

# The Effects of Cervical Mucous Glycodelin-A, Granulocyte Colony-Stimulating Factor and L-Selectin on Pregnancy Outcomes in In-Vitro Fertilization-Embryo Transfer Cycles

## Servikal Mukus Glikodelin-A, Granülosit Koloni Stimüle Edici Faktör ve L-Selektin Seviyelerinin İn Vitro Fertilizasyon-Embriyo Transferi Sikluslarında Gebelik Sonuçları Üzerine Etkisi

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**ABSTRACT Objective:** To investigate the benefits of cervical mucous glycodelin-A, granulocyte colony-stimulating factor (G-CSF) and L-selectin levels as a marker of endometrial receptivity in in-vitro fertilization/embryo transfer cycles (IVF-ET). **Material and Methods:** In this prospective cohort study cervical mucus samples were collected during oocyte pick up and embryo transfer from 56 IVF-ET patients. Blood samples were collected during embryo transfer only. The glycodelin-A, G-CSF and L-selectin levels in cervical mucus and blood samples were measured and the results were compared with the pregnancy rates. **Results:** Cervical mucus G-CSF and L-Selectin levels measured at embryo transfer were significantly higher than those measured during oocyte pick-up ( $p=0.04$  and  $p=0.002$ , respectively). Mean cervical mucous G-CSF levels were significantly higher in pregnant patients compared to non pregnant patients during embryo transfer ( $p=0.024$ ). When 1365 pg/ml was set as cut-off point for G-CSF levels in predicting pregnancy, sensitivity and specificity were 76% and 61.3 %, respectively. However, there were no significant relationship between the glycodelin-A and L-selectin levels in the cervical mucus and pregnancy outcomes. **Conclusion:** Investigating markers of endometrial receptivity in the cervical mucus during embryo transfer is a novel and non-invasive method in IVF cycles. G-CSF may be an important marker in predicting the implantation.

**Key Words:** Fertilization in vitro; granulocyte colony-stimulating factor; L-selectin; PAEP protein, human

**ÖZET Amaç:** Servikal mukus glikodelin-A, granülosit koloni stimüle edici faktör (G-CSF) ve L-selektin seviyelerinin, in vitro fertilizasyon/embriyo transferi (IVF-ET) sikluslarında, endometriyal reseptivite belirteçi olarak yararlılığını araştırmak. **Gereç ve Yöntemler:** Bu Prospektif kohort çalışmasında, 56 IVF-ET hastasından oosit toplama ve embriyo transferi sırasında servikal mukus örnekleri toplandı. Kan örnekleri sadece embriyo transferi sırasında alındı. Servikal mukusta ve kanda glikodelin-A, G-CSF, L-selektin seviyelerine bakıldı ve gebelik sonuçları ile karşılaştırıldı. **Bulgular:** Embriyo transferi sırasında ölçülen servikal mukus G-CSF ve L-selektin seviyeleri oosit toplama sırasında ölçülen değerlere göre anlamlı derecede yüksekti ( $p=0,04$ ,  $p=0,002$  sırasıyla). Embriyo transferi sırasında ölçülen ortalama servikal mukus G-CSF seviyeleri gebe kalanlarda, gebe kalmayanlara göre anlamlı derecede yüksekti ( $p=0,024$ ). Gebeliği öngörmeye G-CSF için kesme noktası 1365 pg/ml alındığında, duyarlılık %76, özgüllük %61,3 olarak bulundu. Buna karşın, glikodelin-A ve L-selektinin servikal mukus seviyeleri ile gebelik sonuçları arasında ilişki bulunmadı. **Sonuç:** Embriyo transferi sırasında, servikal mukusta endometriyal reseptivite belirteçlerinin araştırılması, IVF siklusları için yeni ve girişimsel olmayan bir yöntemdir. G-CSF implantasyonu öngörmeye önemli bir belirteç olabilir.

**Anahtar Kelimeler:** Tüp bebek; granülosit koloni uyarıcı faktör; L-selektin; PAEP protein, insan

Implantation failure is a problem that remains to be solved in the field of assisted reproductive technologies (ART). Successful implantation and pregnancy requires a vital embryo and a receptive endometrium and depend on complex process of interactions between these two factors. Endometrium is receptive to the implanting embryo only for a limited time which is referred as 'window of implantation'.<sup>1</sup> Implantation window is defined as the limited period during which endometrium is receptive to the implantation of the free-lying blastocyst. In natural human cycle blastocyst apposition begins on the 6<sup>th</sup> day of Lutein hormone (LH) peak and completed by the 10th day of LH peak.<sup>2</sup> This putative implantation window is believed to be regulated by locally acting growth factors, transcription factors and cytokines.<sup>3,4</sup>

Although it is possible to evaluate the embryo quality in ART cycles by using morphological methods, methods enabling the non-invasive evaluation of endometrial receptivity are yet to be found.<sup>1</sup>

Glycodelin-A, also known as placenta protein 14 or progesterone associated endometrial protein is a glycoprotein coded on chromosome 9. It is the major protein synthesized by the late secretory endometrium and the gestational decidua but it is also found in other tissues.<sup>5</sup> Due to its immunosuppressive nature, it prevents the rejection of the fetus from the implantation area as a foreign body.<sup>6</sup> Its secretion diminishes during ovulation and enables fertilization while it increases during the luteal phase and enables implantation.<sup>7</sup>

Selectins, the members of the adhesion molecule group, include P-selectin, L-selectin and E-selectin. Selectins are glycoproteins, which consist of at least 30% carbohydrate.<sup>8</sup> L-selectin is secreted by the trophoblasts and oligosaccharide based L-selectin ligand is secreted by the endometrium which serves as a bridge that binds the embryo to the endometrium.<sup>9</sup> L-selectin is secreted more from the luminal epithelium than from the glandular epithelium and reaches its highest levels during the secretory phase and to the lowest during the proliferative phase.<sup>10</sup>

Granulocyte colony stimulating factor (G-CSF) is a cytokine, which belongs to the family of hematopoietic growth factors.<sup>11</sup> Although it is primarily produced by hematopoietic cells, it may also be produced by osteoblasts, smooth muscle cells, endothelial and epithelial cells, ovaries and endometrium.<sup>12</sup> It has been shown that white blood cells and the G-CSF level are increased in cycles, in which ovarian stimulation is applied.<sup>13</sup> Salmassi et al. has determined higher G-CSF levels in the follicular fluid than in serum and reported that G-CSF is important in follicular maturation.<sup>14</sup> Additionally, animal studies have shown that in the absence of certain cytokines including the G-CSF, implantation sites or litter sizes are reduced.<sup>15</sup>

In order to improve the implantation rates in ART cycles, markers that indicate endometrial receptivity with non-invasive methods and without harming the ART cycle are needed. Endometrial biopsy procedure during the embryo transfer cycle may have deleterious effect on the pregnancy rate. Therefore, by using cervical mucus and serum samples in in vitro fertilization embryo transfer (IVF-ET) cycles we measured 3 endometrial receptivity markers, namely glycodelin-A, G-CSF and L-selectin, to find their beneficial value in the prediction of pregnancy.

## MATERIAL AND METHODS

A total of 59 patients who were scheduled for In vitro fertilization intracytoplasmic sperm injection (IVF/ICSI) treatment were included in the study. The approval of the clinical ethics committee of Dokuz Eylül University Medical School was obtained for the study. Written informed consent was signed by all the patients participating in the study.

The patients undergoing IVF-ET were superovulated with gonadotrophins after down regulation with Gonadotropin releasing hormone GnRH analogues as described elsewhere. When 3 follicles with a diameter  $\geq 18$  mm were reached, 10.000 IU human chorionic gonadotropin (hCG; Pregnyl, Netherlands) was administered. Transvaginal oocyte retrieval was performed under intravenous sedation and ultrasound guidance 36 hours after

hCG injection. Embryo transfer was performed approximately 48 to 72 hours after insemination or ICSI. Embryo quality was determined according to the morphological criteria described elsewhere.<sup>16</sup> Luteal phase was supported with vaginal progesterone starting on the day of ovum pick-up until the time of pregnancy test. Biochemical pregnancy was defined as serum  $\beta$ -hCG concentration higher than 30 mIU/ml on the 12<sup>th</sup> day, while clinical pregnancy was defined as the presence of one or more gestational sacs detected with transvaginal ultrasonography 2 weeks after the positive pregnancy test.

Cervical secretions were collected with an ophthalmic sponge (BD visispear eye sponge; BD Visitec, USA) just before irrigating the vagina with sterile saline solution during the oocyte pick-up and embryo transfer procedures. Cervical secretions were collected after exposure of the cervical os with the speculum. The secretions were collected by placing the ophthalmic sponge directly into the cervical os and allowing it to absorb secretions for approximately 1 minute. The cervical secretion samples, which were obtained by this method were placed in lidded plastic tubes and transported to be stored at -80 °C. The blood samples which were collected during embryo transfer were centrifuged at 2000 rpm for 10 minutes and serum was separated. The obtained serums were frozen and stored at -80 °C. The cervical secretion and serum samples were stored until biochemical analysis.

### BIOCHEMICAL ANALYSIS

The ophthalmic sponges were extracted according to the method defined by Castle et al.<sup>17</sup> Each sponge was weighed to determine the amount of secretion absorbed by it. The sponges were incubated in 300mL extraction buffer for 30 min at 4°C. The extraction buffer consisted of the following components: phosphate buffer, NaCl (0.25 M), Aprotinin (100 mg/mL) and Sodium azide (0.001%). Following the incubation, centrifuge was performed again at 16000 g for 15 minutes by using Spin-X centrifuge filter tubes (Corning, Sigma). The identical extraction volume was added again to the extracted volumes and centrifuge was performed immediately. The two extraction volumes were combined

and stored at -80 °C until the analysis. By using the weight of the dry sponges (y) and that of the sponges containing absorbed secretion (x), the dilution factor was calculated according to the following formula:  $[(x-y)+0.6 \text{ g buffer}]/(x-y)$ . The density of the buffer was 1.005 g/mL and the weight of the 0.6 mL buffer was considered as 0.603 g. ELISA method was used via L-Selectin (Bender MedSystems Inc., USA), G-CSF (Biosource, USA) and Glycodelin-A (DRG, Germany) kits. The highest worked-up standard concentration in each test was picked for the recovery experiment. The standard concentrations were added to the sponges at an amount of 100 mL and incubated for 10 minutes. The extraction process that was previously applied to the samples was also applied to the standard buffers and these samples were analyzed in terms of G-CSF, glycodelin-A and L-selectin levels. The following are the respective markers ratio absorbed onto the sponges: L-Selectin: 81.26%, G-CSF: 62.54%, Glycodelin-A: 74.75%. The inter-assay variabilities were 4.66% for L-selectin, 8.64% for glycodelin-A and 5.4% for G-CSF.

### STATISTICAL ANALYSIS

The data were analyzed by using SPSS (Statistical Package for Social Sciences, version 11.0). Parametric Independent T-test was applied to compare the levels of G-CSF, Glycodelin-A and L-selectin during oocyte pick-up and embryo transfer. Two groups were formed: clinical pregnancy positive and clinical pregnancy negative. In order to determine whether there were differences between the groups in terms of Glycodelin-A and L-selectin levels, non-parametric Mann-Whitney U-Test was used.  $p < 0.05$  was considered as statistically significant.

## RESULTS

Of the 59 patients included in the study, 2 patients in whom fertilization did not occur and 1 patient in whom sperm was not obtained during TESE were excluded. The data obtained from a total of 56 patients were analyzed. Biochemical pregnancy developed in 28 (50%) patients while clinical pregnancy developed in 25 (44.6%) patients. The

implantation rate was 20.51%. The distribution of the patients included in the study according to the infertility etiologies were as follows: male factor: 46.4% (n:26), unexplained infertility: 28.6% (n:16), tubal factor: 10.7% (n:6), anovulation: 5.4% (n:3), endometriosis: 5.4% (n:3), and multiple factor: 3.6% (n:2). The clinical and embryological data of the IVF-ET cycles were presented in Table 1. There were no significant differences between the pregnant and non-pregnant patients as far as the clinical parameters are concerned.

The glycodelin-A, G-CSF and L-selectin levels at the time of oocyte pick-up and those at the time of embryo transfer were compared. G-CSF ( $p=0.04$ ) and L-selectin ( $p=0.002$ ) levels were significantly increased during embryo transfer compared to those during oocyte pick-up while no significant difference was observed in glycodelin-A levels (Table 2).

In patients with and without positive clinical pregnancy, the glycodelin-A, G-CSF and L-selectin levels of both the cervical mucus samples at oocyte pick-up and the serum samples collected during embryo transfer were compared (Table 3). Although the G-CSF levels measured in the cervical mucus during embryo transfer were significantly higher in patients with positive clinical pregnancy ( $p: 0.024$ ), glycodelin-A and L-selectin levels were not different.

**TABLE 1:** Cycle characteristics of the patients during in-vitro fertilization and embryo transfer.

n=56	Mean $\pm$ SD	range
Age (year)	31.6 $\pm$ 4.66	21-40
Day 3 E2 (pg/ml)	52.06 $\pm$ 28.73	20-168
Day 3 FSH (mIU/ml)	6.82 $\pm$ 2.55	1,54-16,20
Number of previous IVF cycles	1.35 $\pm$ 0.58	1-3
Total gonadotropin dose (IU)	3117.67 $\pm$ 1833.04	700-9000
Total gonadotropin duration (day)	11.58 $\pm$ 2.47	8-22
Number of total oocytes	12.73 $\pm$ 7.48	1-36
Number of total embryos	8.14 $\pm$ 5.08	1-22
Number of transferred embryos	3.48 $\pm$ 0.91	1-5
Mean transferred embryo grade	1.64 $\pm$ 0.57	1-4

SD: Standard deviation.

**TABLE 2:** The cervical mucus levels of the markers during oocyte pick-up and embryo transfer.

n=56	OPU	ET	p value*
Glycodelin-A ng/ml	261.11 $\pm$ 124.85	270.68 $\pm$ 111.11	0.67
G-CSF pg/ml	1544.98 $\pm$ 1251.12	2057.99 $\pm$ 1783.82	0.046
L-selectin ng/ml	66.39 $\pm$ 58.92	95.23 $\pm$ 58.20	0.002

\* Independent T-test.

The data are presented as mean  $\pm$  standard deviation.

OPU: Oocyte pick-up; ET: Embryo Transfer.

In order to determine the efficiency of CSF levels in the prediction of pregnancy; sensitivity, specificity, positive predictive value, negative predictive value and likelihood ratios were calcu-

**TABLE 3:** Comparisons of the cervical mucous and serum levels of the markers between the pregnant and non-pregnant patients.

	Pregnant (n=25)	Non-pregnant (n=31)	P value*
<b>Cervical mucus levels</b>			
Glycodelin-A ng/ml (ET)	276.91 $\pm$ 19.79	265.65 $\pm$ 113.73	0.77
G-CSF pg/ml (ET)	2461.43 $\pm$ 2021.28	1732.64 $\pm$ 1523.23	0.024
L-selectin ng/ml (ET)	104.87 $\pm$ 57.37	87.46 $\pm$ 58.62	0.24
Glycodelin-A ng/ml (OPU)	258.48 $\pm$ 108.79	263.24 $\pm$ 138.20	0.92
G-CSF pg/ml (OPU)	1534.26 $\pm$ 1090.31	1553.62 $\pm$ 1385.11	0.70
L-selectin ng/ml (OPU)	58.73 $\pm$ 56.40	72.57 $\pm$ 61.09	0.32
<b>Serum levels during ET</b>			
Glycodelin-A ng/ml	9.52 $\pm$ 4.28	9.66 $\pm$ 4.64	0.86
G-CSF pg/ml	57.84 $\pm$ 11.01	60.17 $\pm$ 14.07	0.55
L-selectin ng/ml	2068.64 $\pm$ 626.39	2358.46 $\pm$ 625.30	0.11

\* Mann-Whitney U Test.

The data were presented as mean  $\pm$  standard deviation.

**TABLE 4:** The efficiency of G-CSF levels in predicting pregnancy.

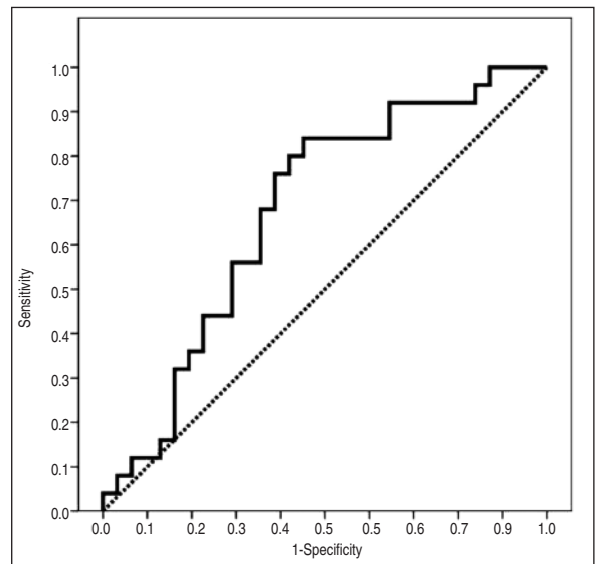
Cut-off	Sensitivity	Specificity	PPV	NPV	Kappa	LHR
871 pg/ml	92%	35.5%	53.5%	84.6%	0.257	1.42
1106 pg/ml	84%	54.8%	60%	81%	0.374	1.85
1365 pg/ml	76%	61.3%	61.3%	76%	0.364	1.96
1595 pg/ml	68%	64.5%	60.7%	71.4%	0.321	1.91

PPV: Positive predictive value; NPV: Negative predictive value; LHR: Likelihood ratio.

lated. The results were presented in Table 4. When 1365 pg/ml was set as the cut-off point for G-CSF levels in predicting pregnancy, sensitivity and specificity were 76% and 61.3%, respectively. The ROC curve generated for the G-CSF was presented in Figure 1.

## DISCUSSION

Embryo implantation is the rate limiting step in IVF success. Successful implantation and pregnancy requires a vital embryo and a receptive endometrium. Although there are ways to define a vital embryo with a high capacity of implantation, what precisely constitutes a receptive endometrium is poorly defined. The human endometrium is receptive to the blastocyst for a limited time period in the mid-luteal phase. The duration of the implantation window is believed to be regulated by the expression of locally acting growth factors, transcription factors, adhesion molecules and cytokines. In recent years, a number of key regulators have been identified which appear to be crucial for implantation in humans. Many clinical markers have been proposed including integrins, selectins, glycodelin, leukemia inhibiting factor and G-CSF. However, none of them have yet shown to be clinically useful and there is no any non-invasive method to measure these endometrial factors without injuring the endometrium.<sup>18</sup> Many years endometrium is assessed by a biopsy, but aspirated cervical mucous at the time of embryo transfer may give valuable information about endometrial secretions without harming the endometrium. Van der Gaast et al. have shown that endometrial secretion aspiration with an insemination catheter attached to a 10 ml syringe prior to embryo transfer does not reduce implantation rates in IVF cycles and that the protein content



**FIGURE 1:** Receiving operating characteristics (ROC) curve constructed for G-CSF in predicting pregnancy.

in the endometrial fluid was sufficient for protein pattern analysis.<sup>19</sup> In our study we used an ophthalmic sponge which is even more non-invasive since there is no entrance to the uterine cavity and no negative pressure with a syringe was applied. Additionally, in the biochemical analysis the material obtained has shown to be effective for measuring the implantation marker levels.

Glycodelin-A is expressed by the endometrium by a cycle dependent manner and there is no detectable glycodelin-A in endometrium during cycle days 5-17 and from cycle day 18 expression increases and reaches a maximum in the late luteal phase. Brown et al. studied endometrial expression of glycodelin-A with serial endometrial biopsies in 15 oocyte donors undergoing controlled ovarian hyperstimulation cycles. They noticed a significantly increased proportion of glycodelin-A staining in en-

endometrial cells in stimulation cycles compared to natural cycles and both groups demonstrated an increasing pattern of glycodeilin-A expression throughout the late luteal phase.<sup>20</sup> Contrary to this, another study did not find any difference in glycodeilin-A levels between the stimulated and natural cycle luteal phase biopsy of oocyte donors.<sup>21</sup> However, we did not detect an increase in glycodeilin levels from the day of OPU to the day of ET. This may be related to 2 to 3 days of time period may be too early to detect this difference, since the glycodeilin levels mainly peak at the time of implantation that is 2 or 3 days after cleavage stage transfer. Additionally, we did not detect a difference in both cervical mucous and serum glycodeilin levels between the pregnant and non pregnant cycles. In the early studies with glycodeilin-A, decreased levels were detected in patients with habitual abortions and in patients with luteal phase insufficiency.<sup>22,23</sup> It has been speculated that ovarian stimulation with gonadotrophins may increase glycodeilin levels and improve pregnancy outcomes.

L-selectin is an adhesion molecule that plays a crucial role in the initial adhesion of the blastocyst to the endometrium. Selectin expression of the embryo occurs after hatching from the zona pellucida and the L-selectin binding oligosaccharide based ligands are expressed by the endometrium in the window of implantation where they slow down the movement of the embryo before the final attachment by the integrins.<sup>1</sup>

In a recent study, Foulk et al. found that patients with recurrent implantation failures who are negative for the glycodeilin ligand did not become pregnant despite an average of five successive embryo transfers.<sup>24</sup> Instead, 75% of the patients with positive L-selectin ligand subsequently conceived. Although there were no differences in the cervical mucous L-selectin levels between the pregnant and non-pregnant patients in our study all the patients had detectable levels of this implantation marker. Other studies relating L-selectin levels in donor IVF cycles mostly found increased levels in the endometrium of pregnant patients compared to non-pregnant women. In a retrospective cohort analysis in donor egg recipients, endometrial L-selectin ex-

pression at the apical surface detected by immunohistochemistry was significantly higher for pregnant patients.<sup>25</sup> However, because of ethical reasons, this biopsy was obtained from a cycle other than that of embryo transfer, although the authors claim that they applied identical hormonal stimulation. In contrast to this study, Lai et al. detected reduced expression of L-selectin in the GnRH antagonist treated donor cycle patients' endometrial samples compared with control group of paraffin-embedded archival endometrial samples.<sup>26</sup> They interpreted these results as that controlled ovarian hyperstimulation is associated with a reduction of L-selectin ligand expression during the luteal phase and luteal phase support does not have a corrective impact on this phenomenon. Whether GnRH antagonist or another component of the stimulation contributed to this effect remains unclear. However, same group reported that in a GnRH antagonist protocol IVF cycle, luteal phase support with micronized progesterone and 17  $\beta$ -estradiol seem to increase endometrial L-selectin ligands in the luminal endometrium.<sup>27</sup>

Colony stimulating factor is a hematopoietic growth factor which stimulates the proliferation of leucocytes.<sup>28</sup> It has been shown that implantation and ongoing pregnancy rates are diminished in mice with absent CSF and correction of CSF concentrations restores fertility.<sup>29-31</sup> These findings suggest a role for CSF for implantation. However, studies relating to G-CSF and implantation are scarce in the literature. Salmassi et al measured G-CSF levels on the day of oocyte retrieval in the serum and follicular fluid of patients undergoing IVF treatment.<sup>32</sup> They observed that G-CSF is higher in the follicular fluid than the serum and there was a gradual increase in the serum from low through moderate and high response to ovulation induction. G-CSF level increased only in pregnant patients from embryo transfer to implantation and gestation. Their results demonstrated that patients with the highest levels in the serum had the best pregnancy rates. Similar to these findings we also observed an increase in the level of G-CSF in the serum of patients undergoing IVF from the day of oocyte retrieval to the day of embryo transfer. Ad-

ditionally, we have found that cervical mucous G-CSF levels were significantly higher in the pregnant patients compared to the non-pregnant ones. Our results show that G-CSF is constantly increased in the cervical secretions from the day of oocyte retrieval to the day of embryo transfer and is associated with better pregnancy rates.

The drawbacks of the study is that we do not know yet whether cervical mucous sampling is representative of the entire endometrium, because there may be difference between the level detected and the functional biochemical marker level. This must be clarified with further studies.

Ideal implantation marker should be easily and non-invasively obtained from the endometrium

and it has to be specific to the endometrium or the implantation process. It must be normal in women with known fertility and should be below a cut off level in patients with an implantation failure. This ideal marker is yet to be found.

With further research, we believe that cervical secretion analysis of implantation markers in the index IVF cycle may give important clues for the receptivity of the endometrium and this will allow a clinical decision for the embryo transfer. For example, in patients with an unfavorable endometrium after an analysis, all the transferable embryos may be frozen and transferred in a subsequent cycle when the endometrium is most receptive. Further studies are needed in this topic.

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