Sperm transport and retention at the fertilization site is orchestrated by a chemical guidance and oviduct movement

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Abstract

In mammals, only a few spermatozoa arrive at the fertilization site. During the last step in the journey to the egg, apart from their self-propulsion, spermatozoa may be assisted by oviduct movement and/or a guidance mechanism. The proportion of rabbit spermatozoa that arrive at the fertilization site was determined under *in vivo* conditions, in which either the ovulation products (secreting chemoattractants) and/or the oviduct movement (causing the displacement of the oviductal fluid) was inhibited. When only one of these components was inhibited, sperm transport to the fertilization site was partially reduced. However, when both the ovulation products and the oviduct movement were inhibited, almost no spermatozoa arrived at the fertilization site. The results suggest that spermatozoa are transported to and retained at the fertilization site by the combined action of a chemical guidance and the oviduct movement. A working model is proposed to explain how these two mechanisms may operate to transport spermatozoa to the fertilization site, probably as an evolutionary adaptation to maximize the chance of fertilizing an egg.

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Introduction

In mammals, after the spermatozoa and the egg enter the oviduct, they still have to reach the fertilization site (Halbert *et al.* 1976, Rodriguez-Martinez 2007). However, this is not an easy journey for the gametes. The ovulated egg is transported through the ampulla (the oviduct region proximal to the ovary) by means of epithelial cell cilia beating (Halbert *et al.* 1976), arriving at the ampullary isthmus junction (AIJ) in about 3–13 min in the rabbit (Harper 1965, Boling & Blandau 1971, Halbert *et al.* 1976). A temporary reduction in oviduct diameter retains the egg at the AIJ for 12–18 h as observed in the rabbit (Halbert *et al.* 1976). Since this oviduct constriction does not affect the movement of spermatozoa and oviductal fluid, the AIJ is thought to be the place where fertilization may take place (Hodgson *et al.* 1976, Bourdage & Halbert 1988).

Although the spermatozoon is a self-propelled cell, its path inside the oviduct to get to the fertilization site is long and tortuous. To be ready for fertilization, spermatozoa must first reside for a lapse of time in the sperm reservoir at the isthmus (oviduct region near the uterus; Suarez & Pacey 2006, Rodriguez-Martinez 2007). There, they undergo several physiological changes known as ‘capacitation’, required to fertilize the egg (De Jonge 2005). The occurrence of the first

capacitated spermatozoa varies according to the specie’s reproductive mode, e.g. 30 min for periodic ovulators, like humans, or 10 h for induced ovulators, like rabbits (Cohen-Dayag *et al.* 1994, Giojalas *et al.* 2004). At any given time, only a few spermatozoa are capacitated (Cohen-Dayag *et al.* 1995, Giojalas *et al.* 2004) and released from the sperm reservoir to the oviduct lumen (Rodriguez-Martinez 2007, Suarez 2008). From there, the capacitated spermatozoa must travel several centimeters to arrive at the fertilization site (Bourdage & Halbert 1988, Yaniz *et al.* 2000).

These observations lead to the assumption that fertilization is more than a casual event, and that the few capacitated spermatozoa released from the reservoir may be additionally assisted (apart from their self-propulsion) to successfully reach the egg. Different types of transport mechanisms have been postulated. One of them may carry spermatozoa by means of oviduct movement (Ito *et al.* 1991), which causes the displacement of the tubal fluid toward the ovarian end of the oviduct (Rodriguez-Martinez *et al.* 1982). The other may orient the movement of capacitated spermatozoa by an increasing gradient of either an attractant molecule (a phenomenon called chemotaxis) or temperature (known as thermotaxis; Eisenbach & Giojalas 2006).

In the last 20 years, several *in vitro* studies on sperm chemotaxis and thermotaxis have been documented

(Eisenbach & Giojalas 2006), suggesting the existence of gradients of either a physiological attractant (Teves *et al.* 2006, Guidobaldi *et al.* 2008) or temperature (Bahat *et al.* 2003) inside the oviduct. However, the involvement of these phenomena in transporting spermatozoa has yet to be defined *in vivo*.

Under *in vivo* conditions, it has been observed that spermatozoa were successfully transported from the isthmus to the fertilization site only in the presence of ovulation products (Harper 1973a, Ito *et al.* 1991), which contain the egg complex and consist of cells with the ability to secrete chemoattractants (Sun *et al.* 2005, Guidobaldi *et al.* 2008, Oren-Benaroya *et al.* 2008). However, since peristaltic activity in the oviduct was not controlled, the identification of the sperm transport mechanism was not conclusive. Nevertheless, both *in vivo* and *in vitro* evidence suggests that more than one mechanism may assist spermatozoa to reach the fertilization site. We hypothesize that spermatozoa arrive at the fertilization site by the combined action of a guidance mechanism and oviduct movement.

Results

Experimental design

Experiments were designed to assess the proportion of spermatozoa that arrive at the fertilization site when the oviduct movement was pharmacologically inhibited and/or the ovulation products were surgically prevented from entering the oviduct (Figs 1 and 2). The study was performed in the rabbit where ovulation is expected to occur around 10 h post mating (Harper 1973a, Overstreet & Cooper 1978a). Therefore, the surgery to block the entrance of ovulation products was performed 6 h post mating, thus ensuring the presence of spermatozoa in the isthmus (Overstreet & Cooper 1978b). One oviduct was surgically tied at the fimbriae level, leaving the contralateral oviduct untied. Since most spermatozoa stay in the isthmus until ovulation (Overstreet & Cooper 1978b), oviduct contractions were inhibited 8 h post mating (hence, prior to ovulation) for a period of 8 h. The surgery to recover spermatozoa from the oviduct was performed 16 h after mating, time when

Figure 1 Experimental design. (A) Either the entrance of the ovulation products or the oviduct movement was handled according to the timing of several biological events triggered by mating (sperm arrival at the oviduct, sperm capacitation, ovulation, and egg fertilization). (B) The table summarizes the different treatments applied as a result of combining the inhibition of the entrance of ovulation products and/or the oviduct muscle contractions and the corresponding sperm transport mechanisms expected to be inhibited. (For further details, see ‘Materials and methods’ and ‘Results’ sections). i.m., intramuscular injection.

Figure 2 Oviductal regions delimitation. The anatomical region of the oviduct (the isthmus and the ampulla) are shown in (A) and the schematic representation of the three experimental regions delimited by ligations in (B). The ampullary isthmic junction (AIJ) can be recognized by observing the place where the oviduct becomes thicker and the ovarian artery (white arrow) supplies the oviduct with a bifurcation.

the maximum level of capacitated spermatozoa *in vitro* (Giojalas *et al.* 2004) and fertilized egg *in vivo* (Harper 1973a) is expected. The oviduct was divided by ligation into three regions defined as the isthmus, the AIJ and the ampulla (Fig. 2), from which spermatozoa were flushed for counting. Sperm redistribution along the oviduct was expressed as the percentage of spermatozoa in each region, considering the total number of spermatozoa recovered in the whole oviduct as 100%. For further technical details, see 'Materials and methods' section.

Oviduct contraction inhibition

Muscle contractions were inhibited with ritodrine (Elea, Buenos Aires, Argentina), a beta-adrenergic agonist (Caritis *et al.* 1990, Vesalainen *et al.* 1999). In the rabbit, the transport of the oocyte-cumulus complex is mediated by cilia of oviductal epithelial cells, and is not affected by muscle contraction inhibition (Halbert *et al.* 1976). In this study, the arrival of the egg in the AIJ region was verified after flushing the oviduct. Although beta-adrenergic agonists inhibit oviduct smooth muscle

contractions (Halbert *et al.* 1976), we verified the effect of ritodrine by video recording the displacement of a dye droplet inside the oviduct. When muscle contractions were not inhibited, the movement of a dye droplet from the isthmus to the ampulla was easily observed (Supplementary Video 1, see section on supplementary data given at the end of this article). However, when an i.m. injection of ritodrine (1 mg/kg) was administered 30 min before the surgery, the dye droplet did not move inside the oviduct. Moreover, the oviduct muscle did not contract even upon a mechanical stimulus (Supplementary Video 2, see section on supplementary data given at the end of this article). Since a secondary effect of ritodrine is to increase heart beat frequency (HBF; Caritis *et al.* 1990, Vesalainen *et al.* 1999), this parameter was used to control the inhibiting effect of the drug during the experiment. Immediately after the i.m. injection of ritodrine, there was a significant increase in HBF, and this was stable for 2 h in comparison with the saline control (Fig. 3A). Moreover, repeated doses of ritodrine every 2 h enabled a constant effect of the inhibitor over time (Fig. 3B). Thus, oviduct contractions were inhibited for ~8 h during the experimental procedure (before ovulation and until the

Figure 3 Heart beat frequency (HBF) variation under ritodrine treatment. The HBF (number of beats per min) was measured by directly listening with a stethoscope under unique (A) or successive (B) doses of 1 mg/kg of ritodrine (diamond). The basal HBF (square) was determined before treatment as a reference value to calculate the relative HBF ($rHBF = HBF / \text{basal HBF}$). Saline solution (circles) was administered in order to discard any stressing effect on the HBF caused by animal manipulation during the injection. ^{a,b}Significant difference vs saline solution; $P < 0.05$.

surgery to recover spermatozoa), and the effect was regularly checked by measuring the HBF, a non-invasive procedure. In addition, ritodrine did not affect sperm motility or velocity under *in vitro* conditions, as shown in [Supplementary Figure 3](#), see section on [supplementary data](#) given at the end of this article. In summary, ritodrine has several advantages such as i.m. administration, external control, and no toxicity on sperm motility. These features make it preferable among other beta-adrenergic agonists (e.g. isoproterenol) previously used in the rabbit ([Halbert *et al.* 1976](#)), which requires constant i.v. administration, stressing the animal.

Sperm transport and retention at the fertilization site

Under the condition where sperm transport was not inhibited (treatment 1), spermatozoa were unevenly distributed along the oviduct. The largest proportion of the cells was found in the isthmus (~86%), followed by the AIJ and ampulla regions with ~7% each, as reported by others ([Overstreet & Cooper 1978b](#)). Treatments did not cause a significant difference in the sperm proportion recovered from the ampulla. However, in the isthmus and AIJ regions, significant differences were observed according to treatment ([Fig. 4](#); the corresponding raw data are shown in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article).

Thus, when the oviduct movement and the ovulation products were simultaneously inhibited (treatment 4), the percentage of spermatozoa in the isthmus significantly increased ($97.9 \pm 1.2\%$), whereas spermatozoa were severely prevented from reaching the AIJ ($0.2 \pm 0.1\%$) compared to the control group (treatment 1). However, when only the ovulation products were not allowed to enter the oviduct that is free to contract (treatment 2), $2.3 \pm 1.1\%$ of spermatozoa arrived at the AIJ, which was significantly different from treatment 1 and treatment 4. By inhibiting contraction in the oviduct containing ovulation products (treatment 3), $3.8 \pm 1.7\%$ of spermatozoa was observed in the AIJ, which was significantly different only from treatment 4. These results suggest that oviduct movement per se did not significantly affect sperm transport to the AIJ and that ovulation products partially inhibited sperm accumulation at the fertilization site. However, the inhibition of both the chemical guidance and the oviduct movement produced almost a complete suppression of sperm transport to the AIJ, suggesting a concerted action of both mechanisms.

When the sperm transport mechanisms were individually inhibited (treatments 2 and 3), the proportion of spermatozoa leaving the isthmus was similar (~6%; [Fig. 4](#) and [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). However, it seems that these two mechanisms caused a differential sperm distribution in the AIJ. In order to know whether spermatozoa leaving the isthmus are

preferentially retained in the AIJ, the relative sperm accumulation in this region was estimated as a ratio between the mean values corresponding to the proportion of spermatozoa in the AIJ and in the ampulla for each treatment. Thus, the more the spermatozoa that are accumulated in the AIJ, the higher the relative sperm

Figure 4 Sperm distribution along the oviduct regions. The proportion of sperm recovered per region for every individual experiment was represented by circles and the mean value by slash. The same letter indicates significant difference between treatments; ^a $P < 0.050$; ^b $P < 0.018$; ^c $P < 0.001$; ^d $P < 0.046$; ^e $P < 0.006$.

accumulation value that is expected. When sperm transport was not inhibited (treatment 1), the relative sperm accumulation value was 0.9. In contrast, the simultaneous inhibition of both the ovulation products and the oviduct movement (treatment 4) caused a null sperm accumulation in the AIJ (0.1). In the absence of the ovulation products (treatment 2), the relative sperm accumulation value in the AIJ was 0.6, but under the inhibition of the oviduct movement (treatment 3), 2.9 times more sperm were accumulated in the fertilization site. This observation suggests that sperm leaving the isthmus are preferentially retained in the AIJ by the ovulation products.

Discussion

Once sperm capacitation is accomplished, spermatozoa are detached from the isthmus epithelium, becoming free to swim in the oviduct lumen (Suarez 2008). How are these few ready-to-fertilize spermatozoa transported to and retained at the fertilization site? This has been a long-standing open question in reproductive biology. Some experimental evidence from *in vitro* and *in vivo* studies suggests that more than one mechanism may assist spermatozoa in reaching the egg. Here, we performed *in vivo* experiments in the rabbit, because its reproductive features allowed us to manage sperm transport according to the timing of gamete biological events. Thus, the study was focused on the way that spermatozoa were redistributed along the oviduct after ovulation, when the oviduct movement and/or the entry of the ovulation products secreting chemoattractants were suppressed.

Earlier *in vivo* studies, where only the entry of the ovulation products but not oviduct contractions was inhibited, suggested that either chemotaxis (Harper 1973a) or oviduct peristalsis (Ito *et al.* 1991) may transport spermatozoa to the fertilization site. However, these reports could not clearly distinguish which of these sperm transport mechanisms was involved, since oviduct contractions were not controlled in the studies. Our results suggest that freely swimming spermatozoa may be assisted to traverse the long complex path from the isthmus to the fertilization site by the combined action of the oviduct movement (which may transport spermatozoa without distinguishing their physiological state), and the ovulation products (which may guide only capacitated spermatozoa toward the egg vicinity). We based our conclusion on several observations as discussed below.

Under oviduct movement inhibition (treatment 3), the apparent decrease in the percentage of sperms accumulated in the AIJ was not statistically significant; however, the sperm transport was apparently reduced to the half and sperms were mainly accumulated in the AIJ. These observations suggest that the ovulation products preferentially accumulate sperms in the AIJ, but it may not be the unique factor affecting sperm transport.

Accordingly, when the ovulation products were prevented to enter the oviduct (treatment 2), the sperm proportion in the AIJ was significantly reduced but some of them still accumulate there, suggesting that probably the oviduct movement may deliver these few sperms to the AIJ in an unspecified manner. These observations suggest that both the ovulation products and the oviduct movement alone may partially contribute to transport spermatozoa in the oviduct. This conclusion is reinforced by the fact that the simultaneous inhibition of both ovulation products and oviduct movement (treatment 4) retained sperms in the isthmus, preventing them from reaching the fertilization site. In this context, the following question arises: is there a need for a concerted action of two different mechanisms to transport spermatozoa in internal fertilizing species? We next propose an explanation for this interrogative.

Sperm 'sweep' by the movement of the oviductal fluid

The lumen of the oviduct is filled with fluid that moves due to two forces applied in opposite directions: the oviduct contractions and the epithelium cilia beating. Oviduct contractions are separated by few seconds of relaxation and, as a consequence, the oviduct fluid moves toward the ovarian end (Supplementary Video 1, see section on supplementary data given at the end of this article, Battalia & Yanagimachi 1979). In contrast, the cilia beating generates a persistent smooth current of fluid in the direction of the uterus (Kolle *et al.* 2009). Our study was carried out around ovulation, a time when the oviduct contractions and cilia beating are intensified due to high estrogen and low progesterone levels in the blood (Battalia & Yanagimachi 1979). The experimental design cannot determine to what extent cilia beating counteracts the force of the oviduct contraction. However, a dye droplet added at the uterine end of the oviduct showed a net ad-ovarian advance (Supplementary Video 1, see section on supplementary data given at the end of this article), as observed by others (Battalia & Yanagimachi 1979). Thus, it is reasonable to think that the movement of the oviduct fluid could transport free-swimming spermatozoa faster than their own propelling velocity. In order to discover whether the ad-ovarian movement of the oviduct fluid participates in sperm transport to the fertilization site, oviduct contractions were pharmacologically inhibited with ritodrine. Although cilia beating is not affected when the oviduct contractions are suppressed (Halbert *et al.* 1976), under ritodrine inhibition the dye droplet did not visibly move toward the uterus (Supplementary Video 2, see section on supplementary data given at the end of this article). Therefore, the current caused by cilia beating did not significantly counteract the sperm transport toward the ovary mediated by oviduct movement. Even though around ovulation the oviduct contractions are strong enough to carry spermatozoa, these cells would be

mechanically swept along the whole oviduct even up to the peritoneal cavity. Therefore, another mechanism would be necessary to retain them at the fertilization site; this possibility is discussed below.

Sperm guidance mediated by the ovulation products

After ovulation, the cumulus cells secrete sperm chemoattractants (Eisenbach & Giojalas 2006). Therefore, one possible role of the ovulation products could be to chemically guide spermatozoa to the egg. In the last 20 years, mammalian sperm chemotaxis has been reported in several *in vitro* studies (Eisenbach & Giojalas 2006), but it has not so far been demonstrated under *in vivo* conditions. Our study is based on the proportion of spermatozoa observed at the fertilization site, which may reflect not only sperms arrived from the isthmus but also those retained near the oocyte–cumulus complex. In any event, an attractant secreted by the cells contained in the ovulation products should diffuse out of the source forming a concentration gradient. However, the distance and length of an attractant gradient depends not only on the continuous supply of the attractant molecules from the source but also on the movement of the oviductal fluid. Assuming that, once at the fertilization site, the ovulation products continuously secrete attractants, how is the attractant gradient preserved under strong oviduct contractions? It is improbable that a unique long-lasting gradient of attractant molecules remains inside an oviduct that is moving. Instead, the chemoattractant gradient could be restored between contractions. Although this hypothesis is difficult to verify *in vivo*, it is based on the following observations: 1) the oviduct movement is intermittent (Supplementary Video 1, see section on supplementary data given at the end of this article; Bourdage & Halbert 1980), which would allow short periods of quiescence between contractions; 2) the cumulus cells could supply attractants while the oocyte–cumulus complex is present in the oviduct (Eisenbach & Giojalas 2006); and 3) the cilia beat in the direction of the uterus (Halbert *et al.* 1976, Kolle *et al.* 2009), generating a smooth oviductal fluid current, which would help to expand the attractant gradient out toward the isthmus. However, the recovery of a long-distance gradient would not be efficient enough; hence, the probability that spermatozoa may be guided from the isthmus to the AIJ by chemotaxis seems to be low. Conversely, the attractant gradient could be better restored in the surroundings of the cumulus surface. Thus, some spermatozoa might be rescued from the ad-ovarian oviductal fluid current mediated by the oviduct movement, increasing the probability of retention near the egg. Indeed, we observed that under a quiescent oviduct, the ovulation products preferentially accumulate spermatozoa in the AIJ than in the ampulla. In contrast, under the inhibition of the ovulation products but not of the oviduct

movement, the sperm cells tend to pass by the AIJ toward the ampulla. This observation supports the idea that spermatozoa may be retained at the fertilization site by chemoattractants secreted by the ovulation products. Interestingly, sperm migration to the rabbit fertilization site was also observed when rat oocyte–cumulus complexes were introduced in a ligated oviduct (Harper 1973b). This observation is in line with the notion that the ovulation products have the ability to recruit spermatozoa.

The experimental approach does not enable us to know the identity of the attractant molecule contained in the ovulation products. However, there are several *in vitro* studies suggesting progesterone as a sperm-attractant candidate. After ovulation, the cumulus cells secrete progesterone (Vanderhyden & Tonary 1995, Chian *et al.* 1999, Yamashita *et al.* 2003, Guidobaldi *et al.* 2008), which may form a concentration gradient by diffusion along and beyond the cumulus matrix (Teves *et al.* 2006, Guidobaldi *et al.* 2008, Oren-Benaroya *et al.* 2008). A gradient of a picomolar concentration of progesterone chemoattracts only capacitated spermatozoa, those ready to fertilize the egg (Teves *et al.* 2006). In addition, progesterone has been identified as the sperm attractant secreted by the oocyte–cumulus complex (Guidobaldi *et al.* 2008, Oren-Benaroya *et al.* 2008). These observations support the idea that ovulation products secrete progesterone that might guide capacitated spermatozoa to the egg.

However, the ovulation products may have other roles apart from sperm chemoattraction that may affect sperm transport, such as sperm detachment from the isthmus and hyperactivation. For example, mouse spermatozoa have to become hyperactivated to detach from the oviduct epithelium (Demott & Suarez 1992). Hyperactivation has been shown to depend on the activation of a specific calcium channel known as CatSper (Ho *et al.* 2009), which was recently suggested to be activated by nM to μ M progesterone concentrations homogeneously supplied to the sperm cells (Lishko *et al.* 2011, Strunker *et al.* 2011). However, it is unlikely that progesterone secreted by the cumulus cells could reach the isthmus at adequate concentrations to stimulate hyperactivation via CatSper and hence sperm detachment. Instead, progesterone supplied by the ovarian artery may stimulate sperm detachment from the epithelium as suggested by Hunter (2008). Similarly, systemic progesterone has been observed to facilitate sperm release from their reservoir inside the chicken female reproductive tract (Ito *et al.* 2011). Moreover, we observed that under the inhibition of either ovulation products or oviduct movement, there is the same proportion of cells released from the isthmus, supporting the idea that sperm detachment was not preferentially affected by the ovulation products. These considerations support the idea that the effect of the ovulation products on

sperm transport more likely occurs near the fertilization site rather than in the whole oviduct.

Other sperm-guiding mechanisms

When oviduct movement and ovulation products were inhibited, almost no spermatozoa arrived at the fertilization site. This observation suggests that these two factors may be involved in sperm transport from the isthmus to the AIJ, whereas other mechanisms (e.g. thermotaxis) may have a minimal effect, if any. Instead, thermotaxis may have a different role under *in vivo* conditions, for example, guiding capacitated spermatozoa along the cumulus matrix. Hyaluronic acid (the main component of the cumulus matrix) is a highly hygroscopic molecule, which preferentially binds water molecules, decreasing local temperature (Hunter & Einer-Jensen 2005). Thus, the gradual distribution of hyaluronic acid (higher concentration in the periphery of the cumulus) could facilitate an increasing temperature gradient toward the oocyte vicinity. However, this hypothesis needs to be tested.

Working model to explain sperm transport and retention at the fertilization site

Our results suggest that the oviduct movement and a chemical guidance are involved in sperm transport and retention at the fertilization site. How do these two mechanisms work together? We propose a working model based on present results (Fig. 5), the hypothesis of attractant gradient recovery mentioned above, and the

notion that, after ovulation, the cumulus cells continuously secrete chemoattractants.

Around ovulation, there is an intensified intermittent contraction of the oviduct, which propels the luminal fluid toward the ovary. Thus, spermatozoa swimming in the oviduct fluid may be carried from the isthmus along the oviduct, without preferential retention at the fertilization site (Fig. 5A). After ovulation, the cumulus cells continuously secrete sperm attractants, which may diffuse, forming a concentration gradient along the viscous matrix of the cumulus and beyond. Capacitated spermatozoa carried by the oviduct movement to the vicinity of the AIJ would be chemically guided toward the oocyte–cumulus complex surface (Fig. 5B). Although oviduct movement may temporarily disrupt the attractant gradient, when oviduct contractions stop, this would be immediately restored in the oocyte–cumulus complex surroundings, due to the continuous supply of attractant molecules by the cumulus cells. Moreover, this attractant gradient could be further expanded toward the isthmus by means of fluid currents generated by the cilia beating. Hence, the guidance of capacitated spermatozoa toward the egg may be intensified during the quiescence periods of the oviduct movement. Thus, most capacitated spermatozoa would be retained by chemical guidance in the periphery of the cumulus surface, while others would be carried toward the ovarian end of the ampulla by oviduct contractions. This cycle between oviduct contractions may be regularly repeated as long as a viable oocyte–cumulus complex is still in the oviduct.

However, capacitated spermatozoa retained at the fertilization site still have to reach the oocyte surface

Figure 5 Working model to explain sperm transport and retention at the fertilization site mediated by chemical guidance and oviduct movement. The model is based on present results and the hypothesis of attractant gradient recovery mentioned in the text. The oviduct movement mechanically propels oviductal fluid droplets containing free-swimming capacitated spermatozoa toward the ovary (A). The egg complex continuously secretes an attractant forming a gradient in the cumulus surroundings which may be expanded toward the isthmus by the cilia beating current. This attractant gradient may chemically guide capacitated spermatozoa toward the egg (B). The attractant gradient disrupted during the oviduct contractions may be restored by the cilia beating during the quiescence period between contractions. These two mechanisms, the chemical guidance and the oviduct movement, would alternate as long as a viable egg complex is available in the oviduct. The schemes representing the oviduct are not drawn to scale – blue spermatozoa: capacitated; green spermatozoa: non-capacitated.

passing through the cumulus mass. This last stage in the route to the oocyte could be stimulated by a progesterone gradient due to a higher hormone concentration in the inner part of the cumulus (Teves *et al.* 2006, 2009), by a different attractant (Eisenbach & Giojalas 2006) and/or by another mechanism (thermotaxis?).

Capacitated mammalian spermatozoa have to travel a long distance from the isthmus to the fertilization site, while the capacitation state is short and transient (Cohen-Dayag *et al.* 1995). Therefore, the combined action of the oviduct movement and the ovulation products to efficiently transport and retain capacitated spermatozoa at the fertilization site may be seen as an evolutionary adaptation to maximize the chance to fertilize the egg.

Materials and Methods

Ethics statement

Experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (ILAR 1996). We received permission to conduct the research from the science promotion agencies that financially supported the study.

Visualization of oviduct muscle contraction inhibition

The female was mated with a fertile male and 6 h later was anesthetized with a mixture of 5 mg/kg Xilazine (Vetec, Buenos Aires, Argentina) and 25 mg/kg Ketamine (Holiday, Buenos Aires, Argentina). Then, a dose of 1 mg/kg of Ritodrine (Elea) was administered. A 10-cm incision was made in the median ventral area, exposing the reproductive tract. Then, 200 µl of Sudan Black in Vaseline were introduced in the oviduct lumen from the uterine end with a 25G needle. The movement of the dye was video recorded with a digital camera.

Ovulation products blockade

Females were mated with a fertile buck, and 6 h later (Fig. 1A) were anesthetized with a mixture of 5 mg/kg Xilazine (Vetec) and 25 mg/kg Ketamine (Holiday). The abdomen was shaved and a 2-cm cut along the median line (2 cm above the posterior nipple) was performed. The ovary was localized by following the uterine horn. A tie was performed with suture thread at the fimbriae end of the oviduct of one side (assigned at random), leaving the contralateral oviduct open to the entrance of the oocyte-cumulus complex. After stitching the body wall, the female was kept isolated and warm, and 1 h later had completely recovered. Oviduct ligation caused a moderate accumulation of tubal fluid in the ovarian end of the ampulla which was indicative of the effectiveness of the tie. In addition, the anesthetics did not affect the motility or viability of spermatozoa, as checked under a wide range of concentrations by videomicroscopy and image analysis (Supplementary Figure 3, see section on supplementary data given at the end of this article). As a whole, this blocking procedure

was fast (15 min), simple and effective, without interfering with physiological ovulation as other procedures may do (e.g. removal of the ovary).

Oviduct flushing and sperm counts

Sixteen hours after mating (at the end of the experimental design, see Fig. 1), the female was euthanized with an i.v. injection of sodium thiopental (150–200 mg; Abbot, Buenos Aires, Argentina), through the internal dorsal vein in the ear. Immediately, a new cut was performed over the suture of the previous surgery, localizing the reproductive tract. The occurrence of ovulation was verified by checking the state of the ovaries (Supplementary Figure 1, see section on supplementary data given at the end of this article). Then, three regions were delimited with suture thread in the following order (Fig. 2): AIJ, isthmus, and ampulla. The limit between the isthmus and the ampulla was easily identified by looking at the site where the oviduct becomes thicker and the ovarian artery supplies the oviduct with a bifurcation (Fig. 2A; Bourdage & Halbert 1988). Once this point was clearly recognized, the AIJ region was limited by two ties with suture thread, positioning each 2 cm from the AIJ in both directions. Then, the isthmus and ampulla regions were closed with an additional tie at the uterine and ovarian ends respectively. The ties for limiting the three oviduct regions were performed first in the ipsilateral oviduct and then in the contralateral one. To recover the spermatozoa contained in the oviduct lumen, each region was flushed in the following order: ampulla, AIJ, and isthmus. The flushing procedure was performed as follows (Supplementary Video 3, see section on supplementary data given at the end of this article): in the proximal end (uterine) of each oviduct region, a small transverse cut was made in the oviduct wall with scissors, introducing a previously blunted 21G needle, which was clamped with forceps. To check whether the needle was inside the oviduct lumen, small amounts of saline were flushed inside. Then, the ovarian end of the region was held with forceps and the tie was cut, washing the wall with saline to avoid blood remnants. The distal end of the region was opened inside a centrifuge tube, where the flushed fluid was recovered by passing 1 ml of saline through. The content of each oviduct region was centrifuged at 5000 *g* for 10 min; the pellet was resuspended in a solution of 5% formaldehyde and 0.1 mg/ml Hoechst 33258 in PBS, for 15 min at room temperature. The samples were washed twice with distilled water at 5000 *g* for 10 min. The pellet was resuspended in distilled water, spreading it on a slide, and left to dry in air. Since red blood cells have no nucleus, the fluorescent dye stained only spermatozoa and epithelial cells, which can easily be distinguished from spermatozoa (Supplementary Figure 2, see section on supplementary data given at the end of this article). The total number of spermatozoa recovered from the oviduct lumen in each region was counted under simultaneous phase contrast and fluorescent illumination at 400×. Then, the percentage of spermatozoa in each region was calculated by dividing the number of spermatozoa in one region by the total number of spermatozoa counted in the three regions of the oviduct times 100.

Statistical analysis

A total of 21 females were used, 13 to set up the experimental design and 8 for the experiments themselves. Sperm distribution in the oviduct was analyzed with a one-way ANOVA, where the dependent variable was the proportion of spermatozoa in each region of the oviduct subjected to different treatments (none, products of ovulation inhibition, oviduct movement inhibition).

Data were normalized by the square root arcsine of the proportion before performing the statistical analysis. Differences between pairs of treatments were determined by a posteriori Duncan test. For HBF experiments, significant differences were determined with one-way repeated-measures ANOVA followed by a posteriori Holm–Sidak test. All the statistical analyses were performed with SigmaPlot software (Systat Software, Inc., San Jose, CA, USA).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0478>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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