (2.92-11.95)	4.28 (2.20-8.59)	0.250
Follicular fluid glycodelin (ng/ml)	6.40 (4.22-8.84)	6.47 (4.04-9.52)	6.40 (4.22-8.84)	0.996
Fertilization rate	%72	%73	%71	0.990

Data are expressed as median (25-75%). Abbreviations: OPU: oocyte pick-up. *p*-value < 0.05 was considered statistically significant.

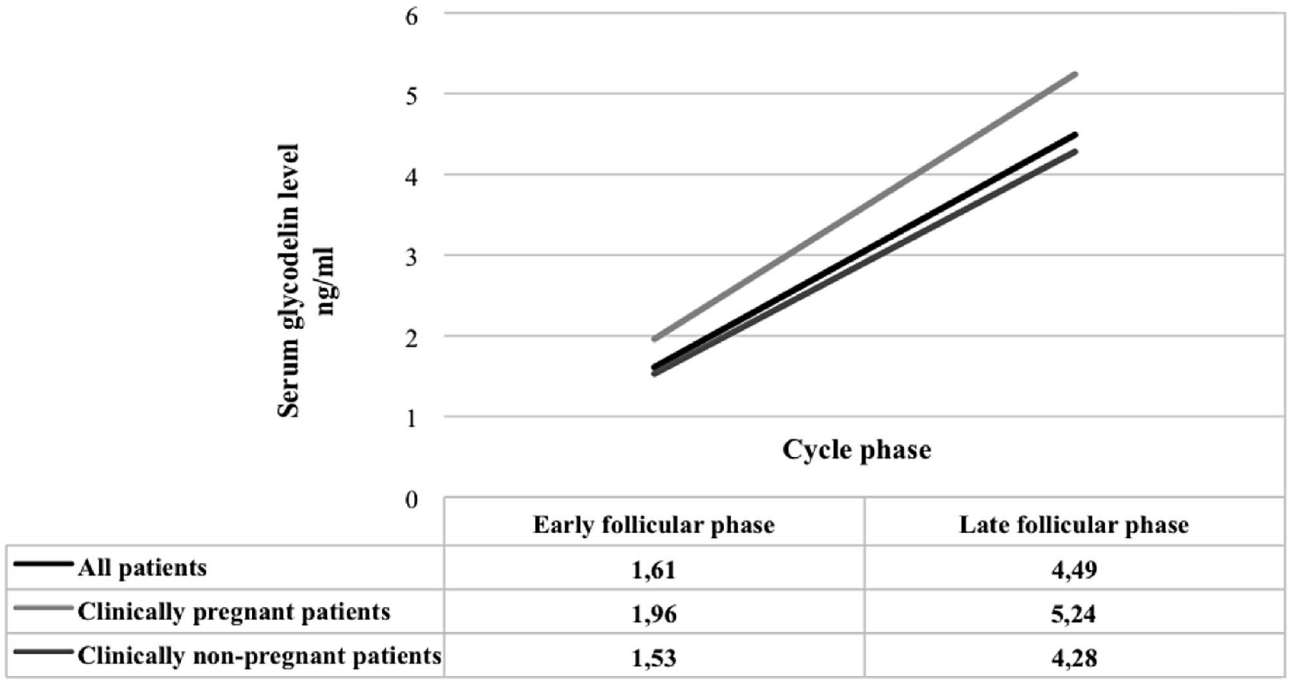

Figure 1. — Median serum glycodelin levels during different phases of GnRH-antagonist ART cycle. In all patients ($p < 0.001$), clinically pregnant ($p < 0.001$), and non-pregnant ones ($p < 0.001$) serum glycodelin increased significantly during the follicular phase.

pregnancy occurred and 4.28 (2.20 - 8.59) ng/ml for the group in which pregnancy did not occur; however, this increase was not significantly different ($p > 0.05$, Table 2).

The day 2-3 and the OPU-day serum glycodelin concentrations for all patients were 1.61 (1.13 - 2.13) and 4.49 (2.47 - 10.02), respectively ($p < 0.001$). The median values were 1.96 (1.40 - 2.66) and 5.24 (2.92 - 11.95) ($p < 0.001$) for patients who became pregnant, and 1.53 (1.09 - 2.0) and 4.28 (2.20-8.59) ($p < 0.001$) for patients in which pregnancy did not occur (Figure 1).

The median value of glycodelin in the FF collected on the OPU-day for all patients was 6.40 (4.22 - 8.84) ng/ml. The median value was 6.47 (4.04 - 9.52) ng/ml for the group in which pregnancy occurred and 6.40 (4.22 - 8.84) ng/ml for the group in which pregnancy did not occur. The FF glycodelin level was slightly higher in patients who became pregnant than in patients who did not; however, the difference was not significant ($p > 0.05$, Table 2).

Discussion

In all patients, whether pregnancy occurred or not, serum glycodelin pattern in GnRH-antagonist controlled cycles was totally different from the natural cycles [10] and the GnRH-agonist controlled cycles [5]; OPU-day serum glycodelin was found to be significantly higher than early follicular serum glycodelin level (Figure 1). Additionally, there were no significant differences in serum glycodelin concentrations in either the early follicular phase or the preovulatory phase between clinically pregnant and non-pregnant patients. In addition, there were no differences in the follicular fluid glycodelin concentrations between the two groups. Therefore, the serum or follicular fluid glycodelin levels were not predictive of clinical pregnancy in GnRH-antagonist cycles.

The ability of normal menstrual cycle serum glycodelin levels to predict pregnancy in IVF cycles was evaluated by Westergaard *et al.* in a study of 19 patients [10]. The authors found that for all of the measurements in various phases of the menstrual cycle, including the early follicular phase, patients with low glycodelin levels exhibited higher pregnancy rates in the subsequent IVF cycle. As stated in the present results, at the beginning of the cycle (day 2-3), serum glycodelin concentrations were higher in patients who became pregnant with the GnRH-antagonist treatment cycle; however, this difference was not statistically significant (1.96 ng/ml vs. 1.53 ng/ml) ($p = 0.060$). Although the present authors did not make serial measurements during the three normal menstrual cycles as did Westergaard *et al.* [10], they evaluated the results of 80 patients at the beginning of the GnRH-antagonist ICSI cycle during the unmedicated period (day 2-3). Therefore, the present findings contradict the widely held belief that low serum glycodelin concentrations in the early follicular phase are correlated with a higher probability of pregnancy. Additionally Westergaard *et al.* reported that glycodelin peaked in the early follicular and late luteal phases during the ovarian stimulation cycle, as well as in natural cycles, and that the levels were lowest in the late follicular and ovulation phases [10]. Again contrary to these findings in literature, median OPU-day glycodelin levels were higher than the median day 2-3 glycodelin levels in all patients (4.49 ng/ml vs. 1.61 ng/ml; $p < 0.001$), in clinically pregnant (5.24 ng/ml vs. 1.96 ng/ml; $p < 0.001$) and non-pregnant ones (4.28 vs. 1.53 ng/ml; $p < 0.001$) in the present study (Figure 1). Furthermore, glycodelin does not decrease towards mid-cycle in GnRH-antagonist cycles as in natural cycles; on the contrary, it increases. So it is probable that serum glycodelin pattern in GnRH-antagonist cycles may not be same as in natural cycles and/or in non-controlled (with GnRH-agonist or GnRH-antagonist) IVF cycles, and GnRH-antagonist may effect serum glycodelin in a totally different way.

In another study with 51 patients who underwent long GnRH-agonist protocol ART-IVF treatments, Bersinger *et al.* demonstrated that, up to seven days prior to the LH peak, the glycodelin was much lower than in patients in natural cycles. Furthermore, they found that the lowest glycodelin level, measured on the OPU-day, was similar to the natural cycle LH peak phase [5]. To the present authors' knowledge, there are no studies in the literature that investigate the glycodelin pattern in GnRH-antagonist cycles or its ability to predict clinical pregnancy. Based on the present results, the OPU-day glycodelin level was higher than the day 2-3 level in patients who underwent ART-ICSI cycles with a fixed-dose GnRH-antagonist protocol (4.49 ng/ml vs. 1.61 ng/ml, $p < 0.001$). These data may contradict other reports in the literature because of the differences in the protocols. Furthermore, glycodelin does not decrease towards mid-cycle in GnRH-antagonist cycles as in GnRH-agonist cycles; instead, it increases. Additionally, OPU-day serum glycodelin levels were slightly higher in patients who became clinically pregnancy after treatment; however, the difference was not statistically significant (5.24 ng/ml vs. 4.28 ng/ml, $p = 0.250$). According to the present results, it is probable that serum glycodelin pattern in GnRH-antagonist COH cycles may be totally unlike from GnRH-agonist controlled COH cycles.

Currently, conducting an ART cycle with a GnRH-antagonist is a more patient-friendly method for initiating ovarian stimulation and provides physicians with a possible alternative to GnRH-agonist treatment. Additionally the emergence of GnRH-antagonists in ART cycles has enabled the development of milder ovarian stimulation protocols that may have a reduced impact on endometrial receptivity [16]. However, concerns remain regarding the possible detrimental effects of GnRH-antagonists on endometrial receptivity [17, 18]. Therefore, although the authors did not study endometrial receptivity, the different serum glycodelin patterns in GnRH-antagonist cycles and the natural and GnRH-agonist cycles may be very important in this re-

gard. This finding may explain the lower pregnancy rates that have been reported in IVF cycles using GnRH-antagonists versus the GnRH-agonist long protocol [11, 19].

One of the major limitation of the present study is that the authors only evaluated the patients with GnRH-antagonist ART cycles and they compared their findings with the literature-derived natural and GnRH-agonist ART results. Therefore, future studies with a three armed study design (natural, GnRH-agonist, and GnRH-antagonist cycles) will give more knowledge on the condition. Additionally, in the follicular phase, the authors only measured serum glycodelin at the early follicular and late follicular phases; more frequent serum glycodelin measurements will provide us with more precise information. However, to the best of their knowledge, this is the first study that presents the serum glycodelin pattern in GnRH-antagonist controlled cycles.

In conclusion, although day 2-3 and OPU-day measurements of serum glycodelin levels were not significant in predicting clinical pregnancy, the serum glycodelin pattern between GnRH-antagonist COH cycles was different than the patterns in natural and GnRH-agonist downregulated cycles. The difference in the serum glycodelin pattern of the GnRH-antagonist cycles may play a role in the lower pregnancy rates that have been reported in GnRH-antagonist cycles, when compared to GnRH-agonist cycles. Additionally, follicular fluid glycodelin levels were not effective in predicting clinical pregnancy in the GnRH-antagonist cycles.

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