

Original research article

Late follicular phase administration of levonorgestrel as an emergency contraceptive changes the secretory pattern of glycodelin in serum and endometrium during the luteal phase of the menstrual cycle

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Abstract

This study examined serum glycodelin concentrations and endometrial expression during the luteal phase following oral administration of levonorgestrel (LNG) at different stages of the ovarian cycle. Thirty women were recruited and allocated into three groups. All groups were studied during two consecutive cycles, a control cycle and the treatment cycle. In the treatment cycle, each woman received two doses of 0.75 mg LNG taken 12 h apart on days 3–4 before the luteinizing hormone (LH) surge (Group 1), at the time of LH rise (Group 2) and 48 h after the rise in LH was detected (Group 3). Serum progesterone (P) and glycodelin were measured daily during the luteal phase, and an endometrial biopsy was taken at day LH +9 for immunohistochemical glycodelin-A staining. In Group 1, serum P levels were significantly lower, serum glycodelin levels rose earlier and endometrial glycodelin-A expression was weaker than in Groups 2 and 3, in which no differences were found between control and treatment cycles. Levonorgestrel taken for emergency contraception (EC) prior to the LH surge alters the luteal phase secretory pattern of glycodelin in serum and endometrium. Based on the potent gamete adhesion inhibitory activity of glycodelin-A, the results may account for the action of LNG in EC in those women who take LNG before the LH surge.

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1. Introduction

Progestins are widely used for contraception in various modes of administration, such as injectables, subdermal implants, intrauterine systems, and peroral tablets [1], including hormonal emergency contraception (EC). Hormonal EC is an effective means to prevent unwanted pregnancies [2]. As a contraceptive, this method does not disrupt an established pregnancy and, among several modalities, the use of steroid hormones has become the method of choice. The two widely used hormonal EC methods are the combined estrogen–progestin and the progestin-only regimens [3,4]. In both, two doses of the contraceptive formulation taken 12 h apart are administered after unprotected intercourse. The contraceptive mechanisms of action of these

methods remain largely unknown. It is likely that multiple mechanisms operate, depending on the timing of drug intake with respect to ovulation. In previous studies, treatment around the ovulatory phase of the cycle has affected the luteinizing hormone (LH) surge and ovulation in some, but not in the majority of cases, suggesting that mechanisms other than anovulation are often involved. Additional possible mechanisms include thickening of the cervical mucus, alterations in sperm penetration or transport, fertilization, and/or interference with fertilization, follicular growth, corpus luteum development and/or implantation [5–11].

Glycodelin-A is a major secretory progesterone (P)-regulated glycoprotein of the human endometrium [12]. During the normal periovulatory phase, glycodelin-A is absent from the endometrium, and it becomes highly expressed during the last week of the luteal phase only [13,14]. The temporal expression is significant because glycodelin-A is a potent inhibitor of sperm–zona binding

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[15]. Through its inhibitory activity on the immune cells, glycodeclin-A is also believed to play a role in fetomaternal defense mechanisms [16–18].

In a previous study [5], we reported a high frequency of anovulation when levonorgestrel (LNG) was taken before the LH surge, whereas ovulation was more frequent among those women who took the drug at the time of or after the LH surge. This too indicated that mechanisms other than anovulation must play a part. Interestingly, no difference was found in endometrial histology in ovulatory cycles of women who took LNG during midcycle. We hypothesized that progestin-only EC may interfere with the normal cyclical pattern of glycodeclin expression. To this effect, we used the same material as in the study by Durand et al. [5] to investigate whether periovulatory intake of LNG may alter the expression pattern of glycodeclin-A in the endometrium and/or the glycodeclin levels in serum.

2. Material and methods

2.1. Subjects and study design

The study was approved by the institutional review board of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, and the subjects signed an informed consent form. The anthropometric and clinical characteristics of all subjects included in this study have been previously described [5]. The material from our previous study was used for this study in a retrospective fashion [5], excluding those women in whom drug intake fell outside the three defined categories (see below). This left a total of 30 participants (mean age, 32 years; range, 29–35 years) with regular menstrual cycles (mean cycle lengths \pm SD of three previous cycles, 28.8 ± 1.9 days; range, 25–32 days). The subjects were allocated into three different treatment groups and the study involved two complete menstrual cycles. A control cycle was used to estimate timing of the LH surge and to guide treatment and endometrial biopsies during the consecutive study cycle. In Group 1, eight women received two doses of 0.75 mg of LNG (Postinor, Gedeon Richter, Budapest, Hungary) taken 12 h apart, with the first dose given prior to the occurrence of the LH surge (between LH -4 and LH -2). In this group, the timing of LNG administration was determined retrospectively once the LH surge in the treatment cycle had been identified. Group 2 involved 11 subjects taking the first pill as soon as the first rise in LH was detected, and 11 women assigned to Group 3 were treated 48 h after the rise in LH was detected. During both cycles, the subjects were asked to monitor urinary LH daily, starting on the 11th day of their menstrual cycle, dated according to the first day of the last menstrual period, until presence of LH was detected. At this time, daily blood samples were obtained until the onset of menses. An endometrial biopsy was taken on day LH $+9$, from the day at which rise in LH was detected, during both control and treatment cycles. Measurements of LH in serum

allowed us to retrospectively determine the time in the cycle at which treatment was initiated and endometrial biopsies were taken. As described previously [5], all the women included in this study were ovulatory in both control and treatment cycles. Ovulation was documented by ultrasonographic findings of follicular rupture and midluteal phase serum P concentrations above 5 ng/mL. The mean length of luteal phase and levels of serum P in study cycles of Group 1 were significantly shorter and lower, respectively, compared to the controls, whereas no differences between control and treatment cycles were observed in Groups 2 and 3.

2.2. Hormone assays

Luteinizing hormone in urine was monitored by a commercially available kit (Ovuquick, Corne SA de CV, Mexico, D.F.). In all subjects, serum concentrations of LH, estradiol (E_2) and P were measured in duplicate by a specific immunoradiometric assay for LH and specific radioimmunoassays for E_2 and P, using commercial reagents (Diagnostic Products, Los Angeles, CA), or with the reagents and protocols provided by the World Health Organization Matched Reagent Programme (Geneva, Switzerland) as previously described [5,19]. The inter-assay coefficient of variation (CV) was less than 10% for all hormones, and intra-assay CVs were 4.9%, 1.63% and 1.33% for LH, E_2 and P, respectively. In the subjects from Groups 1 and 2, serum glycodeclin concentration was measured by a sandwich-type immunofluorometric assay [20]. Since in this study we were interested in the early luteal phase effects of LNG, serum glycodeclin concentrations were not analyzed in Group 3 in which LNG was given after the LH surge. Briefly, glycodeclin immunoreactivity was quantitated in serum using monoclonal antibodies produced against purified glycodeclin-A [21]. The antibodies (F25-9D8) were labeled with Delfia europium-labeling reagent (Wallac, Turku, Finland) and used in the assay at a concentration of 50 ng/0.2 mL per well. For solid phase coating, biotinylated monoclonal antibodies (F23-9G2) were used at a concentration of 0.5 μ g/0.2 mL per well. Standards and serum samples were added to the wells and were incubated overnight at 4°C. These were then washed and further incubated with labeled antibodies for 1 h at room temperature as previously described [22]. The wells were washed four times, and 0.2 mL enhancement solution was added and fluorescence measured using a 1234 Delfia research fluorometer (LKB Wallac). The intra-assay and inter-assay CVs were 3.4% and 7.7% at the level of 21.3 and 22.7 ng/mL, respectively. To avoid systematic inter-assay variation, the samples were randomized and analyzed in a blinded fashion.

2.3. Immunohistochemistry

Endometrial expression of glycodeclin-A was studied in sections (5 μ m) of formalin-fixed, paraffin-embedded

endometrial tissues from all subjects during both control and treatment cycles on day LH +9 as previously described [23,24]. Rabbit anti-glycodelin IgG was used as the first antibody, and biotinylated swine antirabbit IgG (Dako, Glostrup, Denmark) and normal rabbit serum were used as the second and control antibodies, respectively. Another negative control was added using the first antibody, but immunoabsorbed with purified glycodelin-A (16 µg/mL). Endogenous peroxidase activity was blocked by treatment with 0.6% perhydrol in methanol. Immunostaining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole as a substrate. The tissue sections were counterstained with hematoxylin (blue). Glycodelin-A appeared as red staining. Immunohistochemical analysis was performed in a blinded fashion by three observers. Staining intensity was recorded using a semiquantitative scale of 0, 1, 2 or 3 (none, weak, moderate or strong).

2.4. Endometrial histology

Histologic dating of endometrial biopsies was performed using criteria described by Noyes et al. [25] and by Hendrickson and Kempson [26]. Glandular and stromal

elements were dated separately and given equal importance [27]. The degree of histologic maturation was based on the typical changes in glandular and stromal elements. The morphological endometrial characteristics were correlated with the serum LH surge, follicular rupture, and luteal serum concentrations of E₂ and P rather than with the ideal 28-day cycle. A specimen in which glandular maturation was delayed by three or more days from the date of the LH surge was considered to be out of phase.

2.5. Statistical analyses

The differences between untreated and treated cycles were established using the paired *t* test. The one-sided test was used for comparisons since changes in variables in treated cycles were expected to occur in one direction. Analysis of variance was used for comparisons among groups. A *p* value ≤.05 was considered as significant.

3. Results

The secretion profile of glycodelin in serum from subjects belonging to Groups 1 and 2 is shown in Fig. 1. Treatment with LNG before the LH surge shortened by 4 days

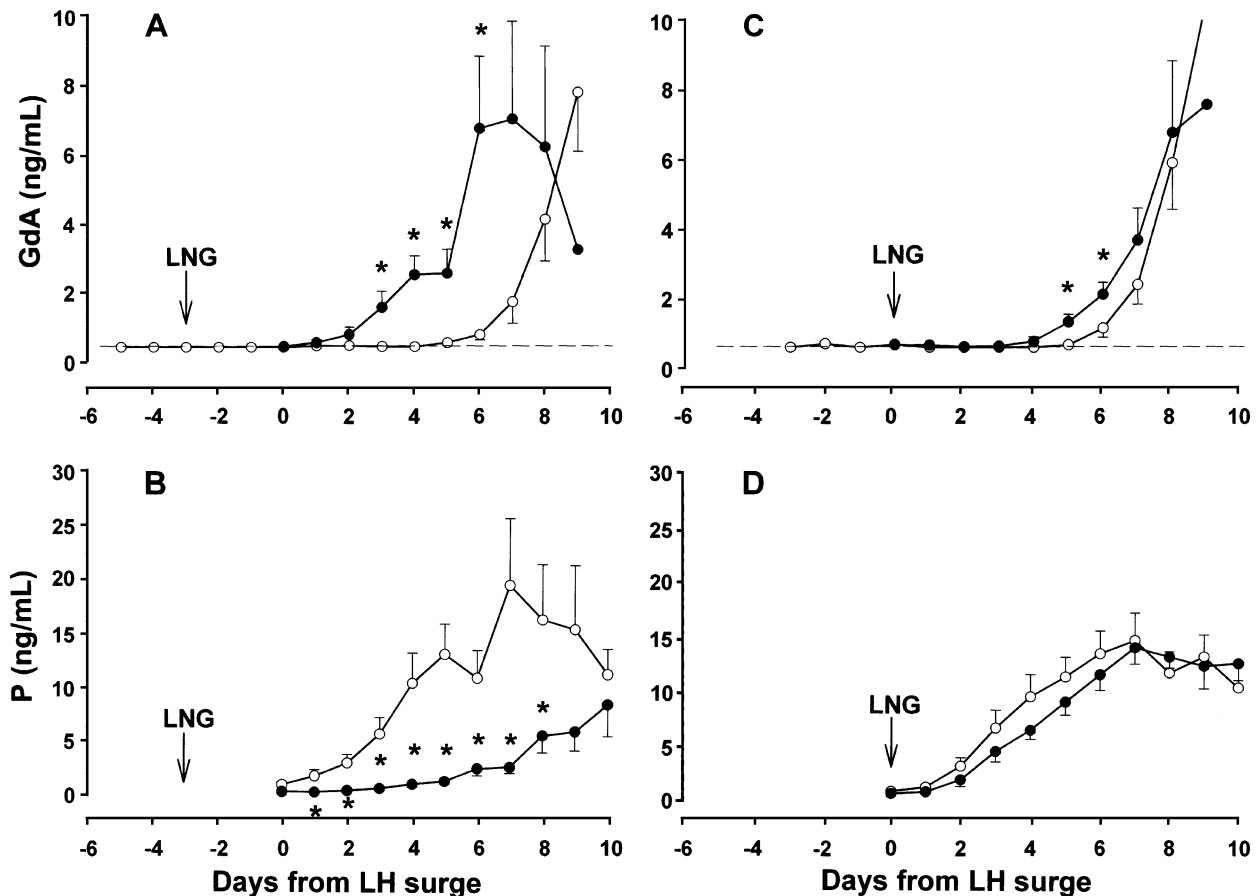


Fig. 1. Daily luteal serum glycodelin (GdA) (panels A and C) and P (panels B and D) concentrations were measured in control (open circles) and LNG-treated cycles (closed circles) prior to (Group 1, panels A and B) or at the time (Group 2, panels C and D) of the LH surge, respectively. Each point represents the mean±SD. **p*<.05 vs. control cycles.

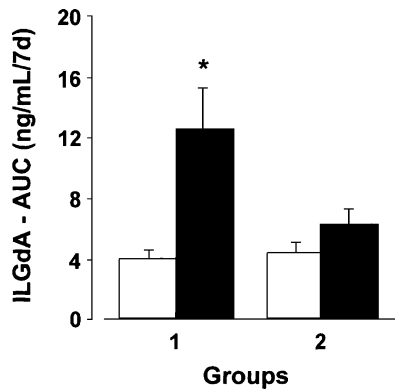


Fig. 2. Mean \pm SE of ILGdA-AUC of individual serum glycodelin-A concentrations during 7 days after the LH surge in control (open bars) and LNG-treated (closed bars) cycles in Groups 1 and 2, respectively. * $p < .05$ vs. control cycles.

the lag period before appearance of glycodelin in serum during the luteal phase ($p < .05$). In these subjects, serum glycodelin levels steadily rose from day LH +2 to days LH +7–8 of the luteal phase after which they declined (panel A, closed circles). Daily serum P concentrations during this time of the luteal phase remained significantly low (panel B, closed circles). In contrast, control cycles showed a significant rise in serum glycodelin concentrations, above the sensitivity limits of the assay (broken line), 7 days after the day of maximum serum LH concentrations (panel A, open circles) and continued to rise toward the end of the luteal phase. The time of appearance of glycodelin in serum of control cycles (panel A, open circles) followed the same pattern as the P concentrations in the luteal phase (panel B,

open circles). Treatment with LNG at the time of the LH surge (Group 2) did not change the secretory pattern of glycodelin in serum in the luteal phase (Fig. 1, panel C). Although both control (open circles) and treated cycles (closed circles) had similar secretion profiles at the midluteal phase, LNG-treated cycles showed slightly higher serum concentrations of glycodelin than did control cycles ($p < .05$). The highest glycodelin serum concentrations occurred later in the luteal phase and showed no temporal correlations with the maximum secretion of P in both control and treatment cycles (Fig. 1, panel D). Since glycodelin in serum was only measured up to 9 days post LH surge, our results do not allow conclusions about whether glycodelin in the late luteal phase would fall in Group 2 as it did in Group 1.

The mean of individual serum integrated luteal phase glycodelin-A area under the curve (ILGdA-AUC) from day LH +1 to LH +7 is shown in Fig. 2. The mean serum glycodelin-A area under the curve was significantly higher in LNG-treated cycles in the subjects of Group 1. No differences were found between control and treatment cycles in Group 2.

Histologically, none of the endometrial biopsies were considered out-of-phase as they corresponded to day LH +8.6 \pm 1.3 and LH +8.9 \pm 0.7 (mean \pm SD) for control and treatment cycles, respectively. The majority of women treated with LNG in Groups 1, 2 and 3 stained positive for glycodelin-A in most cases, but no glycodelin-A was found in 12.5% of the specimens in Group 1. Examples of glycodelin-A staining in biopsy specimens taken on day LH +9 are illustrated in Fig. 3. The relative frequencies of

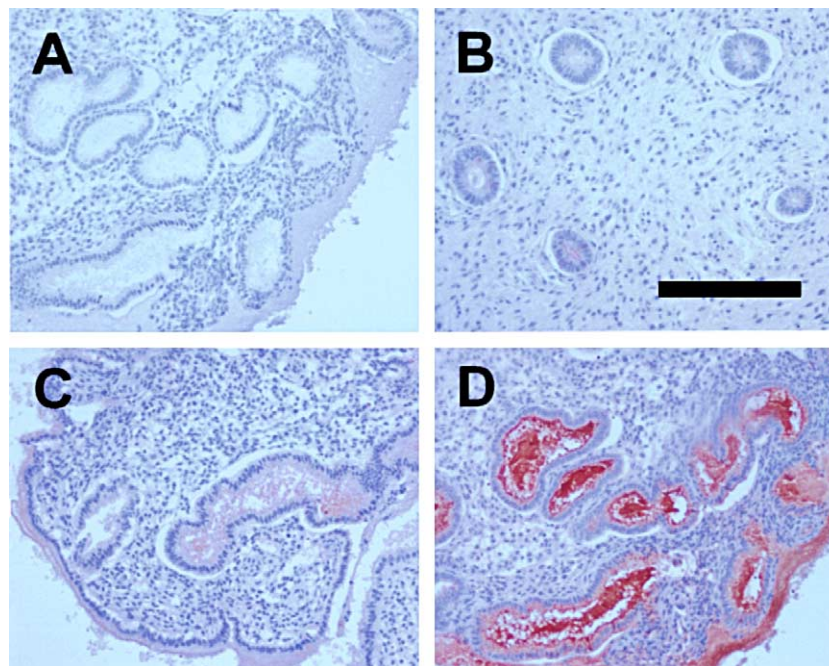


Fig. 3. Representative examples demonstrating glycodelin-A immunostaining intensity in endometrium taken on day LH +9. Negative control (A), weak (B), moderate (C) and strong (D) staining. Bar length, 200 μ m.

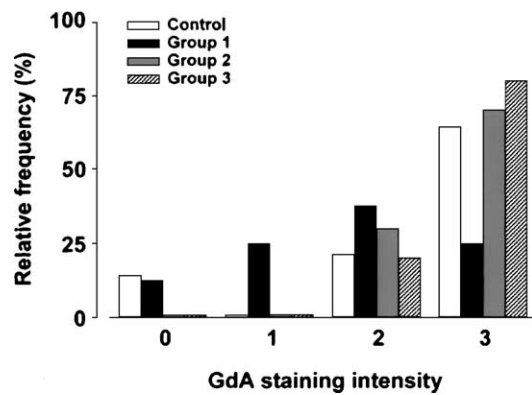


Fig. 4. Relative frequencies of glycodelin-A immunostaining intensities of glandular endometrial cells in the four groups studied during control and treatment cycles: 0=no staining, 1=weak, 2=moderate, and 3=strong.

glycodelin-A immunostaining intensities in the three LNG-treated groups are shown in Fig. 4. The majority of specimens from women in Groups 2 (70%) and 3 (80%) showed the strongest staining for glycodelin-A, whereas only 25% of those from Group 1 had a staining intensity of 3. None of the women from Groups 2 and 3 were negative for glycodelin-A expression or presented with a staining score of 1 in the endometrium. Eighty-seven percent of the specimens from Group 1 were almost equally distributed among the three staining intensities. In the control cycles, most of the endometrial biopsies stained strongly for glycodelin-A, with distribution of immunostaining intensities similar to those of Groups 2 and 3.

4. Discussion

Anovulation explains only a part of the mechanisms of action of hormonal EC. This appears to be the case particularly when medication is taken during the mid to the late follicular phase, before the LH surge [5,8,9,28]. In ovulatory cycles, hostile cervical mucus brought about by progestin only is likely to contribute to contraceptive activity of the regimen, and a short luteal phase is not uncommon [5,7]. However, normal ovulation and even normal corpus luteum function may occur if the treatment is given at the time when the neuroendocrine signals leading to midcycle gonadotropin surge are about to or have been already initiated [5,7]. Therefore, besides delaying or preventing ovulation, additional mechanisms for this period need to be considered. Among possible targets of hormonal EC, histological or biochemical alterations in the endometrium that may interfere with implantation have been subjects of interest. However, the data so far reported have failed to show any consistent biological impact on markers of uterine receptivity [5,29,30]. Inappropriate expression of glycodelin-A by sustained delivery of LNG has been observed in women using LNG-releasing intrauterine system and subdermal implants [31,32], whereas glycodelin-A is absent from endometrium during the normal fertile window.

This observation is significant because glycodelin-A inhibits binding of sperm to the zona pellucida [15]. The concentration required for significant inhibition of sperm-egg binding is about 25 µg/mL [33], that is, several orders of magnitude higher than the levels we found in serum in this study. But, such high concentrations are normally found in uterine fluid during the peri-implantation period [34].

Because glycodelin-A is regulated by P, we addressed the possibility that short-term oral administration of LNG during the periovulatory phase of the menstrual cycle may alter the pattern of endometrial and serum glycodelin-A concentrations during the luteal phase, as studied in biopsies taken within the implantation window. The administration of LNG and serum sampling were timed according to the LH surge rather than the onset of the last menstrual period in order to decrease variability of the results.

This study included only the ovulatory women from the previous study [5]; therefore, it was of interest to note that P production was significantly decreased only in those subjects who took LNG before the LH surge. In these subjects, the early rise in serum glycodelin concentration as well as its expression in the endometrium appears to reflect an effect of LNG more than that of P because the P concentrations were low in the early luteal phase. This observation may also explain why the glycodelin-A concentration in endometrial tissue was significantly lower and the relative frequency of women with the highest staining score in this group was smaller than in the other groups. In addition, maximum serum glycodelin levels were postponed in the luteal phase in these women, and even their maximum levels remained significantly lower than those in the control cycles or in those women who took LNG at the time of the LH surge. In contrast, in Group 2, the treatment cycles showed no early rise of glycodelin-A during the luteal phase, and P production was similar to that in the control cycles. This intergroup difference in glycodelin secretion is most probably related to the time of the cycle at which LNG was administered. The timing in Group 2 was similar to the one used in a previous investigation on the Yuzpe regimen [35] and, interestingly, no difference in serum glycodelin level was found between the test and the control cycles in that study either. Although a number of studies have suggested an extra-endometrial source of glycodelin [12], results from hysterectomized women indicate that the majority of circulating glycodelin in the late luteal phase is of endometrial origin, glycodelin-A [12,36]. The present results break down the effects of LNG according to timing of the drug intake with respect to the LH surge, showing that LNG administration prior to the LH surge does not always prevent ovulation but it has deleterious effects on P production by the corpus luteum. The weak correlation between serum P and glycodelin concentrations is obviously due to the fact that glycodelin secretion lags behind P by 3–4 days [37]. Likewise, there was no correlation between serum glycodelin concentrations and endometrial immunodetection of glycodelin-A, a not

unexpected finding given the wide variability of glycodelin-A expression in different parts of human endometrium [38]. The low staining score for endometrial glycodelin-A in Group 1 indicates that intake of LNG before the LH surge has endometrial effects that are not identified by normal histology [5]. This may reflect either direct effect of LNG in the absence of P or an indirect effect because of insufficient support by the corpus luteum. Considering the inhibitory effects of glycodelin-A on the natural killer cells [17], abundant at the implantation site, reduced endometrial glycodelin-A expression may indicate weakened immunosuppressive microenvironment at the fetomaternal interface at the time of implantation.

In view of the antifertility activity of glycodelin-A, the overall results demonstrating early increase in glycodelin-A secretion make glycodelin-A an interesting model to investigate additional mechanisms by which LNG may act as a postcoital contraceptive. However, this mechanism would apply only to those cases in which LNG is taken before the LH surge. Even under these circumstances, it remains uncertain whether the LNG-induced premature secretion of glycodelin-A at the time of ovulation or during the first days after it would be sufficient and extend over the entire fertile window to inhibit fertilization, particularly because glycodelin-A may be detached from sperm by the corona cells that surround the oocyte [39].

It is concluded that hormonal EC with LNG, taken before the LH surge, alters endometrial glycodelin secretion in two important phases of the cycle. The first is during the fertile window during which an early increase of glycodelin secretion is of interest because of its antifertility activity. The second is the phase of uterine receptivity in which reduced glycodelin expression may reflect weakened immunosuppressive microenvironment within the uterus at the time of implantation [12,16].

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