Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa

By means of a videomicroscopy system and a computer image analysis, we performed chemotaxis assays to detect true chemotaxis in human spermatozoa, in parallel to immunohistochemistry detection of progesterone inside the cumulus cells. Progesterone indeed chemotactically guides mammalian spermatozoa at very low hormone concentrations, and the cumulus oophorus could be a potential place for sperm chemotaxis mediated by progesterone in vivo. (Fertil Steril® 2006;86:745–9. ©2006 by American Society for Reproductive Medicine.)

Sperm chemotaxis is a cell transport mechanism that guides spermatozoa up an attractant concentration gradient (1). In recent years, by means of approaches that differentiate chemotaxis from other cell-accumulation processes, sperm chemotaxis toward follicular fluid was observed in humans (2), mice (3), and rabbits (4) and toward oviductal fluid in mice (3). Further characterization of mammalian sperm chemotaxis showed that the chemotactic response was expressed by a small sperm subpopulation (approximately 10%), consisting of capacitated spermatozoa (4–6).

Follicular and oviductal fluids are mixtures of different molecule types (from peptides to hormones). Therefore, a long-standing question is: Which is/are the chemoattractant molecule(s) in these fluids? Progesterone, the main steroid present in the egg microenvironment by the time of ovulation, has been assayed for human sperm chemotaxis by different groups, giving rise to contradictory results (7–11). Clarification of this issue would shed light on the mechanism of sperm transportation, with relevant implications in reproductive medicine for the treatment of male/female infertility and/or contraception.

In the last decade, four groups reported that P is a chemoattractant for human spermatozoa (7–10), whereas another group suggested that the hormone either is not a sperm chemoattractant or is a weak chemoattractant (11). All these studies based their conclusions on assays run at high concentration ranges of P (μmol/L to mmol/L). Furthermore, those studies claiming a positive chemotactic response to P (7–10) used assays that could not differentiate chemotaxis from other sperm-accumulation processes.

To determine unequivocally whether P is a sperm chemoattractant, we chose experimental conditions that allowed us to clearly determine chemotaxis. These conditions were: [1] a chemotactic parameter (% of oriented cells) that determines chemotaxis independently of variations in the sperm speed or in the acquisition of a hyperactivated motility (4), because the latter processes can also cause sperm accumulation (1); [2] a high number of analyzed spermatozoa per treatment in order to detect the small chemotactic sperm subpopulation, because in mammals approximately 10% of the cells are involved (1); [3] cell suspensions enriched with capacitated spermatozoa, because only those cells are able of chemotactic response (4–6); [4] follicular fluid as a well established positive control (1); and [5] a wide range of P concentrations (0.1 pmol/L to 1 mmol/L) not fully investigated in the past.

The low level of P ranges was chosen because the human sperm chemotactic response to specific attractants such as burgeonal (12) and RANTES chemokine (13) was elicited at low attractant concentrations. Therefore, we assumed that, if a P-mediated chemotactic response does occur in spermatozoa, it should be observed at low attractant levels. For comparison with earlier studies that have given rise to the controversy regarding P sperm guidance, we also assayed higher (though nonphysiologic) P concentrations.

Human spermatozoa chemotactically responded to a concentration gradient generated from 1 to 100 pmol/L of P. The results, which showed the expected bell-shaped dose-response curve (typical of a chemotactic response of any cellular model [1]), were statistically different from the negative control ($P<.001$) but not from the positive control, follicular fluid (Fig. 1A). The maximal level of chemotactic spermatozoa was detected at 10 pmol/L of P, which was elicited by a small sperm subpopulation (8% vs. the control group; Fig. 1A), in line with the mean level of capacitated spermatozoa (12%, data not shown). A smaller second chemotactic peak was observed at about 1 μmol/L of P ($P<.03$; Fig. 1A). Between 0 and 1 μmol/L P there was no significant increase in the sperm velocity nor in the induction of the hyperactivated pattern of movement (Fig. 1B). Similar results were observed in the rabbit, although the maximum chemotactic response was at 100 pmol/L of

Received September 5, 2005; revised and accepted February 3, 2006.
Supported by Secyt (UNC) and Conicet, Argentina. L.C.G. is a member of the National Council of Scientific Research (CONICET); M.E.T. and H.A.G. are fellowship holders from Foncyt and Conicet (Argentina), respectively.
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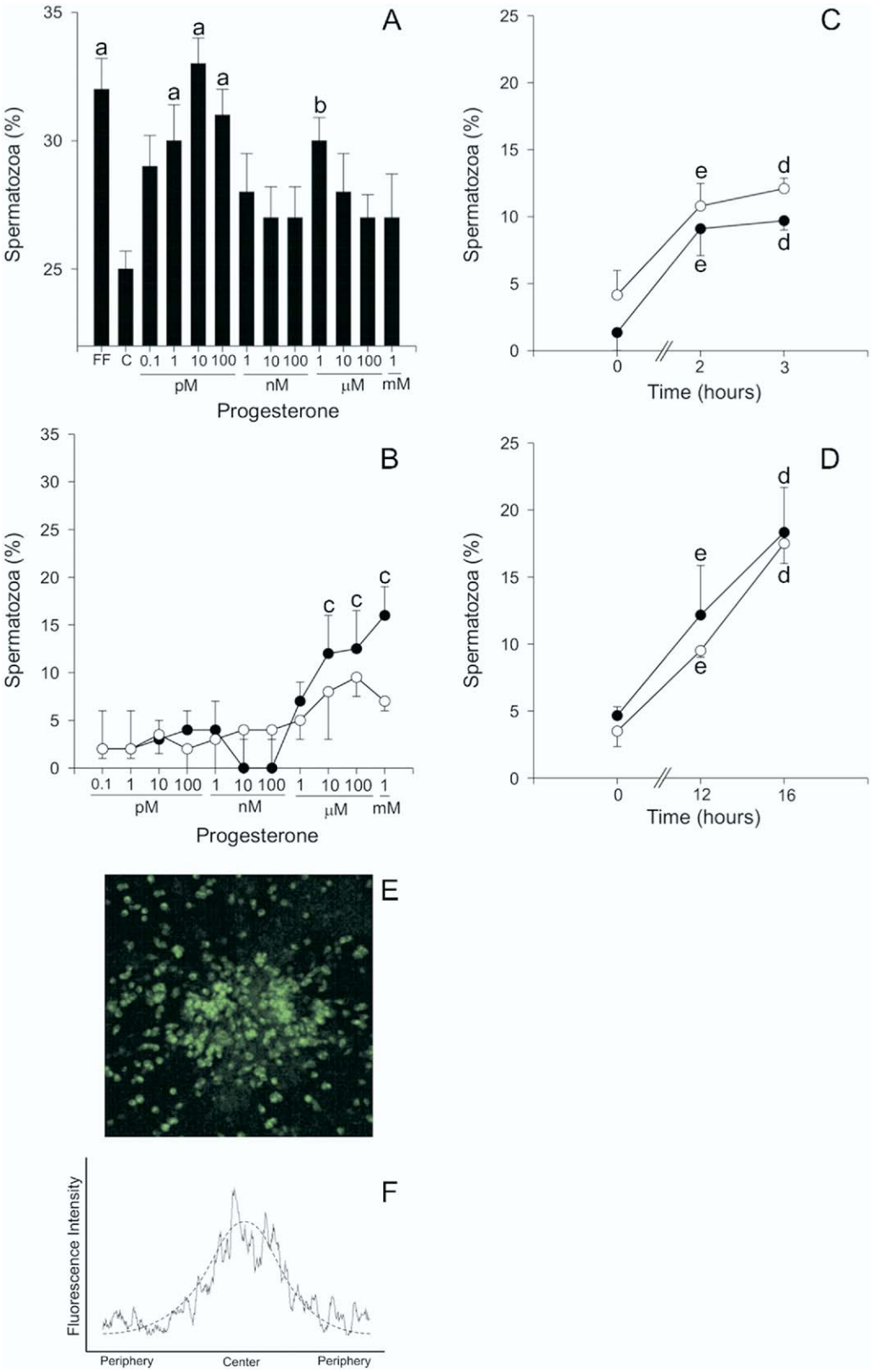
FIGURE 1



Teves. Progesterone chemotactic activity at pM range. Fertil Steril 2006.

FIGURE 1 Continued

(**A** and **B**) Kinematics of human spermatozoa as a function of P concentration: (**A**) chemotaxis, expressed as the percentage of cells traveling in the direction of the P (or culture medium as negative control); (**B**) percentage of chemokinetic spermatozoa (*open circles*)—cells that increase the curvilinear velocity—and hyperactivated spermatozoa (*solid circles*), where the corresponding control values have been already subtracted. Significant differences vs. absence of the hormone (a: $P<.001$; b: $P<.03$) and vs. P concentrations <10 μM (c: $P<.001$); FF = 10^4 bovine follicular fluid positive control; C = negative control. (**C** and **D**) Correlation between the level of sperm chemotaxis mediated by P (*solid circles*) and the A23187-induced acrosome reaction (*open circles*), as a function of the incubation time for capacitation, in humans (**C**) and rabbits (**D**), where the corresponding control value has been already subtracted. Significant differences vs. time zero (d: $P<.001$; e: $P<.05$). Sperm preparation was done as previously described (4, 6). Motility parameters were evaluated in a modified chemotaxis chamber by video microscopy and computer image analysis as previously described in detail (3–4). The percentages of spermatozoa showing chemotaxis, chemokinesis, hyperactivation, and induced acrosome reaction was determined as previously described (4) and are expressed as mean ± standard error. The experiments were performed with ejaculates from different individuals (either human or rabbit), each carried out in three or more repetitions. The minimal number of blindly analyzed spermatozoa per treatment and repetition was 150 cells for kinematic parameters (giving a total cell number of 450–3,000) and 400 cells for the acrosome reaction determination (giving a total cell number of 1,200–8,000). (**E** and **F**) Immunocytochemistry with antiprogesterone (*green fluorescence*) of an egg-cumulus complex: the confocal image (**E**), and the relative fluorescence intensity (**F**) expressed as arbitrary units from the egg (center) to the cumulus periphery. Egg-cumulus complexes were obtained by aspiration from women undergoing in vitro fertilization. Those complexes classified as normal according to the clinic criterion, and not suitable to be included in the IVF program, were processed for P gradient determination by standard immunohistochemistry. The first antibody against P was a rabbit antiprogesterone-11α–bovine serum albumin (BSA) (1:10 in phosphate-buffered solution [PBS] with 0.1% sodium citrate and 0.1% Triton X-100), and the secondary antibody (labeled with fluorescein isothiocynate) was a monoclonal anti–rabbit IgG (1:32 in PBS with 0.1% BSA). In parallel, the negative control was incubated in the same conditions but in the absence of the first antibody. A total of 12 egg-cumulus complexes were observed under a confocal microscope (TCSSP; Leica, Mannheim, Germany), and the image analysis was done with the Scion image software (National Institutes of Health, Bethesda, MD). Human and rabbit assays were performed in accordance with the guidelines for experimentation of the Declaration of Helsinki and the Swiss Academy of Medical Sciences, respectively. All chemicals were from Sigma (St. Louis, MO).

P (data not shown). On the other hand, at high P concentration (10 μmol/L to 1 mmol/L), where no chemotactic signal was detected, a significant increase in the level of human hyperactivated spermatozoa was observed ($P<.001$; Fig. 1B), in agreement with other reports (14).

Earlier studies that reported a positive chemotactic response of human spermatozoa toward P (7–10) used variants of an accumulation assay in which chemotaxis cannot be successfully distinguished from chemokinesis and hyperactivation. In the present study, the hyperactivation effect of P was observed at high hormone concentrations in the absence of a sperm chemotactic response. Therefore, it is probable that the P-stimulated cell accumulation, observed in some of the earlier studies at the μmol/L–mmol/L range, and which was attributed to chemotaxis (7–10), was mainly due to trapping caused by hyperactivation, in line with the suggestion of Jaiswal et al. (11). Although Villanueva-Diaz et al. (8) assayed almost as low chemoattractant concentrations as those at which we found the highest chemotactic activity, they probably missed the chemotactic signal, because they apparently did not use capacitated spermatozoa in their experiments, which is essential for the detection of sperm chemotaxis (4–6) (Figs. 1C and 1D). In contrast, Jaiswal et al. (11) concluded that P either is not a sperm chemoattractant or is a weak one. Although those authors performed the chemotaxis assays with capacitated spermatozoa and determined chemotaxis with strict criteria, they studied those P concentrations claimed in earlier studies (7–10) to cause sperm chemotaxis (1–100 μg/mL); however, the concentration range assayed by those authors is orders of magnitude higher than the P chemotactic dose found in the present study (Fig. 1A).

In summary, sperm chemotaxis toward P was not definitely demonstrated in the past, because extremely low concentrations of the hormone were not assayed in a system that allowed clear chemotaxis identification. Thus, these two different sperm processes (chemotaxis and hyperactivation) are both stimulated by P but at poles-apart concentrations, low for chemotaxis and high for hyperactivation, in agreement with the presence of

high- and low-affinity P receptors on the sperm surface (15).

Humans and rabbits are two mammalian species that show different reproductive strategies: periodic ovulation in humans and mating-induced ovulation in the rabbit. These differences are reflected in the kinetics of capacitation, which seems to be programmed according to the differential timing of their own egg availability (6). In both species, the follicular-fluid chemotactic ability is acquired during the incubation time for capacitation (1). Therefore, we investigated the correlation between the percentage of induced acrosome-reacted spermatozoa and those that chemotactically respond to P along the incubation time. The cells were confronted with the higher chemotactic concentration of P (10 pmol/L and 100 pmol/L for humans and rabbits, respectively) at different time points during sperm capacitation, and the level of A23187-induced acrosome-reacted cells was determined in parallel. A strong significant correlation between the net proportions of both chemotactic and acrosome-reacted spermatozoa was observed along the time (humans: $r^2 = 0.98$; $P<.001$; rabbits: $r^2 = 0.81$; $P<.01$; Figs. 1C and 1D). Therefore, in both species, only capacitated spermatozoa are able to chemotactically respond to P, a sperm guidance mechanism that seems to be rather conserved in mammals.

Our in vitro experiments show that P guides mammalian spermatozoa by chemotaxis. However, this conclusion cannot be extended to what may be happening in vivo. By the time of ovulation, P is almost everywhere in the egg microenvironment, but a stable P concentration gradient should be formed for chemotaxis to occur. Recently, it was suggested that if chemotaxis exists in vivo, the most probable site of its occurrence should be along the cumulus oophorus (16), whose particular viscosity and organization could keep a stable chemoattractant gradient. Following ovulation, the cumulus cells synthesize and secrete P (17) and its carrier protein (18) which makes P soluble once out of the cell. We showed that the cumulus cells producing P are radially distributed (Figs. 1E and 1F), suggesting that, when the hormone is excreted, such cell distribution favors a P gradient formation from the center to the periphery of the cumulus cellular mass. These results raise the possibility that the cumulus oophorus could be a potential place of P-mediated sperm chemotaxis in vivo.

In summary, a possible mechanism of action of P in vivo could be as follows: In the vicinity of the cumulus, the low levels of P activate in the spermatozoon the high-affinity P receptors, and, as a consequence, the spermatozoon swims toward the cumulus mass by chemotaxis. Once inside the cumulus, where the P concentration is higher, the low-affinity P receptors are stimulated in the spermatozoon, leading to the acquisition of a hyperactivated pattern of movement, which helps it to pass across the cumulus mass and the zona pellucida. In parallel, an unknown attractant probably secreted by the egg (19), could guide the spermatozoon to the egg surface.

Acknowledgment: The authors thank Diego Uñates (Ph.D. student, UNC-Argentina) for performing some of the rabbit chemotaxis experiments, Peter Koenig (Ph.D., Institute of Anatomy, University of Giessen, Germany) for confocal images, and Roberto A. Rovasio (Ph.D., UNC-Argentina) for critically reading the manuscript.

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