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Post-coital administration of levonorgestrel does not interfere with post-fertilization events in the new-world monkey *Cebus apella*

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BACKGROUND: Experimental evidence to disprove the belief that emergency contraception with levonorgestrel (LNG) prevents pregnancy by interfering with post-fertilization events is lacking. Here we determined the effect of post-coital and pre-ovulatory administration of LNG on fertility and ovulation, respectively, in the *Cebus* monkey. METHODS: To determine the effect on fertility, LNG 0.75 mg or vehicle were administered orally or s.c. once or twice within the first 24 h after mating occurring very close to the time of ovulation. Females that became pregnant were aborted with mifepristone and re-entered the study after a resting cycle until each of 12 females had contributed, in a randomized order, two LNG and two vehicle-treated cycles. To determine the effect on ovulation, LNG 0.75 mg or vehicle were injected twice coinciding with follicles smaller or larger than 5 mm in diameter. Six females contributed five treated cycles each. RESULTS: The pregnancy rate was identical in vehicle- and LNG-treated cycles. LNG inhibited or delayed ovulation only when treatment coincided with a follicle <5 mm diameter. CONCLUSION: In *Cebus* monkeys, LNG can inhibit or delay ovulation but, once fertilization has taken place, it cannot prevent the establishment of pregnancy. These findings do not support the hypothesis that emergency contraception with LNG prevents pregnancy by interfering with post-fertilization events.

Key words: *Cebus* monkey/emergency contraception/levonorgestrel

Introduction

Levonorgestrel (LNG), a progestin widely used for regular hormonal contraception, is also used for emergency contraception (EC) either alone or combined with estrogen. Hormonal EC is taken after unprotected intercourse in order to prevent pregnancy. The mode of action of EC has become the subject of heated debate in North America and in several Latin American and Caribbean countries. The main question is centred on whether or not EC prevents pregnancy by interfering with post-fertilization events. This issue is of importance for many people who consider that a new human life begins at the time that fertilization is completed. Accordingly, interference with post-fertilization events would lead to loss of human life. In spite of a lack of scientific evidence to support a post-fertilization effect, this possibility is used as an argument to turn legal, political and religious constituencies against the availability and use of EC.

When a woman uses EC, she does not know whether she is taking the pills before or after ovulation and before or after fertilization. For ethical and logistic reasons, it has not been possible to segregate groups of women who take EC after fertilization in order to assess its effect on the establishment of pregnancy. Hence, there is no direct evidence that post-coital treatment with LNG prevents pregnancy by interfering with post-fertilization events. On the other hand, animal experimentation allows the investigator to segregate groups treated before or after critical events, such as ovulation, fertilization and implantation, in order to define the contribution of interference with pre- and post-fertilization events to the contraceptive efficacy of the drug. Although extrapolation of the results to humans has limitations, experiments in animals often shed light on possible mechanisms operating in humans. The occurrence of post-fertilization effects of LNG has been examined in the rat, by administering doses several-fold higher than those used for EC in women. In this species, LNG inhibited ovulation depending on the timing of treatment and/or total dose administered. In contrast, it had no effect on fertilization or implantation when administered shortly before or after mating, or before implantation. The authors concluded that post-coital administration of LNG had no post-fertilization effect that impaired fertility in the rat (Müller *et al*., 2003). Here we report the effect of post-coital administration of LNG upon the establishment of pregnancy and the effect of LNG given in the follicular phase upon the fate of the leading follicle

and upon hormonal parameters in the *Cebus* monkey. The *Cebus* monkey is a primate that has both similarities to and differences from humans. It has a menstrual cycle that lasts 21 days, usually ovulates a single oocyte in each cycle from one of the ovaries and presents oscillations of steroid hormones in serum similar to other primates. It differs from women in that the follicular phase is shorter than the luteal phase, and that mating occurs only very close to ovulation.

Materials and methods

Animals

Cebus apella were housed at the Chilean Primate Center of the Catholic University of Chile. Females were regularly cycling, without history of pelvic surgery, and males were of proven fertility. Animals were caged individually under controlled conditions of 24–27°C, 70% relative humidity, and a 14 h light:10 h dark photoperiod. Tap water was available *ad libitum*, and fresh and dry fruits, pelleted chow, biscuits and cake containing milk, minerals, eggs, honey and corn were provided daily.

Males were 14–26 years old and their body weight was 2.6–4.9 kg. Females were 9–17 years old and their body weight was 2.0–3.1 kg. Before entering the study, each female was submitted to daily vaginal smears in order to detect vaginal bleeding. The first day of bleeding was designated the first day of the cycle. Females entered the study after having experienced at least two consecutive menstrual cycles of normal duration (17–22 days). Vaginal smears and ultrasonography were used to monitor menstrual cycles.

Animal care and experimental procedures were carried out according to the ethical guidelines of the Institutional Ethics Committee.

Bioavailability of LNG after oral and s.c. administration

The menstrual cycles of three females were monitored for administering LNG 0.75 mg once orally (p.o.) or s.c. on the 3rd or 4th day of the periovulatory period. One female contributed to both treatments, leaving one resting cycle between them. Two other females were treated once, one p.o. and the other s.c. Blood samples were taken before treatment and 2, 4, 24, 48, 72 and 96 h after treatment to determine the concentration of LNG by radioimmunoassay (RIA).

Effect of post-coital treatment on the pregnancy rate

The menstrual cycle of 12 females was monitored until a total of 24 mating cycles treated with vehicle and 24 mating cycles treated with LNG were accumulated. Each animal contributed two cycles treated with vehicle and two cycles treated with LNG, in randomized order. Vaginal smears and ultrasonographic examinations were performed before and after mating and immediately before treatment to examine the ovaries and to pinpoint the timing of copulation and treatment with respect to ovulation.

LNG 0.75 mg or vehicle was administered once or twice at 12 h intervals p.o. or s.c. in a randomized order as shown in Table I. Females that did not become pregnant were submitted to a new mating cycle after a resting cycle. Those who became pregnant were aborted with mifepristone and submitted to a new mating cycle after one post-abortion resting cycle. This was carried out until each female had contributed four mating cycles.

Treatment was given within 24 h after mating. The first dose was administered at 9–10 a.m. after confirming the presence of spermatozoa in the vaginal smear, and the second dose was administered 12 h later. The number of pregnancies occurring in these mating cycles was recorded. Pregnancy was confirmed by ultrasonographic examination of the uterus, on day 12 and 15 of the luteal phase.

Table I. Effect of post-coital administration of levonorgestrel (LNG) or vehicle (V) on the probability of pregnancy in *C.apella*

Female	Sequential treatment in four cycles			
	0.75 mg s.c.	0.75 mg p.o.	0.75 mg ×2 s.c.	0.75 mg ×2 p.o.
120	V[a]	LNG	LNG[a]	V
46	LNG	LNG[a]	V	V
174	V	LNG[a]	LNG[a]	V[a]
124	LNG	V	LNG	V
109	LNG	LNG[a]	V[a]	V[a]
140	V[a]	V	LNG	LNG
68	V[a]	LNG[a]	V	LNG[a]
170	LNG[a]	V[a]	V[a]	LNG[a]
8	V[a]	V[a]	LNG[a]	LNG
233	LNG[a]	V[a]	V[a]	LNG[a]
111	LNG[a]	V[a]	LNG	V
49	V	LNG	V	LNG
Total pregnancies	V: 4/6 LNG: 3/6	V: 4/6 LNG: 4/6	V: 3/6 LNG: 3/6	V: 2/6 LNG: 3/6

[a]Conceptional cycles.

Effect of treatment upon the outcome of the leading follicle and hormonal levels

Since administration of LNG in the follicular phase has been shown to interfere with the ovulatory process in rats (Müller *et al*., 2003) and women (Durand *et al*., 2001; Brache *et al*., 2003), we investigated whether or not the same holds true in *Cebus*. For this purpose, LNG was administered in the follicular phase and the fate of the leading follicle was assessed. Eight consecutive cycles of each of six females were monitored. Cycles 1, 2, 4, 6 and 8 were treated cycles and cycles 3, 5 and 7 were resting cycles. The first was a control cycle in which females were injected with vehicle alone. In the following four treated cycles, females were injected with LNG, varying the time of treatment according to the size of the leading follicle.

LNG was administered by s.c. injection of 0.75 mg repeated 12 h later. It was given in one of two periods of the follicular phase: (i) before the leading follicle had reached a diameter of 5 mm; or (ii) when the leading follicle had reached a diameter $\geqslant$5 mm. In control cycles, each animal received two s.c. injections of vehicle 12 h apart coinciding with follicles <5 mm diameter.

Vaginal smears were performed daily to identify the periovulatory period and to determine the length of the follicular and luteal phases. Ultrasonography and blood sampling were done every other day during the follicular phase and daily during the periovulatory period to determine the outcome of follicular development, to measure estradiol (E_2) and progesterone by RIA and to pinpoint the time of ovulation.

The ability of LNG to inhibit or delay ovulation was determined by comparing the fate of the leading follicle in control and LNG-treated cycles of each animal. Ovulation was considered to have occurred normally when a dominant follicle of progressively increasing size, coinciding with increased levels of plasma E_2 and the presence of eosinophilic cells in the vaginal smear, was followed by follicular rupture as determined by a decrease in size. This was always associated with increasing levels of plasma progesterone, a decreased number of eosinophilic cells and the presence of leukocytes. Ovulation was considered to have been suppressed when the follicle did not exhibit a progressive increase in size, did not manifest the expected decrease at the beginning of the luteal phase and vaginal

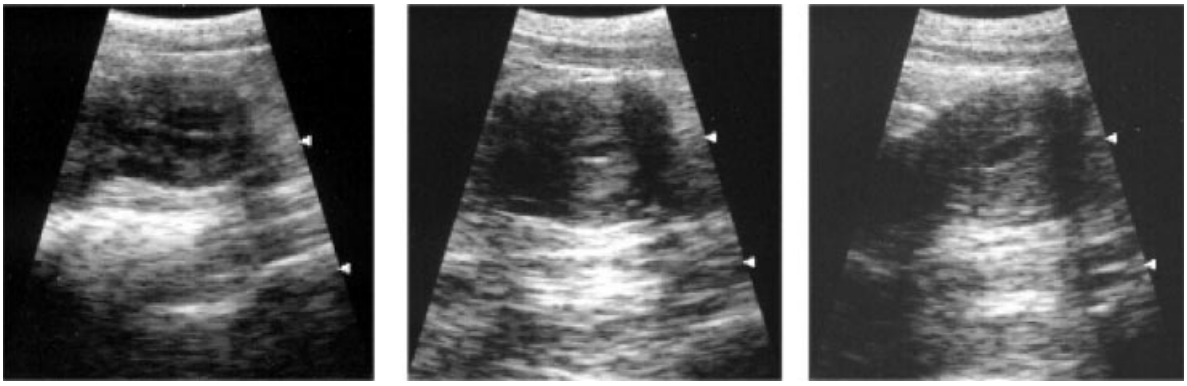

Figure 1. Ultrasonographic images of non-pregnant (**A**) and pregnant uterus on day 12 (**B**) and day 15 (**C**) after ovulation.

smears did not exhibit eosinophilic cells during the advanced follicular phase.

Menstrual cycle monitoring by vaginal smears

The menstrual cycle was monitored by vaginal smears as previously described (Nagle and Denari, 1983). A cotton swab of vaginal secretions was taken every day without removing the animal from the cage. Smears were stained using routine Papanicolau technique and were examined microscopically to look for blood, eosinophilic cells, mucus or leukocytes. The follicular phase was defined as the period between the first day of bleeding and the end of the periovulatory period. The latter is characterized by the presence of large numbers of superficial eosinophilic cells. In turn, decreasing numbers of eosinophilic cells associated with the presence of leukocytes and intermediate cells indicated the beginning of the luteal phase.

Ultrasonographic examination of ovaries

Transabdominal ultrasonographic examination of ovaries was done to identify a leading follicle during the periovulatory period, to determine its size and to pinpoint the time of ovulation. Animals were scanned using a real-time B-mode ultrasound scanner ALOKA SSD-1100 'FLEXUS' fitted with a 7.5 MHz high-density electronic convex probe. Structures as small as 1 mm can be resolved. The dominant follicle was seen as a round or slightly ovoid hypoechoic structure, within the ovary, with a well-delimited border. Follicles >3 mm were measured using omnidirectional calipers. The corpus luteum appears as a lesser hypoechoic ovoid region with an irregular border. The size of follicles was determined from two perpendicular measurements, one of them the largest diameter. The mean of these two diameters was used as follicular diameter. Ultrasounds were performed under ketamine hydrochloride 10 mg/kg i.m. anaesthesia (Ketostop®, Drag Pharma Invetec, Santiago, Chile) supplemented with atropine sulphate 0.04 mg/kg (Atropina sulfato Reg. I.S.P. F-1221-98, Laboratorio Sanderson S.A. Santiago, Chile).

Mating

Nine males and 12 females of proven fertility were available for mating. Each female was transferred to a male cage when the periovulatory period was detected and stayed with the male until mating was confirmed or ovulation had occurred. Mating was confirmed by the presence of spermatozoa in the vaginal smear. Females that did not mate in a given cycle entered a new experimental cycle without a resting period.

Ultrasonographic examination of gestational sac

Pregnancy was diagnosed by ultrasonographic examination of the uterus on day 12 or 13 of the luteal phase. At this time, the gestational sac can be visualized as a hyperechoic vesicle with a 2 mm border in the middle of the uterus. Pregnancy was confirmed by ultrasound examination on days 15 or 16 (Figure 1), and was immediately interrupted by administering a single s.c. injection of 30 mg mifepristone. Following a post-abortion spontaneous menstrual cycle, females re-entered the study.

Hormone assays

Blood samples (0.5 ml) were obtained from the saphenous vein under ketamine–atropine anaesthesia. Blood samples were obtained every other day throughout the cycle, and daily during the periovulatory period for RIA of E_2 and progesterone. After centrifugation, plasma was separated and stored at –20°C until assayed. E_2 and progesterone were measured by a previously validated RIA for *C.apella* (Recabarren *et al*., 1998) and with reagents supplied by the WHO Matched Reagent Program. Assays were done in duplicate from reconstituted aliquots of diethyl ether extracts. The antibody for E_2 was raised against estradiol-6-CMO-BSA, and that for progesterone was a monoclonal antibody raised against progesterone-3-CMO-BSA. The lower limits of sensitivity for E_2 and P were 190 pmol/l and 29 nmol/l, respectively. Inter- and intra-assay coefficients of variation were 11 and 7% for E_2, and 15 and 5% for progesterone.

LNG was measured using reagents and the RIA protocol obtained from Immunometrics Ltd (UK). The precision of the assay was evaluated by determining intra- and inter-assay coefficients of variation in the optimal range of the assay; these were 5.4 and 14%, respectively. To avoid inter-assay variations, all samples of each subject were measured in the same assay run.

Treatment

For s.c. administration, LNG obtained from Norplant® implants (Leiras Oy, Turku, Finland) was dissolved in benzyl benzoate and diluted in corn oil to a final volume of 0.5 ml. The mixture of benzyl benzoate + corn oil was used as vehicle in control cycles treated s.c. For oral administration, Postinor® tablets (Gedeon Richter, Budapest, Hungary) were ground up and mixed with 5 ml of milk with cereal or fruit juice. The same amount of milk or fruit juice was given in control cycles treated orally.

Results

Bioavailability of LNG after oral or s.c. administration

Both treatments rendered concentrations >150 nmol/l within the first 4 h after treatment. Maximum plasma concentrations of LNG after administering 0.75 mg were reached at 2 and 4 h

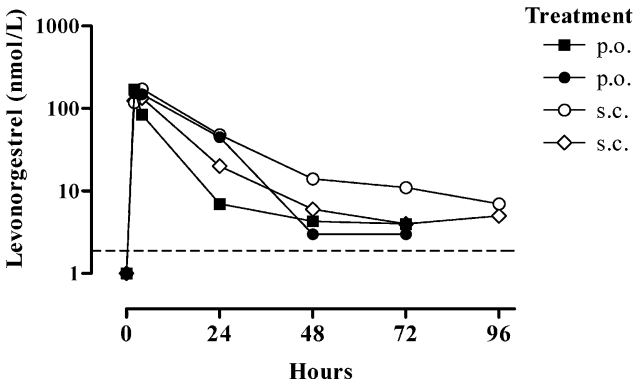

Figure 2. Plasma levonorgestrel concentrations in *Cebus* monkey following oral (p.o.) or s.c. administration of a single dose of 0.75 mg. One female (open and filled circles) contributed to both treatments, leaving one resting cycle between them. Two other females were treated once, one p.o. (filled squares) and the other s.c. (open diamonds).

following p.o. and s.c. administration, respectively. By 24 h, the concentration of LNG had decreased to 15 and 22% of maximum, after treatment p.o. and s.c., respectively. From 24 h on, the concentration tended to remain higher in animals treated s.c. (Figure 2).

Effect of post-coital treatment upon the establishment of pregnancy

Overall, there were 13 pregnancies out of 24 treated cycles in each group. Thus the pregnancy rate was identical (54.2%) in animals treated with vehicle or LNG (Tables I and II). In 41/48, mating took place between 2 days before ovulation and the day of ovulation. In seven cycles, mating preceded ovulation by 3–5 days.

In seven treated cycles and three vehicle cycles, mating was confirmed on day 1 of the luteal phase; therefore, treatment was most probably given after fertilization had already occurred (Ortiz *et al.*, 1995). Among these cycles, six of the seven treated with LNG and two of the three receiving vehicle resulted in pregnancy. This pregnancy rate was not different from, or was slightly higher than, the cycles in which treatment was given before fertilization.

Effect of treatment upon the outcome of the leading follicle and hormonal levels

All 12 cycles treated with LNG coinciding with follicles ⩾5 mm were ovulatory. In 11 of these cycles, ovulation occurred on the same day as, or 1 day earlier than in cycles treated with vehicle. In one cycle, ovulation occurred 5 days later than in the control cycle of the same animal. In control cycles, ovulation occurred between cycle days 7 and 11 (Figure 3A). In cycles treated with LNG coinciding with follicles <5 mm, ovulation was inhibited in four and it was delayed from 1 to 6 days in the remaining eight cycles, with respect to the control cycle of the same animal (Tables III and IV). When ovulation was inhibited, the leading follicle exhibited neither a progressive increase in size nor the abrupt decrease in size that normally occurs at ovulation. Maximal levels of E_2 and progesterone were ~40% lower than those determined in the follicular and luteal phases of the corresponding control cycles (Figure 3B)

Table II. Probability of pregnancy in *Cebus* monkey treated after mating with vehicle or levonorgestrel (LNG) at various intervals from ovulation

Day of treatment relative to ovulation	Pregnant/total mated	
	Vehicle (%)	LNG (%)
0	2/3 (66.6)	6/7 (85.7)
–1	5/8 (62.5)	4/9 (44.4)
–2	4/8 (50.0)	3/6 (50.0)
–3	2/4 (50.0)	0/2 (0)
–4		
–5	0/1 (0)	
Total	13/24 (54.2)	13/24 (54.2)

Table III. Effect of levonorgestrel (LNG) on the ovulatory process when treatment coincided with follicles of different size in *Cebus* monkey

Group	Ovulation Suppressed[a]	Delayed[b]	Normal[a]
LNG <5 mm	4	8	0
LNG >5 mm	0	0	12
Vehicle <5 mm	0	0	6

[a]According to vaginal cytology, ultrasound, and E_2 and progesterone profiles.
[b]With respect to the control cycle of the same animal.

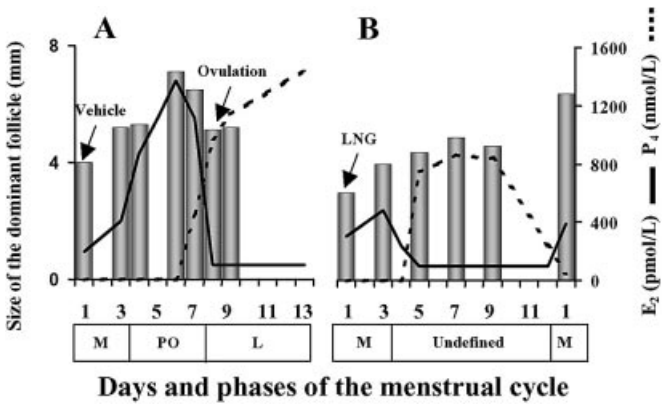

Figure 3. Follicular outcome, hormonal profile and phases of the cycle according to vaginal cytology in a control cycle treated with vehicle (**A**) and in a subsequent cycle of the same monkey, treated with LNG coinciding with a follicle <5 mm (**B**). Note the sluggish increase in size of the dominant follicle and undefined phase of the cycle in the vaginal smear, after treatment with LNG. In the control cycle, a decrease in size of the dominant follicle and a change of the vaginal cytology indicated that ovulation took place on day 8. In the LNG cycle, menses was followed by an undefined vaginal smear until day 12, after which menses heralded the beginning of a new cycle. In this LNG cycle, the follicle failed to exhibit a decrease in size indicative of ovulation, and the maximal levels of E_2 and progesterone were ~60 and 40%, respectively, lower than those seen in the follicular and luteal phase of the corresponding control cycle shown in (A). M = menstruation; PO = periovulatory period; L = luteal phase.

Table IV. Effect of levonorgestrel (LNG) on the duration of the follicular phase of ovulatory cycles when treatment coincided with follicles of different sizes in *Cebus* monkey

Groups	No. of ovulatory cycles[a]/total treated cycles	Duration[b] of the follicular phase mean ± SE (range)
LNG <5 mm	8/12	10.0 ± 0.4 (9–12)[c]
LNG >5 mm	12/12	6.8 ± 0.6 (5–12)[d]
Vehicle <5 mm	6/6	7.3 ± 0.6 (6–10)[d]

[a]According to vaginal cytology, ultrasound, and E_2 and progesterone profiles.
[b]Days.
[c] ≠ [d]$P < 0.05$ (anova).

Discussion

This study shows that post-coital administration of LNG shortly before or after ovulation does not affect the probability of pregnancy in the *Cebus* monkey. Under the experimental conditions utilized, post-coital administration of LNG did not interfere with any post-fertilization process required for embryo implantation, including zygote development and transport to the uterus, acquisition of endometrial receptivity, attachment and trophoblast invasion. This lack of effect on pregnancy rate cannot be attributed to insufficient dose since the dose per kg body weight used was nearly 20 times the dose used in women, and blood levels attained were six times those seen in women (Johansson *et al*., 2002). Furthermore, the same dose inhibited or delayed ovulation when given early in the follicular phase. The fact that an excess of LNG in plasma was sustained for >72 h without interfering with embryo transport, development and implantation is in keeping with the progestational activity of agonistic steroids that act through the progesterone receptor.

In *C.apella*, follicular rupture occurs when the mean follicular diameter reaches 8.2 mm (range 6.4–9.6 mm) and it takes 1–5 days for a leading follicle of 5 mm to attain ovulatory size (Ortiz *et al*., 2004). The fact that LNG inhibited or delayed ovulation only when it was administered before the dominant follicle reached a diameter of 5 mm indicates that LNG interferes with the ovulatory process provided it is not imminent. Mating normally takes place very close to the time of ovulation in *Cebus*. This explains why LNG was unable to inhibit or delay ovulation when given post-coitum.

It was shown previously in a rodent model that post-coital administration of LNG does not affect the rate of embryo implantation, yet pre-ovulatory administration was able to inhibit ovulation, an effect that was markedly dependent on distance between treatment and ovulation. The closer to ovulation, the less effective (Müller *et al*., 2003). The current results show the same holds true in a primate model. Administration of LNG in the follicular phase in women has been shown to interfere with the ovulatory process in a time-dependent fashion (Durand *et al*., 2001; Brache *et al*., 2003).

These results show, for the first time in a primate species, that LNG administered as used in EC in women interferes with the ovulatory process when given early in the follicular phase, but when given soon before or after ovulation it has no impact on fertilization and subsequent processes. However, LNG given post-coitally does not prevent pregnancy in this primate species, apparently because *C.apella* has a different mating pattern from that of humans.

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References

Brache V, Croxatto HB, Cochon L, Massai R, Alvarez F, Forcelledo ML, Faundes A, Paves M and Salvatierra AM. (2003) Efecto de la administración de levonorgestrel solo como anticoncepción de emergencia (AE) sobre la función ovulatoria. Rev Cubana Salud Pública 29 (Suppl 1),R111.

Durand M, Cravioto MC, Raymond EG, Durán-Sanchez O, Cruz-Hinojosa ML, Castell-Rodriguez A, Schiavon R and Larrea F (2001) On the mechanisms of action of short-term levonorgestrel administration in emergency contraception. Contraception, 64,227–234.

Johansson E, Brache V, Alvarez F, Faundes A, Cochon L, Ranta S, Lovern M and Kumar, N (2002) Pharmacokinetic study of different dosing regimens of levonorgestrel for emergency contraception in healthy women. Hum Reprod 17,1472–1476.

Müller AL, Llados C, Croxatto HB (2003) Postcoital treatment with levonorgestrel does not disrupt postfertilization events in the rat. Contraception, 67,415–419.

Nagle CA and Denari JH (1983) The Cebus monkey (*Cebus apella*). In Hearn JP, ed. Reproduction in New World Primates, New Models in Medical Sciences. MTP Press Ltd, Lancaster-Boston, The Hague, pp. 39–67.

Ortiz ME, Gajardo G, León CG, Herrera E, Valdez E and Croxatto HB (1995) Sperm migration through the female genital tract of the new-world monkey *Cebus apella*. Biol Reprod 52,1121–1128.

Recabarren MP, Valenzuela GJ and Serón-Ferré M (1998) Proteic–caloric restriction during pregnancy affects the adrenal–placental axis and decreases birth weight in a primate, *Cebus apella*, without affecting gestational length. Prenat Neonat Med 3,309–313.

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