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Study of the effect of ulipristal acetate on human sperm ability to interact with tubal tissue and cumulus-oocyte-complexes☆,☆☆

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Abstract

Objective: Ulispristal acetate (UPA) is a selective progesterone receptor modulator widely used for emergency contraception (EC). The described main mechanism of action is by inhibiting or delaying ovulation; however, the postovulatory effects of the drug are still on debate. Therefore, the aim of this study was to determine whether UPA could interfere with human sperm fertilizing ability.
Study design: Human motile spermatozoa were incubated under capacitating conditions with or without UPA, and then used to inseminate human tubal explants, mouse cumulus-oocyte complexes and zona-free hamster eggs. The ability of UPA to interact with human sperm progesterone (P)-binding sites was investigated by incubating the cells with fluorescent-labeled P and analyzing them by fluorescence microscopy.
Results: UPA did not affect the ability of human sperm to bind to human tubal tissue explants surface or to penetrate the mouse cumulus mass and the zona-free hamster eggs. In addition, concentrations of UPA much higher than those present in the plasma of EC pill users were required to bind to human sperm P-binding sites.
Conclusions: Our study supports a lack of an agonist or antagonist action of UPA on different functional parameters associated with the fertilizing ability of human sperm.
Implications: This study provides new functional evidence supporting that the contraceptive action of UPA is not related to effects on human sperm cells, contributing to a better understanding of the mechanism of action of UPA as EC.

Keywords: Ulipristal acetate; Emergency contraception; Human spermatozoa; Progesterone; Fertilization

1. Introduction

Progesterone (P) plays a fundamental role during the whole reproductive process; the reason why many pharmacological compounds aimed to block P activity have been developed for contraceptive purposes [1]. P receptor (PR), a member of the nuclear receptor superfamily transcription factors, mediates most of P effects [2]. Since spermatozoa are transcriptionally silent cells, P acts on sperm through membrane nongenomic receptors [3,4] triggering signaling cascades that lead to calcium influx, tyrosine phosphorylation of proteins, hyperactivation, chemotaxis and acrosome reaction (AR) [5].

Ulipristal acetate (UPA), a selective PR modulator (SPRM) with both agonist and antagonist activities on PR [6], is available as an emergency contraceptive (EC) pill after unprotected sexual intercourse [7]. The main mechanism of action by which UPA prevents pregnancy is to inhibit or delay ovulation [8]. However, postovulatory effects of the drug cannot be discarded. In this regard, although *in vitro*

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studies showed no effect of UPA on human embryo viability and attachment [9], exposure of spermatozoa to UPA within the female genital tract could affect their function [10].

A direct effect of UPA on sperm function has not yet been reported, and the possibility that UPA interacts with superficial human sperm PRs interfering with their function cannot be excluded. Previous studies have shown that incubation of human spermatozoa with UPA at concentrations similar to those expected to be present in plasma after EC pill intake (100–200 ng/mL [11]) affected neither protein tyrosine phosphorylation during capacitation nor the occurrence of the AR [12]. Recently, we described that UPA has no effect on mouse fertilization or early embryo development under *in vitro* conditions and that while the injection of UPA at the moment of hCG administration reduces the number of ovulated oocytes, it has no effect on fertilization when administered at the moment of mating [13]. In humans, whereas previous studies analyzed the effect of UPA on capacitation-associated parameters, there is no information regarding the effects of the drug on the fertilizing ability of sperm. In view of this, in the present study, we investigated the effect of UPA on functional events directly associated with the fertilizing ability of human sperm. The results obtained provide convincing evidence supporting that UPA does not have direct effects on human sperm.

2. Materials and methods

2.1. Sperm capacitation

The local ethics committee approved the protocols involving human samples. All semen and oviductal tissue donors signed an informed consent form. We conducted all procedures involving human semen in accordance with the World Health Organization recommendations [18]. Semen samples were obtained from healthy normospermic donors (n=20). We recovered motile spermatozoa by standard swim-up technique, diluted them to a final concentration of 10×10^6 spermatozoa/mL and incubated them 18 h at 37 °C in Ham F10 in a humidified atmosphere of 95% air and 5% CO_2. For functional assays with heterologous gametes, we incubated sperm in Biggers Whitten Whittinhgham medium (BWW) medium [14]. For the experiments described below, we incubated sperm with 1000 ng/mL (2.1 μM) of UPA or 0.1% (v/v) ethanol (vehicle) under different *in vitro* conditions to cover the different exposure times that sperm could be exposed to the drug *in vivo* (*i.e.*, presence of UPA during *in vitro* capacitation and gamete co-incubation (co-incub) to mimic intake of the pill immediately after intercourse or presence of UPA only during *in vitro* gamete co-incub to mimic a pill intake several hours after intercourse). We chose a concentration of 2.1 μM to cover and even exceed the women's serum concentrations after pill intake [11].

2.2. Oviductal explants binding experiments

We obtained fallopian tubes from regularly menstruating women (n=3) without clinical history of infection, scheduled for hysterectomy due to nonmalignancy disease. We calculated the menstrual cycle day of the surgery based on the first day of last menses, and we confirmed it by histological evaluation of the endometrium. As previously described [15], fallopian tubes were placed into Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 100-mcg/mL streptomycin and 100-IU/mL penicillin. We sectioned the isthmic and ampullary regions to expose the inner epithelial surface, and we carefully cut them in 1-mm^3 cubes. We cultured the explants (four to six pieces) in DMEM/Ham F12 medium, supplemented with antibiotics (Gibco), and fetal bovine serum (FBS; Notocor, Córdoba, Argentina, 10% v/v) for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Explant viability and ability to synthesize new proteins under these *in vitro* conditions have already been reported [15].

After culturing, explant fragments were incubated in the presence or absence of 1000 ng/mL of UPA for 1 h in serum-free Ham's F-10 supplemented with Bovine Serum Albumin (35 mg/mL). Explants were then inseminated (final concentration: 1.0×10^5 motile sperm/mL), incubated for 4 h, washed to eliminate loosely adhered sperm, fixed with 4% paraformaldehyde in Phosphate-Buffered Saline and mounted for evaluation at 40× with a confocal microscope (Nikon Eclipse TE-2000-E2; Nikon, Tokyo, Japan) coupled to an image analyzer (SPOT Version 3.5). Only fields in which at least one bound spermatozoon was detected were digitalized for further image analysis. We performed a double blind scoring of the number of sperm bound to the luminal surface and expressed results as the mean number of bound spermatozoa/mm^2. We assessed the acrosomal status of unbound spermatozoa present in the conditioned medium by epifluorescence microscopy (Primo Star iLED™ Carl Zeiss Microimaging, Oberkochen, Germany) at 100×. using Fluoresceine isothiocyanate-conjugated *Pisum sativum* agglutinin (PSA) (50 mcg/mL) as previously described [16].

2.3. Cumulus penetration analysis

The local ethics committee approved the experiments involving animals. We followed the Guide for Care and Use of Laboratory Animals approved by the National Institutes of Health. The cumulus penetration assay was performed as previously described [17]. Hybrid C57BL/6xBALB/cF1 young adult (30–60 days old) female mice were superovulated (5 IU of eCG followed by 5 IU of hCG 48 h later, Sigma–Aldrich Inc., St. Louis, MO, USA). Cumulus-oocyte complexes (COCs) were recovered from the oviducts, washed in BWW and distributed in the experimental groups. We added Hoechst loaded spermatozoa (1×10^4) to the COC and incubation continued for 15 min in the presence or

absence of 1000 ng/mL of UPA. Then, COC were washed, fixed and mounted on slides for examination under a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics (250×) to score the number of fluorescent sperm heads within each *cumulus oophorus*.

2.4. Zona-free hamster oocyte penetration assay (HOPT)

HOPT was performed as previously described [18]. Immature (4–7 weeks old) female hamsters (*Mesocricetus aureatus*) were superovulated [eCG: 30 IU (ip), hCG: 35 IU ip 48 h later]. COC were recovered from the oviducts and zona pellucida (ZP)-free eggs were obtained after treatment of COC with hyaluronidase and trypsine (Sigma). An aliquot of motile capacitated spermatozoa (2×10^5 cells) incubated in the presence of UPA or vehicle was added to hamster eggs either alone or containing UPA. After 2.5 h of co-incub, cells were fixed and stained, and we recorded the number of eggs presenting either decondensing sperm heads or pronuclei and sperm tails in the ooplasm. We expressed results as the percentage of penetrated eggs and the number of spermatozoa/egg in each treatment.

2.5. Effect of UPA on P-binding sites on human spermatozoa

Spermatozoa were capacitated as described above in the presence of different concentrations of P [0–20,000 ng/mL (40 μM)] or UPA [0–10,000 ng/mL (21 μM)]. Afterwards, Progesterone-Bovine Serum Albumin complex labelled with Fluoresceine Isothiocyanate (P-BSA-FITC) was added (final concentration 20 mcg/mL, Sigma), and incubation continued for 30 min. Samples were then mounted and evaluated under the epifluorescence microscope at 1000×. We scored the different fluorescent labeling patterns in at least 200 cells per treatment.

To correlate P-BSA-FITC binding and acrosomal status, we exposed capacitated spermatozoa for 30 min to 10-μM calcium ionophore A23187 to induce AR, washed, and incubated for 30 min with P-BSA-FITC. We then permeabilized sperm with methanol (30 s) and stained the cells for 30 min with tetramethylrhodamine isothiocyanate-conjugated PSA (Tetramethylrodamine (TRITC), 50 mcg/mL, Vector Laboratories, Burliname, CA, USA) at room temperature. Finally, smears were mounted and analyzed under a laser scanning confocal microscope (Nikon Eclipse TE-2000-E2, Nikon, Japan) using the EZ-C1 3.20 Free Viewer program for acquisition of the data.

2.6. Statistical analysis

Statistical analysis was performed with the GraphPad Prism Software (San Diego, CA, USA) using Student's *t*, one-way Analysis of Variance and Tukey–Kramer tests as appropriate. To evaluate the association between P-binding sites and AR, the two-sided Fisher's Exact Test was performed. Results are expressed as mean ± standard error of the mean (SEM). Significance was established at $p<.05$.

3. Results

We first examined the impact of UPA on the sperm ability to interact with human tubal tissue explants. The presence of UPA did not affect the number of bound spermatozoa/mm^2 of explant luminal surface compared to control (UPA: 462±157 sp./mm^2; control: 510±135 sp./mm^2). Evaluation of the acrosomal status of unbound spermatozoa present in the explant conditioned media revealed no differences in the percentage of acrosome-reacted spermatozoa between groups (UPA: 9.6±5.1%; control: 7.3±3.3%).

Evaluation of the ability of UPA-treated human spermatozoa to penetrate the mass of cumulus cells that surrounds the egg showed no differences in the number of fluorescent spermatozoa within the cumulus mass between UPA-treated and control groups under any of the conditions tested (Fig. 1). The analysis of the effect of UPA on human sperm–egg interaction by using zona-free hamster eggs revealed that UPA affected neither the percentage of penetrated eggs (Fig. 2a) nor the number of penetrating spermatozoa/egg (Fig. 2b) compared to controls.

We next evaluated the ability of UPA to interact with human sperm P-binding sites. In the absence of UPA, most (65%) spermatozoa exposed to the P-fluorescent probe were unlabeled (UN), whereas the remaining population (35%) exhibited a uniform labeling in either the whole acrosomal cap (CAP) or the equatorial segment (ES) (Fig. 3a). In all cases, the tail remained UN. The specificity of the binding assay was tested by incubating sperm with different concentrations of UN P prior to their exposure to the probe. Whereas concentrations of P of 5000 ng/mL or higher produced a significant ($p<.001$) decrease in the percentage of labeled sperm (which cannot be attributed to the occurrence of the AR by the presence of P during capacitation) (see Fig. A1), concentrations higher than 1000 ng/mL of UPA were required to obtain a decrease ($p<.001$) in the percentage of labeled cells (Fig. 3b).

4. Discussion

In women users of UPA as EC, spermatozoa could be exposed to different concentrations of the drug during their transit through the female tract, with potential effects on their function. Whereas previous results from our laboratory showed that *in vitro* exposure of human spermatozoa to UPA does not affect sperm viability, capacitation-associated protein tyrosine phosphorylation or AR, no information was available on the effect of UPA on other events leading to fertilization such as the interaction of spermatozoa with the oviduct and the cumulus-oocyte complexes. In addition, as UPA is an SPRM and P plays a critical role in the

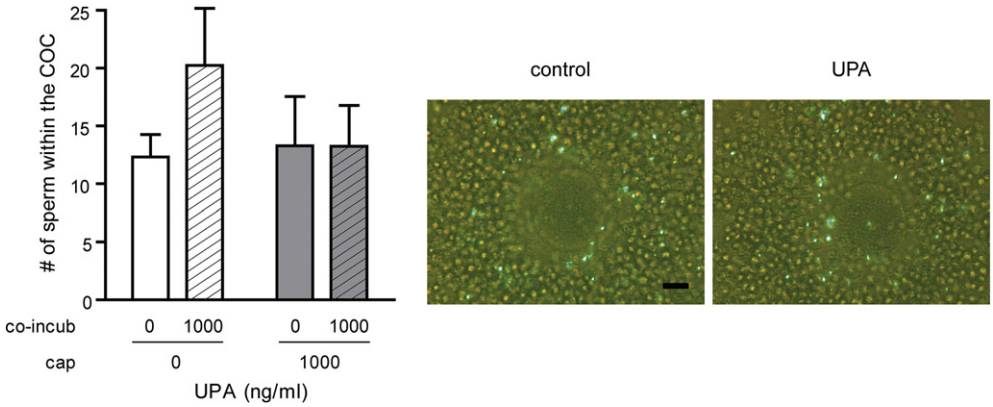

Fig. 1. Effect of UPA on sperm cumulus penetration. Spermatozoa were incubated 18 h under capacitating conditions (cap) in the presence (1000 ng/mL) or in the absence of UPA and stained with 3-mcg/mL Hoechst 33,342 for 10 min. Spermatozoa were added to COC and co-incub proceeded for 15 min in the presence (1000 ng/mL) or absence of UPA. The number of fluorescent human sperm heads within the *cumulus oophorus* was counted. Results are expressed as mean number of spermatozoa/COC ± SEM (n=3). The images on the right show representative merged fluorescent and phase-contrast micrographs of COCs inseminated with either control (left) or UPA-treated (right) spermatozoa (Hoechst stained). Bar: 25 μm.

fertilization process, it is also important to establish whether UPA interferes with the ability of P to interact with its binding sites in spermatozoa.

During their transit through the oviduct, spermatozoa bind to the epithelium and develop hyperactivation, a vigorous motility that releases them from the epithelium and is critical for penetrating the egg coats. Although it has been shown that P [19] and other progestins [20] suppress *in vitro* tubal cilliary beating and contractibility, as far as we know, no data are available on the effect of UPA on sperm–epithelium binding. The lack of effect of this drug on the ability of spermatozoa to interact with the tubal explants supports the idea that sperm binding to the oviduct would not depend on the cilliary beating and contractibility previously shown to be affected by UPA [20]. Furthermore, UPA does not modify the occurrence of the AR in spermatozoa exposed to oviductal-conditioned media in agreement with our previous observations showing that a wide range of UPA concentrations do not affect the AR *in vitro* [12].

After being released from the lower oviductal epithelium, spermatozoa move toward the ampulla containing the COC. While penetrating the cumulus mass, spermatozoa are exposed to a gradient of P [from 1 to 10 mcg/mL (3 to 30 μM) [21,22]], secreted by the cumulus cells which participates in guiding spermatozoa toward the oocyte [23]. Our results showing that UPA does not affect the ability of spermatozoa to penetrate the cumulus support the idea that the drug does not interfere with P-regulated events

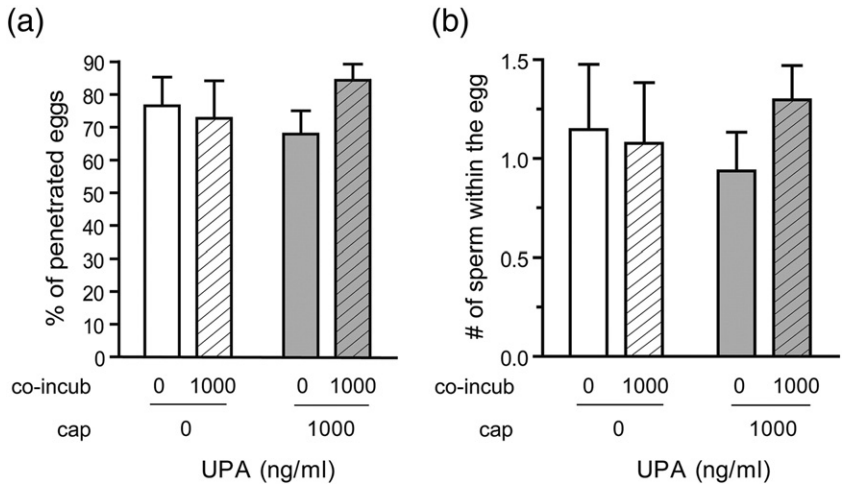

Fig. 2. Effect of UPA on sperm zona-free oocyte penetration. Spermatozoa were incubated 18 h under capacitating conditions (cap) in the presence (1000 ng/mL) or in the absence of UPA and inseminated to zona-free hamster eggs in the presence (1000 ng/mL) or absence of UPA during gamete co-incub. After 2.5 h, the percentage of eggs presenting either decondensing sperm heads or pronuclei and sperm tails in the ooplasm (a), and the number of decondensing sperm heads per eggs (b) were recorded. Results are expressed as mean percentage of penetrated eggs ± SEM (a) and number of sperm/egg ± SEM (b), (n=4).

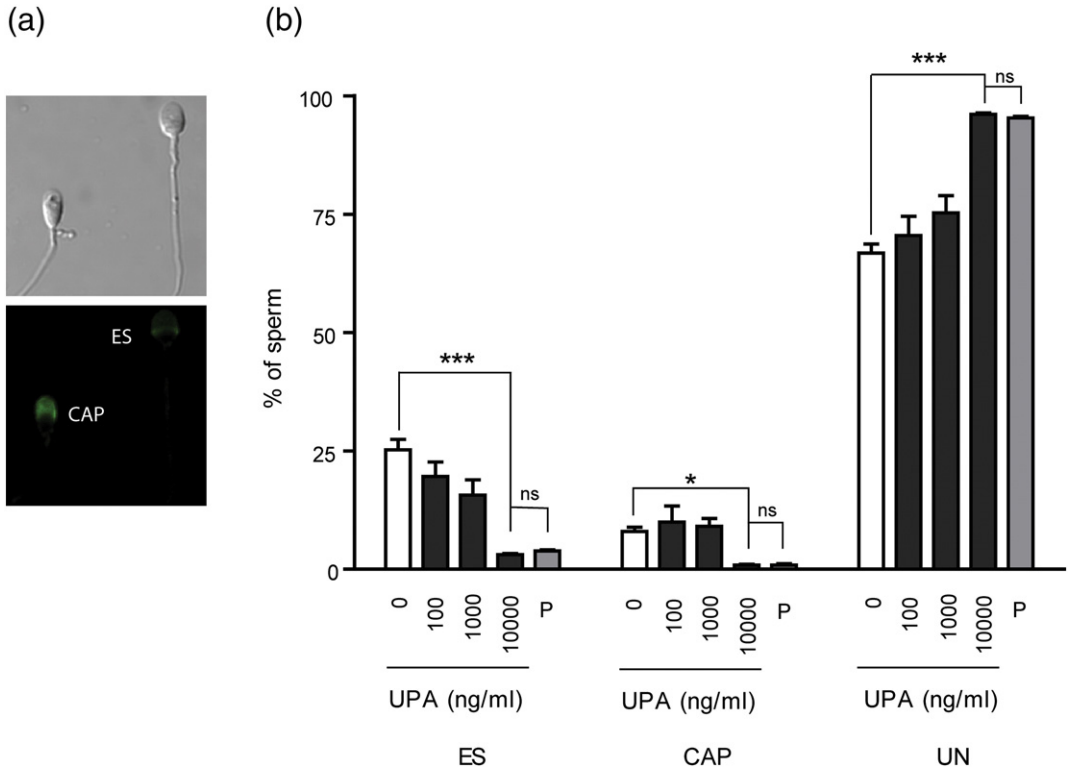

Fig. 3. Effect of UPA on binding of P to spermatozoa. (a) P-binding sites localization in human spermatozoa. Swim up-selected spermatozoa were incubated 18 h in Human Tubal Fluid and incubated for the last 30 min with 20-mcg/mL P-BSA-FITC. Samples were mounted and evaluated for fluorescent labeling. Most spermatozoa were UN, whereas the subpopulation that binds the P-BSA-FITC showed two different patterns of labeling: a uniform labeling of the whole acrosomal region (CAP) and the equatorial region only (ES). No labeling was detectable in the tail in any case. Bar: 5 μm. (b) Spermatozoa were incubated under capacitating conditions in the presence of UPA (0–10,000 ng/mL) or P (5000 ng/mL) for 18 h. Sperm were then incubated with P-BSA-FITC (20 mcg/mL, 30 min), and the number of labeled sperm as well as the pattern of labeling were scored. Spermatozoa were either UN or exhibited labeling on the whole acrosomal region (CAP) or the equatorial region (ES). Viability of spermatozoa in culture was checked during the whole binding experiment (>88% viable cells). The values represent the mean ± SEM ***p<.001 compared to 0 ng/mL (n=7).

other than AR such as sperm guidance and hyperactivated motility required for penetration of the cumulus mass [24,25]. Considering the ethical reasons involved in the use of human oocytes for research, we used the ZP-free hamster oocytes assay to evaluate the effect of UPA on the development of fusion ability. The lack of effect of UPA on both the percentage of penetrated hamster eggs and the number of incorporated spermatozoa per egg reveals that UPA does not affect human sperm fusion ability. These functional studies were performed under different experimental conditions to cover the different time periods that sperm may be exposed to UPA in the *in vivo* situation. The lack of effect of UPA on sperm function when present throughout both *in vitro* capacitation and fertilization supports the idea that the drug would not have effects when given immediately after intercourse and even less when given long after intercourse.

As an approach to understand the mechanisms underlying the lack of effect of UPA on sperm function, we investigated whether this compound was able to interfere with the interaction of P with its binding sites in sperm. Approximately 35% of the cells exhibited labeling for a P fluorescent probe on their head surface. The persistence of labeling in acrosome-reacted sperm confirmed the specificity of this binding. The low percentage of labeling and the localization patterns detected in sperm exposed to the probe are in agreement with previous reports using a similar probe [3] and support that only a small subpopulation of spermatozoa has the ability to respond to P, probably due to the asynchrony of the capacitation process [26].

Our results show that UPA is capable of interacting with the sperm P-binding sites as judged by the reduction in the percentage of labeled cells observed when UPA is present at concentrations higher than 1000 ng/mL. Our findings provide a convincing explanation for the lack of effect of UPA on different functional parameters evaluated here and in previous works [12,27]. Thus, our observations also indicate that UPA does not interfere with the binding of P to spermatozoa at the concentrations expected to be present in the plasma of EC users.

UPA is used worldwide for EC due to the high contraceptive efficacy [7], and the proposed mechanism is by blocking or delaying ovulation when administrated shortly before ovulation [8]. Although potential effects of

UPA on endometrium cannot be discarded [28], this study supports that the pharmacological contraceptive action of UPA is not related to prefertilization effects of the drug on human sperm function, contributing to a better understanding of the mechanism underlying the role of UPA in EC.

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