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In vitro effect of levonorgestrel on sperm fertilizing capacity and mouse embryo development

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Abstract

The objectives of this study were to assess the expression of α-D-mannose binding sites in human spermatozoa, human sperm–oocyte interaction and the development of early stages of mouse embryo in the presence of levonorgestrel (LNG). Semen samples were obtained from 16 normozoospermic men. Spermatozoa were separated by Percoll gradient and incubated overnight for capacitation. The kinetic analysis of the expression of α-D-mannose binding sites was determined at 0, 4 and 22 h and in 22 h-capacitated spermatozoa that had been exposed to 1, 10 or 100 ng/mL of LNG or to a control medium for 30 min. Sperm binding sites for α-D-mannose were detected using commercial α-D-mannosylated bovine serum albumin conjugated with fluorescein isothiocyanate. To evaluate sperm–oocyte interaction, each oocyte was placed in a 100-μL droplet containing one of the three doses of LNG or control medium and inseminated with 1.0×10^5 motile spermatozoa/mL, after which the number of bound spermatozoa was evaluated. A total of 157 two-cell embryos recovered from eight mice was pooled and assigned randomly to treatment (1, 10 or 100 ng/mL of LNG) or control groups. There was a significant increase in the expression of specific α-D-mannose binding sites (Patterns II and III) during the incubation of spermatozoa under capacitating conditions. In the presence of LNG, results showed that there was no significant difference in the expression of specific α-D-mannose binding sites (Patterns II and III) at any LNG concentration tested compared with those spermatozoa in control medium. None of the LNG concentrations were capable of modifying the number of spermatozoa tightly bound to the human zona pellucida. There was no association between the presence or absence of LNG or the different doses of LNG evaluated and mouse embryo development. In conclusion, the hypothesis that in vitro exposure to LNG could interfere with sperm function and could contribute to the mechanism of action of this form of contraception was not confirmed but cannot be ruled out by the results of this study.
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Keywords: Human spermatozoa; Levonorgestrel; D-mannose; Embryo development

1. Introduction

Levonorgestrel (LNG) is a progestin that has been widely used for emergency contraception (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 [1].

In rats, Nikkanen et al. [2] observed that local application of LNG in the cauda epididymis impaired in vivo fertilizing potential, suggesting that the drug has a direct effect on spermatozoa. In addition, it is well known that progesterone stimulates sperm capacitation, hyperactivation, acrosomal reaction (AR), binding of sperm to the zona pellucida (ZP) and sperm penetration of the oocyte [3]. Although the synthetic progestin norgestrel has been shown to be a weak agonist of the progesterone sperm receptor [4,5], we have recently found that the addition of concentrations of LNG ranging from 200 to 800 ng/ml stimulates the occurrence of AR in capacitated spermatozoa [6].

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Considering that acrosome-reacted spermatozoa no longer bind to the ZP and that this is an indispensable prerequisite for fertilization [7,8], we may speculate that part of the contraceptive effect of LNG could be its ability to modify the interaction of the spermatozoa with the oocyte. This hypothesis was tested by Yeung et al. [9], who studied doses of LNG comparable with those observed in the serum of women after ingestion of LNG for EC as well as doses 10-fold higher and 10-fold lower. They reported that LNG had no effect on sperm AR but that it affected sperm motility and spermatozoa–oocyte fusion and that this effect was more evident at high concentrations of the steroid.

In order to contribute to the understanding of the mechanisms involved in the contraceptive effect of LNG (as EC) on human spermatozoa, we investigated the expression of α-D-mannose binding sites, sperm–oocyte interaction and the development of early stages of the mouse embryo in the presence of LNG at the same concentrations previously tested by Yeung et al. [9].

2. Materials and methods

The study was conducted at the Laboratory of Reproductive Studies, Department of Clinical Biochemistry, School of Biochemical and Pharmaceutical Sciences, Universidad Nacional de Rosario (Rosario, Argentina) and at the Human Reproduction Unit, Department of Obstetrics and Gynecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP, Campinas, Brazil). The Institutional Review Board of the Universidad Nacional de Rosario approved the study. All volunteers gave their signed informed consent prior to their admission to the study.

2.1. Semen samples and sperm processing

Semen samples were obtained from 16 normozoospermic donors, collected by masturbation after 3 to 5 days of sexual abstinence. After complete liquefaction, semen analysis was performed according to the World Health Organization's guidelines [10] and strict morphological criteria [11,12]. Seminal plasma was removed by layering 1 mL of semen on top of a discontinuous 90–50% Percoll gradient (Sigma Chemical, St. Louis, MO, USA). Each tube was centrifuged for 20 min at 275×*g*. The bottom layer, containing the motile sperm fraction, was then washed by centrifugation for 10 min with 2 mL of HAM-F10 medium (ICN Pharmaceuticals Biochemicals Division, Aurora, OH, USA). Sperm concentration was adjusted to $2–8\times10^6$/mL and cells were incubated for capacitation in HAM-F10 medium supplemented with 35 mg/ml of bovine serum albumin (BSA fraction V, Sigma Chemical) for 22 h (overnight) at 37°C under 5% CO_2 in air. Postincubation sperm viability was assessed by mixing one drop of sperm suspension with one drop of eosin Y solution [0.5% in phosphate-buffered saline (PBS), ICN Pharmaceuticals Biochemical Division] on a slide and examining 100 spermatozoa at 400× as previously described [10].

2.2. Preparation of LNG solution

A stock solution (2 mg/mL) of LNG (Schering, São Paulo, Brazil) was prepared by dissolving the hormone in pure ethanol (ICN Pharmaceuticals Biochemical Division) and conserving it at 4°C until use. On the day of the experiments, aliquots of the stock solution were serially diluted with HAM-F10 (1:100 followed by a 1:20 dilution). From the latter, aliquots were adjusted to the desired concentration (1, 10 or 100 ng/mL) for each treatment group.

2.3. Effect of LNG on the expression of α-D-mannose binding sites

In order to confirm by kinetic analysis that mannose receptor expression increases with incubation under capacitating conditions, different aliquots from sperm suspensions were taken at 0, 4 and 22 h and binding sites for α-D-mannose were detected using a commercial α-D-mannosylated BSA conjugated with fluorescein isothiocyanate (Man-FITC-BSA, Sigma Chemical) [11]. Spermatozoa were washed twice with core buffer (30 mM HEPES, 0.5 mM $MgCl_2$, 150 mM NaCl, BSA 10 mg/mL, pH 7.0) supplemented with 20 mM $CaCl_2$ and subsequently incubated with 100 μg/mL Man-FITC-BSA for 30 min at 37°C, 5% CO_2 in air. After labeling, spermatozoa were washed twice with calcium-free core buffer and placed onto 70% ethanol cleaned slides, air dried and mounted in a glycerol–PBS medium (9:1). Overnight-capacitated spermatozoa were exposed for 30 min to 1, 10 or 100 ng/mL of LNG or control medium and the expression of α-D-mannose binding sites determined. Sperm viability was assessed by eosin Y dye exclusion at the beginning and at the end of the labeling protocol according to WHO guidelines [10].

Specimens were examined at 100× using a microscope equipped with epifluorescence illumination or at 63× by a Confocal Laser Scan Microscope (Zeiss, Germany). At least 200 spermatozoa were evaluated on every slide. Each spermatozoon was categorized according to the pattern of the fluorescent signal as: nonspecific pattern, I (tail and midpiece); specific pattern, II (whole head plus midpiece); or III (equatorial/postequatorial plus midpiece) [13].

2.4. Effect of LNG on the sperm–ZP binding test

Unfertilized human oocytes recovered from an in vitro fertilization (IVF) program were stored (for less than 1 month) in a saline solution containing 1.5 M $MgCl_2$, 0.1% polyvinylpyrrolidone (MW, 36 000) and 40 mM HEPES in PBS at pH 7.2 [14]. On the day of the experiment, oocytes were washed for 15–20 min in HAM-F10 medium at room temperature. Each oocyte was placed in a 100-μL droplet containing 1, 10 or 100 ng/mL of LNG or control medium (both supplemented with 35 mg/mL BSA) and inseminated with 1.0×10^5 progressively motile spermatozoa/mL. The droplets containing three to four oocytes/treatment were covered with saline-saturated mineral oil (ICN Pharmaceuticals Biochemical Division) and

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