In vivo assessment of the human sperm acrosome reaction and the expression of glycodelin-A in human endometrium after levonorgestrel-emergency contraceptive pill administration

Josiane A.A. do Nascimento[1], Markku Seppala[2], Antero Perdigão[1], Ximena Espejo-Arce[1], Maria José Munuce[3], Laura Hautala[2], Riitta Koistinen[2,4], Liliana Andrade[5] and Luis Bahamondes[1,6]

[1]*Human Reproduction Unit, Department of Obstetrics and Gynaecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil;* [2]*Department of Clinical Chemistry, Helsinki University Central Hospital, Biomedicum Helsinki, Finland;* [3]*Laboratory of Reproductive Studies, Department of Clinical Biochemistry, School of Biochemical and Pharmaceutical Sciences, Universidad Nacional de Rosario, Rosario, Argentina;* [4]*Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Biomedicum Helsinki, Finland;* [5]*Department of Pathology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil*

[6]Correspondence address. Caixa Postal 6181, 13084-971 Campinas, São Paulo, Brazil. Tel: +55-19-3289-2856; Fax: +55-19-3289-2440; E-mail: bahamond@caism.unicamp.br

BACKGROUND: The objectives were firstly to assess acrosome reaction (AR) status of spermatozoa following uterine flushing, secondly to measure levonorgestrel (LNG) levels in serum and in uterine flushing fluid and finally to measure endometrial glycodelin-A expression after administration of LNG as a form of emergency contraception (EC). METHODS: Forty-eight experiments were conducted on 15 regularly menstruating women. Four groups were formed based on different intercourse to treatment interval and treatment to recovery of spermatozoa and the biopsies. RESULTS: Twenty-four and forty-eight hours after treatment, there were $14.5 \pm 3.9 \times 10^6$ and $17.3 \pm 6.8 \times 10^6$ sperm recovered from the uterus, respectively. There were no differences between the AR rate and the endometrial glycodelin-A staining intensity in an LNG or placebo treated cycles. The LNG in uterine flushing medium represented 1.38% of the values observed in serum 24 h after the LNG intake. CONCLUSIONS: Twenty-four and forty-eight hours after administration of EC, neither the proportion of AR sperm, nor the glycodelin-A level was influenced by 1.5 mg of LNG. LNG did not impair the cervical mucus either because viable spermatozoa were found in the genital tract 36–60 h after coitus and 24–48 h after LNG intake. The mechanism of action of LNG as EC remains unknown.

Keywords: emergency contraception; levonorgestrel; human spermatozoa; acrosome reaction; glycodelin-A

Introduction

Levonorgestrel (LNG) is widely used as an emergency contraceptive (EC). It is usually administered in two oral doses of 0.75 mg, given 12 h apart, or in a single dose of 1.5 mg (WHO, 1998; von Hertzen *et al.*, 2002; Devoto *et al.*, 2005; Cheng *et al.*, 2004; Gainer *et al.*, 2006). The mechanism of action of LNG as an EC is still poorly understood (WHO, 1998; Croxatto *et al.*, 2003; Müller *et al.*, 2003; Gemzell-Danielsson and Marions, 2004). The postulated mechanisms of action include an effect on the luteinizing hormone (LH) surge and ovulation, follicular or corpus luteum development, thickening of the cervical mucus affecting sperm penetration and transport and interference with fertilization (Swahn *et al.*, 1996; Durand *et al.*, 2001; Hapangama *et al.*, 2001; Croxatto *et al.*, 2001; Marions *et al.*, 2002).

Another proposed mechanism is that LNG interferes with endometrial function or with endometrial protein expression. One of such protein is glycodelin-A, a secretory progesterone (P)-regulated glycoprotein (Seppala *et al.*, 2002). Inappropriate expression of glycodelin-A by sustained delivery of LNG has been observed in women using LNG-releasing intrauterine system (LNG-IUS) and subdermal contraceptive implants (Mandelin *et al.*, 1997, 2001). Additionally, the administration of LNG for EC prior to the LH surge alters the luteal phase expression pattern of glycodelin-A in serum and endometrium (Durand *et al.*, 2005).

Because it has been reported that the local application of LNG into the tail of the epididymis of rats impairs the *in vivo* fertilizing potential, suggesting that the drug has a direct effect on spermatozoa (Nikkanen *et al.*, 2000), our

group has been working on the hypothesis that LNG as EC could interferes with the fertilizing capacity of spermatozoa (Bahamondes *et al.*, 2003; Brito *et al.*, 2005; Munuce *et al.*, 2005). However, we have seen no effects of *in vitro* exposures to LNG.

The duration of the fertilizing capacity of human spermatozoa in the woman is unknown (Gould *et al.*, 1984). Previous studies based on the recovery of spermatozoa from the cervical mucus, uterus, oviducts and peritoneal fluid reported a maximal fertile life of 24–48 h, with a motile life from 48 h to 7 days (Perloff and Steinberger, 1964; Gould *et al.*, 1984; Zinaman *et al.*, 1989; Bielfeld *et al.*, 1992; Williams *et al.*, 1993). In humans, reliable estimates of the duration of spermatozoa fertility are not available due to the ethical and logistic constraints, and to the technical difficulties associated with retrieving the contents of the uterus (Williams *et al.*, 1993). Spermatozoa deposited in the vagina during intercourse may remain in the endocervix for many hours or even days before ascending to the Fallopian tubes, and only these cells have the ability to fertilize (Croxatto, 1996). Consequently, spermatozoa deposited in the vagina of women who receive LNG for EC could be exposed to unknown concentrations of LNG for hours or days, and this may influence their fertilizing capacity.

Since the mechanism of action of EC is still poorly understood, the objectives of this study were to assess the acrosome reaction (AR) status of spermatozoa recovered *in vivo* following flushing of the human uterine cavity after sexual intercourse or artificial insemination, to measure the LNG concentrations in serum and uterine flushings in respect of the endometrial glycodelin-A expression after the administration of LNG as EC, or placebo.

Subjects and Methods

The study was conducted at the Human Reproduction Unit, Department of Obstetrics and Gynaecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil. Women aged 30–41 years old were invited to participate in the study. The admission criteria included surgical sterilization, regular menstrual periods (25–35 day intervals), negative screening for Chlamydia and gonorrhea, no use of any hormone therapy, no breast feeding or pregnancy in the 3 months preceding the study and a partner with a normal semen analysis performed two weeks before the experiments, assessed according to the World Health Organization Manual (WHO, 1999). All the couples gave their written informed consent and the study protocol was approved by the Institutional Ethical Committee.

Study design

This was a double blind, placebo-controlled study. The participants were randomly assigned to four groups (see below) and the difference between them in respect of the intercourse to treatment interval and the time-elapsed to spermatozoa recovery from the uterine cavity was addressed. All the groups were studied during two consecutive cycles, a control cycle (placebo administration) and the treatment cycle with 1.5 mg of LNG (Postinor-2, Ache, São Paulo, Brazil). Placebo pills were provided by a specialized pharmaceutical company, which also prepared the vials. For randomization, sealed envelopes were used and the pills were prepared in opaque vials by

a person not involved in the study. The coded envelopes were kept outside of the institution.

Forty-eight experiments were conducted, six with placebo and six with LNG in each group. In group I, the women took LNG or placebo 12 h after coitus and the uterine flushing was performed 24 h after pill intake. Group II was given LNG or placebo 12 h after coitus and the uterine flushing was performed 48 h after the pill administration. Group III received the LNG or placebo treatment 36 h after coitus and the uterine flushing was performed 24 h after the pill administration. Group IV received the treatment (LNG or placebo) 24 h after artificial vaginal insemination and the uterine flushing was performed 24 h after the pill administration.

Experiments

In all women, follicular development was monitored daily by ultrasound using a 5.0 MHz vaginal probe (Justavision 400, Toshiba, Toshigi-Ken, Japan), according to the characteristics of the cervical mucus as defined in the WHO Manual (WHO, 1999) and by serum progesterone. The couples were asked to abstain from sexual intercourse during the 5 days preceding the experiment, a behaviour confirmed by absence of spermatozoa in daily assessment of the cervical mucus. The volunteers were instructed to have sexual intercourse on the evening of the day when the greatest follicular diameter showed positive correlation with cervical mucus i.e. spinnbarkheit >10 cm and crystallization >2+ (WHO, 1999). In group IV, they were asked to have an artificial insemination the next morning. On the following morning, the cervical mucus sample was taken in order to carry out a post-coital test (PCT) and the experiment was performed only if the PCT was adequate according to the WHO definition (WHO, 1999).

At 12 or 36 h after sexual intercourse or 24 h after insemination, LNG or placebo was administered at the clinic. Uterine flushing was performed 24 or 48 h after pill intake. All experiments were performed in the middle of the menstrual cycle before ovulation. To confirm this event, a daily blood sample was collected and the serum was separated. Presence of the follicular phase of the cycle was confirmed by daily serum progesterone levels below 3 ng/ml (Israel *et al.*, 1972). The samples of serum were stored at $-20°C$ until the measurement of LNG.

Uterine flushing was performed using the following technique. To minimize discomfort, one tablet of 5 mg of midazolam (Dormonid®, Roche, São Paulo, Brazil) was administered 20 min before the procedure. Next, the cervix was exposed using a speculum, and the cervical mucus was gently removed using a syringe and placed in a sterile tube with 3 ml of human tubal fluid medium (HTF; GIBCO, BRL, Life Technologies, Inc., Grand Island, NY, USA). The cervix was cleaned with saline solution and then its diameter was estimated. A cervical adaptor (Wisap, Munich, Germany) of appropriate size was fixed to the cervix by vacuum. The uterus was then flushed by gently introducing 5 ml of HTF medium (GIBCO) and recovering it by aspiration with a syringe. The "dead space" in the cannula was estimated to be about 2.5 ml. This procedure was repeated five times using the same fluid, to ensure removal of a maximal number of spermatozoa. The pressure applied to the vacuum syringe and to the aspiration syringe of the cervix adapter used for flushing was moderate to prevent bleeding, which could have contaminated the material.

Sonographic monitoring of uterine flushing was performed using a 3.5 Mhz abdominal probe (Justavision 400, Toshiba) to assure that the fluid had gone through the uterine cavity and that it had been totally removed. Whenever we observed retained fluid in the uterus, a neonatal feeding tubes number 6 (Embramed, São Paulo, Brazil) were gently inserted through the cervix to remove the fluid. The recovered fluid was placed in a sterile tube. The tubes containing

2191

the cervical mucus and fluid recovered after flushing were placed at 37°C under CO_2 for 1 h to stabilize the temperature of the HTF medium and to allow the migration of spermatozoa into the medium (Gould *et al.*, 1984; Zinaman *et al.*, 1989). Following this procedure, two centrifugation cycles were performed using phosphate-buffered saline (PBS) (Dulbecco's, GIBCO) and the pellet was diluted in 1 ml of the PBS. Ten µl were placed into a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) to assess the sperm concentration. When the material was very thick, due to the condition of the mucus, it was passed several times through a syringe with a fine needle to break the mucus filaments before centrifugation and no enzymatic treatment was used to solubilize the cervical mucus (WHO, 1999).

Endometrial biopsy

After uterine flushing, endometrial specimens were obtained by biopsy (Pipelle de Cornier®, Prodimed, Neuilly-en-Thelle, France), trying to obtain a representative specimen from the uterine cavity. The specimens were fixed in 10% buffered neutral formalin, dehydrated, embedded in paraffin and cut into 4 µm blocks, deparaffinized, rehydrated, stained with hematoxylin and eosin according to standard protocols and submitted to histology evaluation and immunohistochemical analysis.

Glycodelin-A immunohistochemical staining

Paraffin-embedded tissue sections were deparaffinized and hydrated in graded ethanol. Then, processed as described, including microwave heat treatment (Kamarainen *et al.*, 1996). Endometrial expression of glycodelin-A was evaluated in 5 µm sections of endometrial tissue. Rabbit anti-glycodelin IgG was used as the first antibody, and biotinylated swine anti-rabbit IgG (Dako, Glostrup, Denmark) and normal rabbit serum was used as the second and the control antibody, 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. The staining was carried out using EnVision®+ System-HRP (DakoCytomation, Carpinteria, CA, USA) and rabbit anti-glycodelin IgG (1.2 µg/ml, 20 h, +4°C). Preimmune IgG from the same rabbit was also used as a negative control. The tissue sections were counterstained with haematoxylin (blue). Staining intensity was recorded using a semi quantitative scale of 0, 1, 2 or 3 (none, weak, moderate or strong, respectively).

Assessment of AR status

After the sperm concentration had been evaluated, the fluorescent probe fluorescein isothiocyanate-labelled *Pisum sativum* lectin (FITC-PSA) was used to evaluate the AR status. Two slides were prepared from the sperm suspension, air-dried at room temperature and protected from light. After drying, they were immersed in cool absolute methanol for 30 s. After that, the slides were stained by immersing them in FITC-PSA for 30 min at a concentration of 40 µg/ml in PBS and protected from light at room temperature. After incubation, the slides were washed in PBS and stored in the dark until evaluation for AR and vitality. Evaluation was done using a fluorescent microscope (Zeiss, Axioplan II, Jena, Germany) equipped with a specific filter for the FITC-PSA method (with 494-blue excitation, 520 emission, 510–514 barrier). Two hundred cells were evaluated in the fields chosen at random. The only spermatozoa that were considered acrosome-reacted were those with the following patterns: (i) patchy fluorescence of the acrosomal region (partially acrosome reacted) and (ii) fluorescence of the equatorial band only (acrosome reacted) (Cross *et al.*, 1988).

LNG and progesterone assay

The concentration of serum and uterine LNG was determined by using a validated method of high performance liquid chromatography coupled to a tandem mass spectrometry. All procedures were carried out in compliance with Good Laboratory Practices approved by the Brazilian National Authority on Sanitary Surveillance. The standards of LNG used in the experiments were USP (Rockville, MD, USA). Biological specificity of the method was checked by processing independent plasma samples and blank samples obtained from women not using any kind of hormones and with the use of pure HTF medium.

Briefly, the bioanalytical assay for quantification of LNG was developed using an online SPE method (Spark Holland model Symbiosis Generic, Emmen, Netherlands). The mass spectrometer (Sciex/Applied Biosystems, model API5000, Toronto, Canada) was equipped with a photoionization source (APPI) running in positive ion mode was set up in Multiple Reaction Monitoring for the transition m/z 313.3 → 245.1 for LNG. Toluene was used as solvent at the flow rate of 0.15 ml/min. The lowest limit of quantification of the method was 20 pg/ml and it was linear over the range of 20–5000 pg/ml. The run time was 5.5 min and the retention time of the LNG was 3.8 (±0.3) min (Theron *et al.*, 2004).

The determination of progesterone was performed in duplicate using a commercial electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) with a measurement range of 0.030–60.0 ng/ml and an inter-assay coefficient of variation (CV) of 2.4% and an intra-assay CV of 2.7%.

Statistical analysis

The total number of spermatozoa recovered from the uterine cavity and from the cervical mucus was compared using ANOVA and the Tukey–Kramer test for paired samples. The level of significance was established at $P < 0.05$. All values are shown as mean ± SEM. As this was an experimental (pilot) study we did not estimate the sample size before conducting the study. However, we calculated a retrospective power analysis to identify if the changes on AR rate or glycodelin-A expression had value. The analysis showed that the study was able to detect differences from 58 to 67% of the SEM.

Results

Spermatozoa recovery from uterine cavity

A total of 33 women were invited and enrolled in the study; however, 18 women were discontinued due to problems during the experiments (see below). Fifteen women contributed with the cycles referred to in this study. All of them contributed at least 2 cycles ($n = 30$ experiments) and the other 18 experiments were performed in three out of the 15 women. The women had a mean (±SEM) age of 35.1 ± 3.2 years (ranged 30–41) and parity of 2.6 ± 0.2. A total of 86 cycles were initiated; however, 38 experiments were canceled because there was inadequate cervical mucus (11 cycles), an inadequate PCT (12 cycles) according to WHO definitions (WHO, 1999), premature ovulation (9 cycles) or personal problems with the couple (6 cycles). Only 48 (56%) experiments were successfully completed. No adverse events were reported. The procedures were initiated when the greatest follicular diameter was 14.3 ± 0.7; 13.1 ± 0.5; 14.0 ± 0.7 and 13.0 ± 0.7 mm for the experiments performed in groups I, II, III and IV, respectively.

The number of recovered spermatozoa was $14.5 \pm 3.9 \times 10^6$ cells/ml (range: $0.2–104.9 \times 10^6$ motile spermatozoa/ml) and $17.3 \pm 6.8 \times 10^6$ cells/ml (ranged $0.1–66.4 \times 10^6$ motile spermatozoa/ml) in the groups in which uterine flushing was performed either 24 or 48 h after the pill, respectively, without differences between the cycles treated with LNG or placebo. The characteristics of cervical mucus after the LNG or placebo administration were not changed significantly.

AR status

The AR status was evaluated in the spermatozoa separately in the samples recovered from the cervical mucus and the uterine cavity after uterine flushing. The values in each treatment group are shown in Table 1. The AR ranged 9.3–11.0% in those spermatozoa obtained from cervical mucus in the placebo groups and 8.0–12.5% in the LNG treated groups. The spermatozoa AR percentage obtained in uterine flushing ranged 6.2–10.3% and 7.8–13.0% in placebo- and LNG-treated groups, respectively. There were no statistical significant differences between the AR rates in LNG or placebo treated cycles at different times of LNG exposure after sexual intercourse, or after artificial insemination.

Endometrial biopsies and glycodelin-A staining

All the biopsies showed a proliferative pattern. Glandular and stromal elements were considered separately and given equal importance. Treatment with LNG did not change the expression pattern of glycodelin-A. There were no differences between the glycodelin-A staining intensity on single dose of 1.5 mg of LNG- or placebo-treated cycles when the biopsies were taken at 24 or 48 h after treatment. However, when the biopsies were obtained at 48 h after pill intake, the results were affected by the small number of cases available for the analysis (Fig. 1).

Table 1: AR status of spermatozoa recovered from cervical mucus and uterine flushing, in the different groups

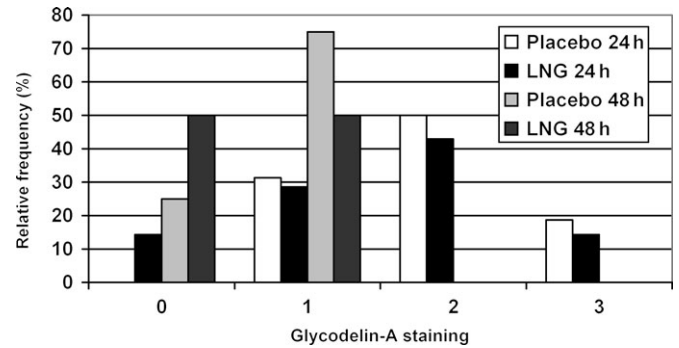| Group	Treatment	Spermatozoa from	AR (mean $\pm$ SEM)
I	Placebo	Mucus	11.0 ± 2.1
		Flushing	10.3 ± 2.7
	LNG	Mucus	11.0 ± 0.9
		Flushing	12.7 ± 2.6
II	Placebo	Mucus	10.2 ± 2.0
		Flushing	6.2 ± 1.5
	LNG	Mucus	10.0 ± 1.8
		Flushing	13.0 ± 3.0
III	Placebo	Mucus	9.8 ± 3.1
		Flushing	7.0 ± 0.8
	LNG	Mucus	12.5 ± 2.5
		Flushing	7.8 ± 1.1
IV	Placebo	Mucus	9.3 ± 3.3
		Flushing	7.3 ± 2.3
	LNG	Mucus	8.0 ± 0.7
		Flushing	10.0 ± 1.5

Figure 1: Glycodelin-A immunostaining intensities of glandular endometrial cells (in percentage) in LNG and placebo treated groups at different intervals between treatment and biopsy: 0, no staining; 1, weak; 2, moderate and 3, strong

LNG concentrations

The LNG concentration was measured only on the day of the experiment. The LNG concentration in the uterine flushing medium after 24 h of LNG administration was 1.38% of the total value observed in serum (47.9 versus 3463 pg/ml). At 48 h after the LNG intake the hormone was no longer detected in uterine flushings ($\leq$20 pg/ml). In serum, the value at 48 h after LNG intake was almost the 50% of those observed at 24 h after the LNG intake (Table 2).

Discussion

LNG is a progestin that has been widely used for EC although its mechanism of action is still unclear. In a randomized clinical trial comparing LNG with the Yuzpe regimen, LNG was shown to prevent significantly more pregnancies than the Yuzpe regimen and its effectiveness increased the closer the drug was administered to the time of coitus (WHO, 1998). Some studies have shown that LNG affects the preovulatory events (Swahn *et al.*, 1996; Durand *et al.*, 2001; Hapangama *et al.*, 2001; Croxatto *et al.*, 2001; Marions *et al.*, 2002), and it was postulated that probably it affects also ovulation, thickening of the cervical mucus, sperm migration, penetration or transport, fertilization and endometrial function (Swahn *et al.*, 1996; Trussell and Raymond, 1999; Croxatto *et al.*, 2001; Durand *et al.*, 2001; Hapangama *et al.*, 2001, Marions *et al.*, 2002; Gemzell-Danielsson and Marions, 2004).

In order to contribute to the understanding of the mechanisms of action involved in the contraceptive effect of LNG as an EC, our primary objective was to explore if the drug

Table 2: LNG concentrations in serum and uterine flushing at different time points after 1.5 mg of LNG intake

	24 h after LNG administration		48 h after LNG administration
	Serum ($n = 18$)	Uterus flushing ($n = 8$)	Serum ($n = 6$)
Mean	3462.9 pg/ml	47.9 pg/ml	1458.8 pg/ml
SEM	275.5	10.6	88.6
Range	(1790–5495)	(9.4–12.1)	(1123–1760)

could modify the *in vivo* status of spermatozoa retained in the endocervix and in the uterine cavity after sexual intercourse or artificial insemination. The results showed that a single dose of 1.5 mg of LNG given before the follicular rupture did not influence the AR status of spermatozoa after different time spans between coitus and the pill intake (12, 24 or 36 h). In addition, the AR rate was similar when it was evaluated at different times between LNG or placebo administration and the recovery of spermatozoa (24 or 48 h).

Although the LNG concentration had reached 3463 ± 276 and 48 ± 11 pg/ml in serum and uterine flushing, respectively, after 24 h of LNG intake, no change in the AR status was detected in the recovered spermatozoa. AR was evaluated previously *in vivo* in spermatozoa recovered from cervical mucus without any treatment (Zinaman *et al.*, 1989). Additionally, our finding in AR rate was similar to previous results with human spermatozoa recovered from the cervix at 72 h after coitus without any drug treatment (Bielfeld *et al.*, 1992) and a little higher than the findings from Zinaman *et al.* (1989) after artificial insemination. The present results are in agreement with results from the previous *in vitro* experiments showing that LNG at 1 ng/ml was unable to induce the AR (Brito *et al.*, 2005; Munuce *et al.*, 2005).

The speculation that LNG as EC could affect the cervical mucus and sperm penetration was based on a well documented effect of progestin and the main mechanism of action of progestin-only contraceptive pill (Moghissi *et al.*, 1973). It was also based on a previous study by Kesseru *et al.* (1974) showing that, after a single dose of LNG intake as an EC, there is a decrease of spermatozoa recovered from the uterus. This has been observed at 3 h after coitus and more significantly at 7 h. Immobilization of spermatozoa due to alkalinization of the uterine fluid and increased viscosity of the cervical mucus after 9 h of coitus may account for this change. Those mechanisms may have contributed to the lack of spermatozoa penetration.

Our findings contradict those results because we observed that a single 1.5 mg dose of LNG did not impair the quality of cervical mucus and spermatozoa penetration. It was possible to recover an adequate number of viable and motile human spermatozoa both from the cervix and the uterine cavity at 36, 48 or 60 h after coitus. The differences between our results and those of Kesseru and his coworkers (1974), could result from the different doses of LNG used, the times between coitus and evaluation, or even the methodology of spermatozoa recovery that was used >30 years ago. Therefore, the initial hypothesis that LNG could interfere with sperm function and penetration and could contribute to the mechanism of action in EC was not confirmed in our *in vivo* and *in vitro* studies (Brito *et al.*, 2005; Munuce *et al.*, 2005).

Additionally, LNG given before follicular rupture did not influence the expression of glycodelin-A in endometrial biopsies taken at 24 or 48 h after pill intake. The administration of LNG for EC prior to the LH surge does not appear to affect endometrial histology or chronological dating of endometrial maturation (Durand *et al.*, 2001). A second study from the same group (Durand *et al.*, 2005) evaluated only ovulatory women and observed an early rise in serum glycodelin-A concentration and its expression in the endometrium when the drug was administered before the LH peak. They observed that a maximum glycodelin-A endometrial expression was significantly lower when LNG was administered at the time of the LH peak compared to drug intake before the LH surge.

We studied the pattern of glycodelin-A expression due to its anti-fertility activity and because the study from Durand *et al.* (2005) showed an effect when LNG was administered before the LH surge. However, our results did not show any effect. It may be relevant that, in the Durand study, endometrial biopsies were taken under native conditions, whereas in this study, the biopsies were taken after endometrial flushing that obviously decreases the amount of secreted glycodelin in the uterine cavity. The effect of endometrial flushing on immunolocalization of glycodelin in endometrial tissue has not been determined. Nevertheless, for the purposes of the present study, the effects of prior endometrial flushing were not likely to cause any bias because comparison was made from the biopsies taken after flushing in the same way from women who had taken either LNG or placebo. Another explanation for the difference is that, although LNG was administered before the follicular rupture, the drug could have been administered close to the LH peak. Finally, because the biopsies were taken 24 or 48 h after LNG intake, the exposure time could have been too short for any significant effect to be observed in the glycodelin synthesis.

The previous results on glycodelin-A expression in the endometrium of users of an LNG-IUS (Mandelin *et al.*, 1997) or LNG-releasing contraceptive implants (Mandelin *et al.*, 2001) cannot be extrapolated to users of LNG as EC because the length of LNG exposure was different. The lack of an association between AR and glycodelin-A expression in the endometrium was not surprising because, unlike glycodelin-F from follicular fluid, glycodelin-A does not interfere with the AR (Yeung *et al.*, 2006), and the spermatozoa recovered from uterine flushings are not likely to have been in contact with the inhibitory glycodelin isoform F.

In conclusion, LNG administered as a form of EC at a single dose of 1.5 mg, 24 or 48 h after sexual intercourse or artificial insemination, did not influence the AR status or endometrial expression of glycodelin-A, suggesting that they were probably not part of the mechanism of action of this kind of EC. Additionally, the administration of the drug had no effect on the quality of cervical mucus or in the penetration of spermatozoa to the uterine cavity. Therefore, the mechanism of action of LNG as an EC remains unknown. However, the clinical significance of AR rate or glycodelin-A expression for health providers probably is small important, since the effectiveness is more important than the real mechanism of action.

References

Bahamondes L, Nascimento JA, Munuce MJ, Fazano F, Faundes A. The in vitro effect of levonorgestrel on the acrosome reaction of human spermatozoa from fertile men. *Contraception* 2003;**68**:55–59.

Bielfeld P, Graf M, Jeyendran RS, Zaneveld LJ. No change in acrosome reaction of human spermatozoa during storage in cervical mucus. *Andrologia* 1992;**24**:83–86.

Brito KS, Bahamondes L, Nascimento JA, de Santis L, Munuce MJ. The in vitro effect of emergency contraception doses of levonorgestrel on the acrosome reaction of human spermatozoa. *Contraception* 2005;**72**:225–228.

Cheng L, Gulmezoglu AM, Oel CJ, Piaggio G, Ezcurra E, van Look PF. Interventions for emergency contraception. *Cochrane Database Syst Rev* 2004;CD001324.

Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. *Biol Reprod* 1988;**38**:235–244.

Croxatto HB. Gamete transport. In: Adashi EY, Rock JA, Rosenwaks Z (eds). *Reproductive Endocrinology, Surgery, and Technology*. Philadelphia: Lippincott-Raven, 1996, 385–402.

Croxatto HB, Devoto L, Durand M, Ezcurra E, Larrea F, Nagle C, Ortiz ME, Vantman D, von Hertzen, Vega M. Mechanism of action of hormonal preparations used for emergency contraception: a review of the literature. *Contraception* 2001;**63**:111–121.

Croxatto HB, Ortiz ME, Muller AL. Mechanism of action of emergency contraception. *Steroids* 2003;**68**:1095–1098.

Devoto L, Fuentes A, Palomino A, Espinoza A, Kohen P, Ranta S, Hertzen H. Pharmacokinetics and endometrial tissue levels of levonorgestrel after administration of a single 1.5-mg dose by the oral and vaginal route. *Fertil Steril* 2005;**84**:46–51.

Durand M, Cravioto CM, Raymond EG, Duran-Sanchez O, De la Luz Cruz-Hinojosa M, Castell-Rodriguez A, Schiavon R, Larrea F. On the mechanism of action of short-term levonorgestrel administration in emergency contraception. *Contraception* 2001;**64**:227–234.

Durand M, Seppala M, Cravioto M del C, Koistinen H, Koistinen R, Gonzalez-Macedo J, Larrea F. 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. *Contraception* 2005;**71**:451–457.

Gainer E, Kenfack B, Mboudou E, Doh AS, Bouyer J. Menstrual bleeding patterns following levonorgestrel emergency contraception. *Contraception* 2006;**74**:118–124.

Gemzell-Danielsson K, Marions L. Mechanisms of action of mifepristone and levonorgestrel when used for emergency contraception. *Hum Reprod Update* 2004;**10**:341–348.

Gould JE, Overstreet JW, Hanson FW. Assessment of human sperm function after recovery from the female reproductive tract. *Biol Reprod* 1984;**31**:888–894.

Hapangama D, Glasier AF, Baird DT. The effects of peri-ovulatory administration of levonorgestrel on the menstrual cycle. *Contraception* 2001;**63**:123–129.

Israel R, Mishell DR, Jr, Stone SC, Thorneycroft IH, Moyer DL. Single luteal phase serum progesterone assay as an indicator of ovulation. *Am J Obstet Gynecol* 1972;**112**:1043–1046.

Kamarainen M, Leivo I, Koistinen R, Julkunen M, Karvonen U, Rutanen EM, Seppala M. Normal human ovary and ovarian tumors express glycodelin, a glycoprotein with immunosuppressive and contraceptive properties. *Am J Pathol* 1996;**148**:1435–1443.

Kesseru E, Garmendia F, Westphal N, Parada J. The hormonal and peripheral effects of dl-norgestrel in postcoital contraception. *Contraception* 1974;**10**:411–424.

Mandelin E, Koistinen H, Koistinen R, Affandi B, Seppala M. Levonorgestrel-releasing intrauterine device-wearing women express contraceptive glycodelin A in endometrium during the midcycle: another contraceptive mechanism? *Hum Reprod* 1997;**12**:2671–2675.

Mandelin E, Koistinen H, Koistinen R, Arola J, Affandi B, Seppala M. Endometrial expression of glycodelin in women with levonorgestrel-releasing subdermal implants. *Fertil Steril* 2001;**76**:474–478.

Marions L, Hultenby K, Lindell I, Sun X, Stabi B, Gemzell Danielsson K. Emergency contraception with mifepristone and levonorgestrel: mechanism of action. *Obstet Gynecol* 2002;**100**:65–71.

Moghissi KS, Syner FN, McBride LC. Contraceptive mechanism of microdose norethindrone. *Obstet Gynecol* 1973;**41**:585–594.

Munuce MJ, Nascimento JA, Rosano G, Faundes A, Saboya-Brito K, Bahamondes L. In vitro effect of levonorgestrel on sperm ferti-lizing capacity and mouse embryo development. *Contraception* 2005;**72**:71–76.

Nikkanen V, Söderström K, Tuusa S, Jaakkola UM. Effect of local epididymal Levonorgestrel on the fertilizing ability of male rat, a model for post-testicular contraception. *Contraception* 2000;**61**:401–406.

Perloff WH, Steinberger E. In vivo survival of spermatozoa in cervical mucus. *Am J Obstet Gynecol* 1964;**88**:439–442.

Seppala M, Taylor RN, Koistinen H, Koistinen R, Milgrom E. Glycodelin: a major lipocalin protein of the reproductive axis with diverse actions in cell recognition and differentiation. *Endocr Rev* 2002;**23**:401–430.

Swahn ML, Westlund P, Johannisson E, Bygdeman M. Effect of post-coital contraceptive methods on the endometrium and the menstrual cycle. *Acta Obstet Gynecol Scand* 1996;**75**:738–744.

Theron HB, Coetzee C, Sutherland FCW, Wiesner JL, Swart KJ. Selective and sensitive liquid chromatography-tandem mass spectrometry method for the determination of levonorgestrel in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;**813**:331–336.

Trussell J, Raymond EG. Statistical evidence about the mechanism of action of the Yuzpe regimen of emergency contraception. *Obstet Gynecol* 1999;**93**:872–876.

von Hertzen H, Piaggio G, Ding J, Chen J, Song S, Bartfai G, Ng E, Gemzell-Danielsson K, Oyunbileg A, Wu S *et al.* Low doses of mifepristone and two regimens of levonorgestrel for emergency contraception: a WHO randomised trial. *Lancet* 2002;**360**:1803–1810.

Williams M, Thompson LA, Li TC, Mackenna A, Barratt CL, Cooke ID. Uterine flushing: a method to recover spermatozoa and leukocytes. *Hum Reprod* 1993;**8**:925–928.

World Health Organization. WHO laboratory manual for examination of human semen and semen-cervical mucus interaction. Cambridge (UK): Cambridge University Press, 1999.

World Health Organization Task Force on Postovulatory Methods of Fertility Regulation. Randomized controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. *Lancet* 1998;**352**:428–438.

Yeung WSB, Lee K-F, Koistinen R, Koistinen H, Seppälä M, Ho PC, Chiu PCN. Roles of glycodelin in modulating sperm function. *Mol Cell Endocrinol* 2006;**250**:149–156.

Zinaman M, Drobnis EZ, Morales P, Brazil C, Kiel M, Cross NL, Hanson FW, Overstreet JW. The physiology of sperm recovered from the human cervix: acrosomal status and response to inducers of the acrosome reaction. *Biol Reprod* 1989;**41**:790–797.