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Mouse sperm begin to undergo acrosomal exocytosis in the upper isthmus of the oviduct

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ABSTRACT

Recent evidence demonstrated that most fertilizing mouse sperm undergo acrosomal exocytosis (AE) before binding to the zona pellucida of the eggs. However, the sites where fertilizing sperm could initiate AE and what stimuli trigger it remain unknown. Therefore, the aim of this study was to determine physiological sites of AE by using double transgenic mouse sperm, which carried EGFP in the acrosome and DsRed2 fluorescence in mitochondria. Using live imaging of sperm during in vitro fertilization of cumulus-oocyte complexes, it was observed that most sperm did not undergo AE. Thus, the occurrence of AE within the female reproductive tract was evaluated in the physiological context where this process occurs. Most sperm in the lower segments of the oviduct were acrosome-intact; however, a significant number of sperm that reached the upper isthmus had undergone AE. In the ampulla, only 5% of the sperm were acrosome-intact. These results support our previous observations that most of mouse sperm do not initiate AE close to or on the ZP, and further demonstrate that a significant proportion of sperm initiate AE in the upper segments of the oviductal isthmus.

1. Introduction

Mammalian spermatozoa are not able to fertilize oocytes immediately after ejaculation; they must first undergo a complex process called capacitation in the female reproductive tract or *in vitro* (Austin, 1951; Chang, 1951). These changes include the development of hyperactivated motility and the ability to undergo acrosomal exocytosis (AE) in response to specific stimuli (Buffone et al., 2014; Suarez, 2008). AE is essential for fertilization. Mice and men that produce sperm lacking acrosomes are sterile (Dam et al., 2007; Kang-Decker et al., 2001; Lin et al., 2007). The occurrence of AE allows IZUMO1, a protein that is essential for sperm–egg fusion, to relocalize to the equatorial region of mouse sperm head (Sosnik et al., 2009).

Not long ago, it was broadly accepted that sperm undergo AE upon interaction with the zona pellucida (ZP) of the egg, and many of the advances in our knowledge of this process were derived from in vitro studies using solubilized ZP (Cherr et al., 1986; Florman and Storey, 1982; Storey et al., 1984). However, recent evidence acquired using transgenic mice that produce sperm carrying enhanced green fluorescent protein (EGFP) in the acrosome and Ds-Red2 red fluorescence in the mitochondria of the flagellar midpiece (Hasuwa et al., 2010) suggest that sperm binding to the ZP is not sufficient to induce AE. Real-time imaging of in vitro fertilization of cumulus-oocyte complexes (COCs) showed that most fertilizing sperm undergo AE before contacting the ZP (Jin et al., 2011). In fact, most acrosome-intact sperm in that study were seen to move away from the ZP without penetrating it. A subsequent study demonstrated that acrosome-reacted sperm recovered from the perivitelline space of oviductal CD9$^{(-/-)}$ oocytes, which cannot fuse with sperm, are able to fertilize other cumulus enclosed oocytes in vitro (Inoue et al., 2011; Kuzan et al., 1984). This confirmed an earlier report that rabbit sperm recovered from the perivitelline space can fertilize zona-intact oocytes (Kuzan et al., 1984). When taken together with the observations that AE of sperm is minimal or nonexistent when the ZP proteins are assembled in the native three-dimensional structure (Baibakov et al., 2007; Buffone et al., 2009), these findings strongly suggest that ZP is not the primary physiological inducer of the acrosome reaction (Yanagimachi, 2011). Furthermore, the findings raise the question of where fertilizing spermatozoa initiate AE.

The mtDsRed2/Acr-EGFP mouse offers the opportunity to

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monitor the status of the acrosome in live, motile sperm swimming within the oviduct, because the fluorescence of the sperm can be clearly detected through the walls of the oviduct. It also provides the opportunity to use ejaculated sperm. Most in vitro fertilization studies with mice have been performed using epididymal sperm, rather than ejaculated sperm; those sperm had not been exposed to secretions of the male accessory sex glands, which could play a role in preparing sperm for fertilization (McGraw et al., 2015). Therefore, the aim of our study was to determine the physiological sites of AE in sperm migrating through the female reproductive tract and penetrating the cumulus matrix by using the fluorescent sperm in a combination of in vitro and ex-vivo approaches.

2. Materials and methods

2.1. Animals

Double-gene knockin males mice [BDF1-Tg (*CAG-mtDsRed2, Acr-EGFP*) RBGS0020sb], expressing EGFP in the acrosome and Ds-Red2 in the flagellar midpiece mitochondria, and 8-week-old F1 wild type females (C57BL/6 J × BalBc) were maintained at 23 °C with a 12 h light:12 h dark cycle. Animal experimental procedures were reviewed and approved by the Ethical Committee of IBYME. Experiments were performed in strict accordance with the Guide for Care and Use of Laboratory Animals approved by the National Institutes of Health (NIH).

2.2. Mating

Double-gene knockin males [BDF1-Tg (*CAG-mtDsRed2, Acr-EGFP*) RBGS0020sb], expressing EGFP in the acrosome and Ds-Red2 in the flagellar midpiece mitochondria, were mated with 8-week-old superovulated F1 wild type females (C57BL/6 J × BalBc) to detect and localize sperm within the oviduct. Superovulation was induced using pregnant mare serum gonadotropin (5IU, PMSG; Calbiochem) at 6:30 PM, followed 48 h later by human chorionic gonadotropin (5 IU, hCG; Calbiochem). Females were placed with males at 6:20 AM the following morning, and mating was allowed until 7:00 AM. The end of the mating period was considered as $t=0$. Mice were sacrificed by CO_2 asphyxiation and oviducts were collected 1.5 h and 4 h after the end of the mating period.

In addition to hormonal stimulation of superovulation, some [B6D2F1-Tg (CAG/su9-DsRed2, Acr3-EGFP) RBGS002Osb] males were mated with females undergoing natural estrous cycles. Visual assessment of cycle stage was used to select (C57BL/6 J × BALB/c) wild type F1 females, which were then caged with males at 6:00 AM for 30 min. Mating was considered to have occurred if a vaginal plug was found afterward. In some experiments, females were caged with males right after hCG injection and then, the occurrence of mating was confirmed at 4:00 AM (pre-ovulatory mating). Then, females with a vaginal plug were separated and the oviducts analyzed at different time point post ovulation (7:00 AM was considered time = 0).

2.3. Sperm capacitation

In vitro capacitation was performed as previously described (Jin et al., 2011). Briefly, spermatozoa from the caudae epididymides of [B6D2F1-Tg (CAG/su9-DsRed2, Acr3-EGFP) RBGS002Osb] males were induced to capacitate by suspending them in a 100-µL droplet of human tubal fluid (HTF)–BSA medium at ~10^5 cells/mL and then incubating them for 1–3 h at 37 °C under an atmosphere of 5% CO_2 and 95% air. Insemination was performed by placing about 1 µL of capacitated sperm suspension (about 100 sperm) at the edge of a coverslip overlaying a slightly compressed cumulus–oocyte complex. If no spermatozoa reached the immediate vicinity of the oocyte within 20 min of insemination, then, another aliquot of capacitated spermatozoa was added.

2.3.1. Preparation of cumulus–oocyte complexes (COCs)

When first retrieved from the ovary, oocyte–cumulus complexes are separate. However, in the oviduct, they adhere to form a single large cumulus mass. Because this mass was too large to detect fertilizing spermatozoa within it, we separated the mass into smaller individual cumulus–oocyte complexes using a brief (0.5–2.0 min) treatment with bovine testicular hyaluronidase (Sigma-Aldrich; 80 units/ml at 37 °C) with gentle pipetting. In some cases, a large cumulus mass was left in hyaluronidase-free HTF–BSA medium for 1 h (5% CO_2, at 37 °C), allowing the mass to dissociate spontaneously into several individual cumulus masses. These were washed four times with Hepes-buffered HTF (pH 7.35 ± 0.1) containing 0.3% BSA, followed by a final wash with HTF–BSA.

2.4. Oviduct preparation

Female reproductive tracts were dissected out after copulation of C57BL/6 J × BALB/c wild-type females with CAG/su9-DsRed2, Acr3-EGFP double knock-in males (Hasuwa et al., 2010). The uterine horn was tied off and dissected out together with the oviduct and the ovary. Oviducts were gently washed in Whitten´s medium (100 mM NaCl, 4.4 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM sodium lactate, 5,4 mM glucose, 0.8 mM sodium pyruvate and 20 mM HEPES, pH 7.4) to remove sperm attached to the outside wall, mounted on slides and covered with coverslips and immediately observed under the microscope.

2.5. Real time imaging of sperm inside the oviduct

To evaluate the migration of sperm, oviducts were imaged using an inverted epifluorescence microscope (TE-2000U, Nikon) connected to a CoolSNAP HQ cooled CCD camera (Roper Scientific) and driven by MetaMorph 7.0 (Universal Imaging Corporation). A microscope stage chamber (Harvard Apparatus) was used to maintain a humid atmosphere of 5% CO_2 in air and the temperature at 37 °C. The behaviors of sperm within each region of the oviduct were recorded and the number of sperm in each Confocal Z-stack were counted manually using ImageJ (National Institutes of Health).

2.6. Assessment of acrosomal exocytosis

Evaluation of AE in sperm passing through cumulus-oocyte complexes (COCs) was performed as previously described (Jin et al., 2011). Briefly, COCs were immobilized on a glass bottom-culture dish under a 3×3 mm^2 coverslip supported by silicon grease. The depth of the preparation was adjusted to 100 µm using a stereo microscope. The culture dish was placed in an incubator chamber at 37 °C and 5 % CO_2, 5% O_2, 90% N_2 (Peltier-4&CO_2, Taiei Electric). The concentration of sperm in the each drop was adjusted to $1–5 \times 10^4$/ml. To image sperm passing through the cumulus, a combination of transmitted light and epifluorescence was used. Mechanical shutters (VS25, Uniblitz), controlled by a pulse generator (VMM-D3J, Uniblitz), were placed at the mercury lamp for epifluorescence optics and at the halogen lamp for transmitted light. The time required for shutters to transition between open and closed states was ~3 ms. EGFP and Ds-Red2 fluorescence were excited at 460–500 nm and the images collected through a dual emission filter by a sensitive video camera (NC-R550b, NEC)

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