ORIGINAL ARTICLE

Sperm Biology

Participation of the inositol 1,4,5-trisphosphate-gated calcium channel in the zona pellucida- and progesterone-induced acrosome reaction and calcium influx in human spermatozoa

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The acrosome reaction is a prerequisite for fertilization, and its signaling pathway has been investigated for decades. Regardless of the type of inducers present, the acrosome reaction is ultimately mediated by the elevation of cytosolic calcium. Inositol 1,4,5-trisphosphate-gated calcium channels are important components of the acrosome reaction signaling pathway and have been confirmed by several researchers. In this study, we used a novel permeabilization tool BioPORTER® and first demonstrated its effectiveness in spermatozoa. The inositol 1,4,5-trisphosphate type-1 receptor antibody was introduced into spermatozoa by BioPORTER® and significantly reduced the calcium influx and acrosome reaction induced by progesterone, solubilized zona pellucida, and the calcium ionophore A23187. This finding indicates that the inositol 1,4,5-trisphosphate type-1 receptor antibody is a valid inositol 1,4,5-trisphosphate receptor inhibitor and provides evidence of inositol 1,4,5-trisphosphate-gated calcium channel involvement in the acrosome reaction in human spermatozoa. Moreover, we demonstrated that the transfer of 1,4,5-trisphosphate into spermatozoa induced acrosome reactions, which provides more reliable evidence for this process. In addition, by treating the spermatozoa with inositol 1,4,5-trisphosphate/BioPORTER® in the presence or absence of calcium in the culture medium, we showed that the opening of inositol 1,4,5-trisphosphate-gated calcium channels led to extracellular calcium influx. This particular extracellular calcium influx may be the major process of the final step of the acrosome reaction signaling pathway.

INTRODUCTION

The acrosome reaction has been recognized as a prerequisite for fertilization since 1958.[1] The acrosome reaction is essential for allowing spermatozoa to release acrosomal enzymes and enables them to penetrate the zona pellucida (ZP).[2] Spermatozoa with a defective acrosome reaction have reduced or no ability to penetrate the ZP and fertilize oocytes either *in vivo* or *in vitro*.[3,4] Following the acrosome reaction, the structural modification of related proteins located in the sperm plasma membrane and exposure of the inner acrosomal membrane also occur; these procedures play important roles in the fusion of spermatozoa and oocytes.[5,6] Spermatozoa have been believed to undergo an acrosome reaction upon interaction with the egg's ZP for many years. Knowledge of this process is derived from *in vitro* studies using solubilized ZP.[7–10] However, Inoue and coworkers suggested that most fertilizing mouse spermatozoa undergo the acrosome reaction before binding to the ZP.[11,12] Although the ZP might not be the main inducer of the mouse sperm acrosome reaction, *in vitro* studies using solubilized ZP still indicate ZP protein involvement in the acrosome reaction of human spermatozoa. Progesterone is also a well-known physiological inducer of the acrosome reaction.[13]

In the last few decades, the signaling pathway of the acrosome reaction has interested many researchers. Numerous studies and hypotheses have been published, most of them based on animal models, such as sea urchins[14–16] and mice.[17–21] Regardless of the type of inducer, the acrosome reaction induced by these factors is ultimately mediated by the elevation of cytosolic calcium. Calcium depletion in the acrosome, which is caused by the opening of the inositol 1,4,5-trisphosphate (IP3)-gated calcium channel, activates a store-operated calcium (SOC) channel in the sperm plasma membrane, resulting in a rapid elevation of cytosolic calcium leading to the acrosome reaction. This model has been established and confirmed by numerous researchers as the final step of the progesterone- and ZP-induced acrosome reaction in mammalian sperm.[13,22–25]

Several permeable specific inhibitors, such as xestospongin C and 2-APB, have been used to support the presence and role of the acrosomal IP3 receptor (IP3R) in mammalian sperm physiology.[26–28] Evidence of IP3R involvement in the acrosome reaction of human sperm has also been reported.[29] The IP3R family has three members, and the existence of IP3R types 1 (IP3R1) and 3 (IP3R3) has been shown in human spermatozoa by immunoblot analyses.[30] Immunohistochemical observations suggested

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that IP3R1 is localized in the anterior portion of the sperm head. After the acrosome reaction, the expression of IP3R1 in spermatozoa is decreased, as shown by blot visualization, and is also detected in vesiculated membrane fragments (which are released by the fusion of the plasma membrane and the outer acrosomal membrane during the acrosome reaction). In contrast, IP3R3 is observed in the posterior portion of the sperm head, midpiece, and tail, but little change is found even after the reaction. These results suggest that IP3R1 is involved in the regulation of the IP3-gated calcium store of spermatozoa.[30,31] Therefore, IP3R1 antibody is a potential inhibitor that may provide evidence for the acrosome reaction signaling pathway. In addition, we may obtain reliable evidence if we could stimulate IP3R directly by IP3. Unfortunately, both molecules have low cell membrane penetration. Mayorga's group established a streptolysin O (SLO) permeabilization protocol that allows the incorporation of exogenous proteins, lipids, and ions into the cytosol.[25,32–35] In this study, we used another novel permeabilization tool named BioPORTER® to introduce IP3R1 antibody and IP3 into spermatozoa. BioPORTER® is an effective macromolecule delivery tool with a lipid-based formulation. It can encapsulate macromolecules and make them directly available to cells. The effectiveness of BioPORTER® in delivering active molecules into cell lines has been reported by many researchers.[36,37] However, there are no related reports in the literature on the use of BioPORTER® in spermatozoa.

In this study, we probed the effectiveness of BioPORTER® in spermatozoa and showed that it can be used to provide direct evidence of IP3-gated calcium channel involvement in the acrosome reaction in human spermatozoa. In addition, we treated spermatozoa with IP3/BioPORTER® in the presence or absence of calcium in the culture medium to explore the importance of extracellular molecules in the acrosome reaction signaling pathway.

MATERIALS AND METHODS
Reagents and chemicals
BioPORTER® QuikEase™ Protein Delivery Kit, pluronic F127, progesterone, IP3R1 antibody (rabbit), and fluorescein isothiocyanate-labeled peanut (*Pisum sativum*) agglutinin (FITC-PSA) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Earle's balanced salt solution (EBSS), phosphate-buffered saline (PBS), and penicillin–streptomycin were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Sodium pyruvate and triethanolamine were purchased from Amresco, Inc. (Solon, OH, USA). Hoechst 33258 and polyvinylpyrrolidone-40 (PVP-40) were obtained from Shanghai Maokang Biotechnology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and glutaraldehyde were purchased from Sangon Biotech Co. (Shanghai, China). Cysteine and chlortetracycline (CTC) were obtained from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Rabbit IgG was purchased from Abcam Plc. (Cambridge, MA, USA). Fluo-3AM was obtained from Beyotime Biotechnology (Shanghai, China). The calcium ionophore A23187 was obtained from 4A Biotech Co., Ltd. IP3 was purchased from MedChem Express LLC (Monmouth Junction, NJ, USA). The FITC labeling kit was obtained from Elabscience Biotechnology Co. (Wuhan, China). All other chemicals were reagent grade.

Ethical approval
The research protocol was approved by the Ethics Committee of the Shanghai First Maternity and Infant Hospital.

Semen sample collection and processing
Normozoospermic semen samples[38] were collected from men visiting the Andrology Laboratory at Shanghai First Maternity and Infant Hospital. The samples were obtained by masturbation and liquefied at 37°C for 30–60 min. Then, highly motile spermatozoa were isolated by swim-up protocol in EBSS supplemented with 2.4 mmol l^{-1} calcium chloride, 0.3 mmol l^{-1} sodium pyruvate, 1% (*v/v*) penicillin–streptomycin, and 3% (*w/v*) BSA at 37°C in an atmosphere of 5% CO_2 in air for 1 h. Next, highly motile spermatozoa (20×10^6 ml^{-1}) were incubated with EBSS/3% BSA for 3 h for capacitation.

Chlortetracycline (CTC) staining[7] was used to determine the capacitation status of spermatozoa. CTC staining solution (750 μmol l^{-1} CTC in 20 mM tris [hydroxymethyl] aminomethane hydrochloride, pH 7.8) was supplemented with 130 mmol l^{-1} NaCl and 5 mmol l^{-1} cysteine. The fixative consisted of 12.5% (*v/v*) glutaraldehyde in 1 mol l^{-1} tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 7.8. The staining solution was prepared daily and maintained in the dark at 4°C until use. After incubation with EBSS/3% BSA for 3 h, the spermatozoa were washed and resuspended with EBSS/0.3% BSA. A total of 20 μl sperm suspension (5×10^6 ml^{-1}) was mixed with 20 μl CTC staining solution, and then 2 μl fixative was added. The mixture was smeared on clean slides and observed in a fluorescence microscope (Nikon, Tokyo, Japan) at ×600 magnification with a filter set consisting of an excitation filter BP450-490, a chromatic beam splitter FT510, and a barrier filter LP520. The capacitation status of 100 spermatozoa in randomly selected fields was evaluated. According to the method of Perry *et al*.[39] the CTC staining of the sperm head was divided into five patterns: CTC1, a fluorescent band in the postacrosomal region; CTC2, a bright fluorescent head with a non-fluorescent postacrosomal region; CTC3, a bright fluorescent head with a nonfluorescent thin band in the postacrosomal region; CTC4, uniform head fluorescence; and CTC5, decreased or loss of uniform fluorescence over the head. CTC1-3 represents the uncapacitated patterns, CTC4 is the capacitated pattern, and CTC5 is the acrosome-reacted pattern. After 3-h of incubation, the mean percentage of capacitated spermatozoa was 57.8 ± 5.1 (mean ± s.d.), which is consistent with previously reported data.[10] The capacitated spermatozoa were resuspended in EBSS (0.3% BSA).

Preparation of solubilized ZP
ZP was obtained from unfertilized human oocytes from the assisted reproduction program at Shanghai First Maternity and Infant Hospital. The ZP was separated from the human oocytes using a 1-ml syringe needle observed in a microscope and stored in ZP cryoprotectant (consisting of 25 mmol l^{-1} triethanolamine, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$, 1% [*w/v*] polyvinylpyrrolidone, pH 7.8) at −80°C. The ZP was then thawed and heat solubilized at 70°C in 5 mmol l^{-1} NaH_2PO_4 buffer (pH 2.5) for 90 min on the day of experimentation. The prepared ZP was centrifuged at 15 000 *g* for 10 min at 4°C, and the supernatant was collected.[10]

Delivering anti-inositol 1,4,5-trisphosphate type-1 receptor and inositol 1,4,5-trisphosphate into spermatozoa by BioPORTER®
Capacitated spermatozoa were washed and resuspended in EBSS (0.0% BSA). The sperm suspension (20×10^6 ml^{-1}) was treated with anti-IP3R1/BioPORTER® or IP3/BioPORTER® according to the operating instructions. Briefly, 100 μl anti-IP3R1 or IP3 (100 μg ml^{−1}) was added to a BioPORTER® QuikEase™ tube and pipetted up and down gently 3–5 times. The samples were then incubated at room temperature for 5 min and vortexed gently at a medium speed for 5 s. A total of 400 μl EBSS (0.0% BSA) was added to bring the final volume to 0.5 ml.

For the detection of the transfer efficiency by flow cytometry, anti-IP3R1 and IP3 were labeled by FITC according to the operating instructions of the FITC labeling kit. The FITC-anti-

IP3R1/BioPORTER® and FITC-IP3/BioPORTER® mixtures were incubated with the sperm suspension for different time periods (3 h and 4 h) and in different proportions (for FITC-anti-IP3R1/BioPORTER®: 8×10^5, 16×10^5, 32×10^5 and 48×10^5 cells per tube; for FITC-IP3/BioPORTER®: 16×10^5, 24×10^5 and 32×10^5 cells per tube) to determine the optimal experimental conditions. After FITC-anti-IP3R1/BioPORTER® and FITC-IP3/BioPORTER® treatment, the spermatozoa were washed three times and resuspended in PBS. Flow cytometry (BD Biosciences, San Jose, USA) was utilized to detect the ratio of FITC-positive spermatozoa and to determine the transfer efficiency. Except for the part detected by flow cytometry, the processed spermatozoa were smeared on clean slides and observed in a fluorescence microscope at ×600 magnification. The filter set used for FITC consisted of an BP450–490 excitation filter, an FT510 chromatic beam splitter, and a LP520 barrier filter, whereas that for Hoechst consisted of a G365 excitation filter, an FT395 chromatic beam splitter, and a LP420 barrier filter. After the optimal experimental conditions were determined, capacitated sperm suspensions were treated with anti-IP3R1/BioPORTER® and IP3/BioPORTER® in relevant proportions for 3h at 37°C. The relevant proportions are 32×10^5 cells per tube for anti-IP3R1/BioPORTER® and 24×10^5 cells per tube for IP3/BioPORTER®. The spermatozoa treated with anti-IP3R1/BioPORTER® were divided into two parts, one for calcium influx detection and the other for acrosome reaction determination. IP3/BioPORTER®-treated spermatozoa were divided into two groups during IP3/BioPORTER® treatment. The first group was cultured in regular culture medium containing 2.4 mmol l⁻¹ calcium chloride, and the second group was cultured in culture medium without calcium. The acrosome reaction was determined for these two groups.

Determination of the effects of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on ZP-, progesterone-, and calcium ionophore-induced calcium influx

The anti-IP3R1/BioPORTER®-treated spermatozoa were washed and loaded with 5 μmol l⁻¹ Fluo-3AM (supplemented with 0.05% [*w/v*] pluronic F127) for 30 min at 37°C. The loaded spermatozoa were washed three times and resuspended in PBS. Before detection, the suspension was balanced for 30 min at 37°C. The relative levels of intracellular calcium within the spermatozoon were measured by flow cytometry according to the ACEA flow cytometry instructions (ACEA Biosciences, Inc., San Diego, CA, USA) with modifications. In brief, baseline fluorescence was collected for 2 min; the collection was paused, and ZP, progesterone, the calcium ionophore A23187 or EBSS (0.0% BSA) were added. The final concentration in the tube was 0.33 ZP μl⁻¹ for the ZP group, 3 μmol l⁻¹ for the progesterone group, and 10 μmol l⁻¹ for the calcium ionophore group. Fluorescence collection was resumed for an additional 4 min. After collection, the gate was set for every 30 s and the median time of each gate was used as the horizontal ordinate. The mean FITC fluorescence intensity of each gate and the initial 2 min were recorded as "F" and "$F_{baseline}$", respectively. The raw intensity values were normalized from the equation $\Delta F = (F - F_{baseline})/F_{baseline}$, ΔF was used as an ordinate.

Determination of the acrosome reaction

Acrosomal status was assessed by FITC-PSA and Hoechst 33258 staining.[7,40] Before determination of the acrosome reaction, anti-IP3R1/BioPORTER®-treated spermatozoa were washed and incubated with ZP (final concentration: 0.33 ZP μl⁻¹), progesterone (final concentration: 3 μmol l⁻¹), and the calcium ionophore A23187 (final concentration: 10 μmol l⁻¹) for 60 min at 37°C. The spermatozoa were washed and incubated with Hoechst 33258 (4 μg ml⁻¹) for 10 min at a proportion of 9:1 to achieve a final concentration of 0.4 μg ml⁻¹ and then centrifuged with 2% (*w/v*) PVP-40 in PBS. The supernatant was then removed and resuspended in PBS, smeared on clean slides, and fixed for 30 min in 95% (*v/v*) ethanol after air drying. After fixation, each slide was washed with distilled water and air dried. Staining consisted of 0.025 mg ml⁻¹ FITC-PSA in PBS for 2 h at 4°C. The slides were observed in a fluorescence microscope at ×600 magnification to determine the fluorescence patterns of 200 spermatozoa in randomly selected fields. The filter set used for FITC-PSA consisted of the excitation filter BP450–490, the chromatic beam splitter FT510, and the barrier filter LP520, whereas that for Hoechst consisted of the excitation filter G365, the chromatic beam splitter FT395, and the barrier filter LP420. Hyperchromatic Hoechst staining indicated dead spermatozoa. Only those live spermatozoa without FITC-PSA staining or FITC-PSA staining at the equatorial segment were identified as those with acrosome reactions.

Statistical analysis

All numerical data are expressed as the mean ± s.d. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The data were analyzed with the use of IBM SPSS Statistics vision 22.0 (IBM.INC, Armonk, NY, USA). For all experiments, the nonparametric repeated measures ANOVA with a rank test for multiple comparisons was used. If the data were normally distributed, Tukey's test or a parametric Student's *t*-test was used where appropriate as the *post hoc* tests. $P < 0.05$ was regarded as statistically significant. The flow cytometry data were analyzed by FlowJo 7.6 (Becton Dickinson, Franklin Lakes, NJ, USA).

RESULTS

Determination of the optimal experimental conditions for BioPORTER®

With 2×10^5–8×10^5 cells per tube, the transfer efficiencies of the 3-h and 4-h treatments were similar. When the proportion was 16×10^5 cells per tube, the transfer efficiency of the 4-h treatment significantly decreased. For the 3-h treatment, the data were relatively stable (**Figure 1a** and **Supplementary Figure 1a**). These findings indicate that 3 h is the optimal treatment time. This treatment time was used in the relevant subsequent experiments.

To determine the availability of BioPORTER®, we used FITC-anti-IP3R1 and FITC-IP3 without BioPORTER® to treat spermatozoa. This analysis was very helpful in eliminating false positives, which may be caused by molecule adhesion to the sperm surface or inadequate washing after treatment. For FITC-anti-IP3R1, the transfer efficiency approached 90% and was significantly higher than that of the negative control, which was treated with FITC-anti-IP3R1 without BioPORTER® (8×10^5 and 32×10^5 cells per tube: $P < 0.001$; 16×10^5 cells per tube: $P = 0.001$; 48×10^5 cells per tube: $P = 0.015$). The actual transfer efficiency is represented as the difference between the anti-IP3R1/BioPORTER® group and the negative control, which was approximately 60% at 32×10^5 cells per tube (**Figure 1b** and **Supplementary Figure 1b**). For FITC-IP3, the actual transfer efficiency was approximately 70% and a dramatic decline was observed at 32×10^5 cells per tube (**Figure 1c**). Moreover, as negative controls, non-FITC-conjugated anti-IP3R1 and IP3 without BioPORTER® were used. No positive FITC fluorescence was recorded (**Supplementary Figure 2**). This finding indicates that BioPORTER® was an effective tool for delivering anti-IP3R1 and IP3 into spermatozoa. For anti-IP3R1, 32×10^5 cells per tube may be the maximal available level (**Figure**
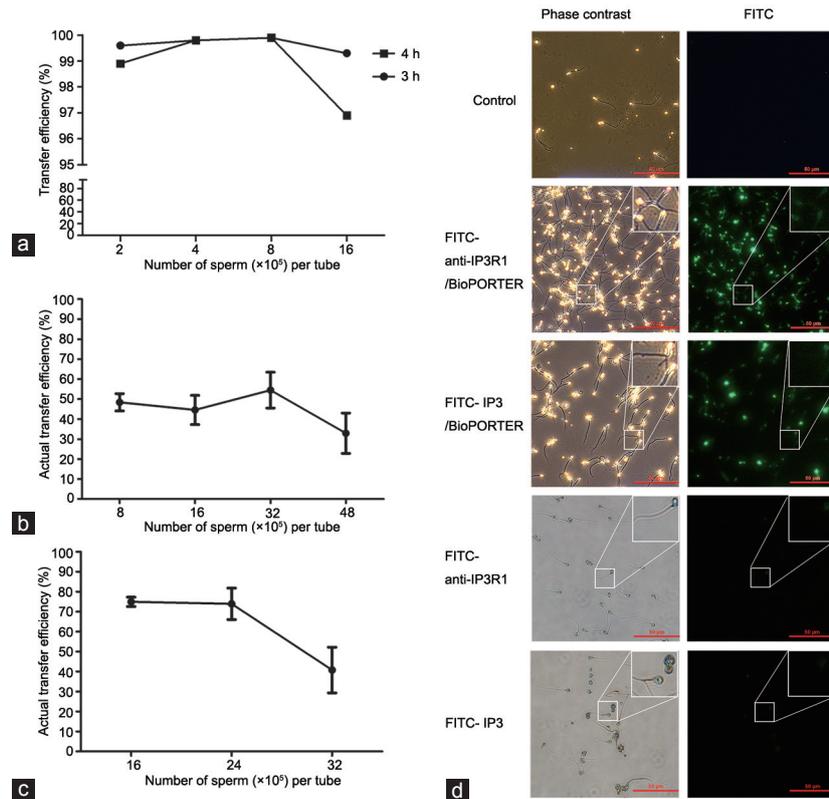
Figure 1: Determination of the optimal experimental conditions for BioPORTER®. (**a**) BioPORTER® transfer efficiency of anti-inositol 1,4,5-trisphosphate type-1 receptor at different treatment times. The FITC-labeled anti-IP3R1 was transferred into spermatozoa by BioPORTER®, and the transfer efficiency is represented as the FITC-positive spermatozoa. (**b**) BioPORTER® transfer efficiency of anti-inositol 1,4,5-trisphosphate type-1 receptor at different concentrations. The actual transfer efficiency is represented as the difference between the groups with or without BioPORTER® treatment. The data are expressed as the mean ± s.d., *n* = 3. (**c**) BioPORTER® transfer efficiency of inositol 1,4,5-trisphosphate in different proportions. The data are expressed as the mean ± s.d., *n*=3. (**d**) Fluorescent images of sperm. Scale bars = 50 µm. FITC: fluorescein isothiocyanate; IP3R1: inositol 1,4,5-trisphosphate type-1 receptor; s.d.: standard deviation.

1b and **Supplementary Figure 1b**). For IP3, 24×10^5 cells per tube showed satisfactory transfer efficiency (**Figure 1c**). After transfer of FITC-anti-IP3R1 and FITC-IP3 into spermatozoa using BioPORTER®, we observed the processed spermatozoa in a fluorescence microscope. For the experimental groups, fluorescence was clearly observed and was chiefly located in the posterior portion of the sperm head and midpiece. Weak fluorescence was also observed with FITC-anti-IP3R1 and FITC-IP3 without BioPORTER® treatment (**Figure 1d**).

Effect of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on progesterone-, ZP-, and calcium ionophore–induced calcium influx and acrosome reaction

Progesterone, ZP, and the calcium ionophore A23187 induced calcium influx. A23187-induced fluorescence intensity amplification was the strongest of the three inducers, and ZP was the least significant (**Figure 2a**). The curve of the calcium ionophore group indicated that the maximum fluorescence intensity occurred approximately 45 s after A23187 was added and subsequently remained constant (**Figure 2d**). For the progesterone group, the fluorescence peak was reached approximately 135 s after treatment (**Figure 2b**). ZP-induced calcium influx showed a slow upward trend, and the curve did not reach a plateau in the limited recording time (**Figure 2c**). Pretreatment with anti-IP3R1/BioPORTER® significantly reduced the calcium influx regardless of the inducers (**Figure 2** and **Supplementary Figure 1c**). The area under the curve was used for statistical analysis (Group progesterone: *P* = 0.048; Group ZP: *P* = 0.022; and Group A23187: *P* = 0.039).

The acrosome reaction rates induced by ZP and the calcium ionophore were similar (*P* = 0.205), and both rates were significantly higher than that induced by progesterone (*vs* ZP: *P* = 0.026; *vs* A23187: *P* = 0.001) (**Figure 3**). No difference in sperm viability were noted among the groups. Although the ZP-induced calcium influx was the weakest of the three inducers in the limited recording time, the curve presented a slow upward trend and did not reach a plateau. We assume that the fluorescence intensity of the ZP group would reach a peak after some time, and the maximum fluorescence intensity may be close to that in the calcium ionophore group. Regardless of induction by progesterone, ZP, or calcium ionophore, anti-IP3R1/BioPORTER® pretreatment significantly reduced the acrosome reaction (group progesterone, group ZP, and Group calcium ionophore: *P* < 0.001) (**Figure 3**). To confirm the effect of anti-IP3R1, we also pretreated spermatozoa with IgG/BioPORTER® under the same conditions. The pretreatment had no effect on the acrosome reaction induced by progesterone, ZP, or calcium ionophore (Group progesterone: *P* = 0.102; Group ZP: *P* = 0.109; and Group calcium ionophore: *P* = 1.000) (**Supplementary Figure 3a**). Moreover, to eliminate the possible impact of the transport tool, PBS/BioPORTER® was used. PBS/BioPORTER® had no effect on the acrosome reaction (*P* = 0.285) or the calcium influx (the area under the curve was used for statistical analysis: *P* = 0.109) induced by progesterone (**Supplementary Figure 3**). The calcium curves in this study were generated for each group and based on the average calcium concentration of the population. Anti-IP3R1 suppressed the second
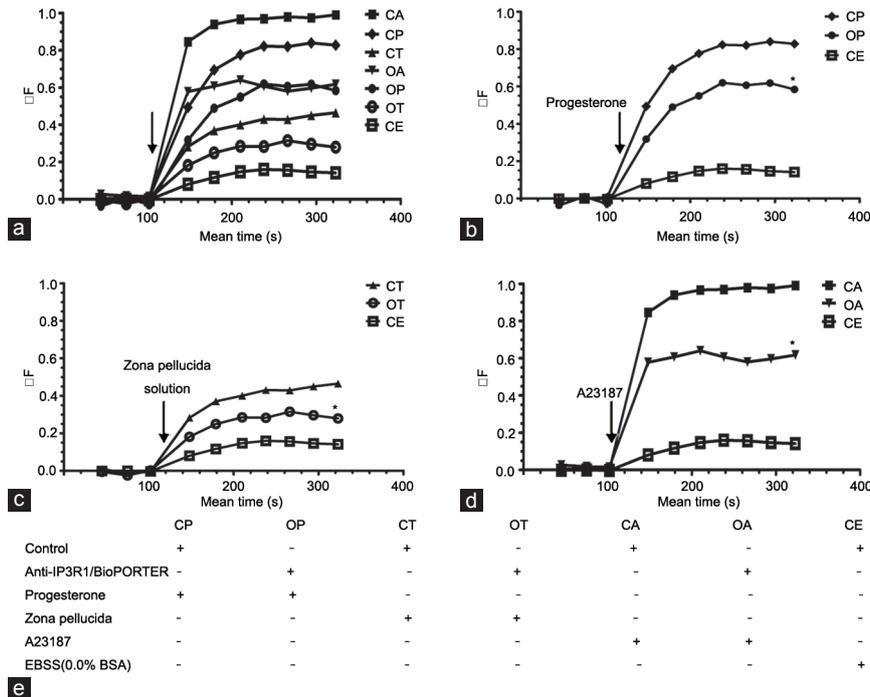
Figure 2: Effect of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on progesterone-, zona pellucida-, and calcium ionophore-induced calcium influx. (**a**) Summary graph. The calcium influx curves in this figure represent the change in the average calcium concentration of the groups. Black arrows indicate when inducers were added. The raw intensity values were normalized as ΔF, $\Delta F = (F - F_{baseline})/F_{baseline}$. "F" represent the mean FITC fluorescence intensity of each gate and "$F_{baseline}$" represents of the mean FITC fluorescence intensity of the initial 2 min. The data are expressed as the mean, $n = 3$. (**b**) Effect of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on progesterone-induced calcium influx. The "*" represents this curve, and the versus curve treated with inducer only was significantly different. The data are expressed as the mean, $n = 3$. (**c**) Effect of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on zona pellucida-induced calcium influx. The data are expressed as the mean, $n = 3$. (**d**) Effect of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on calcium ionophore-induced calcium influx. The data are expressed as the mean, $n = 3$. (**e**) Description of the legends in **a**, **b**, **c** and **d**. Figure legends are given a series of two-letter groupings. The first letter indicates the pretreatment reagent, and the second letter represents the inducers. For the first letter, "C" indicates Earle's balanced salt solution supplemented with 0.0% BSA (EBSS [0.0% BSA]), and "O" represent anti-IP3R1/BioPORTER®. For the second letter, "P" indicates progesterone, "T" represents ZP, "A" indicates A23187 and "E" indicates EBSS (0.0% BSA). IP3R1: inositol 1,4,5-trisphosphate type-1 receptor; ZP: zona pellucida.
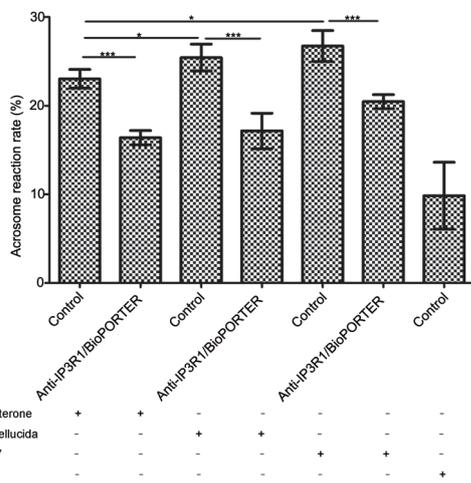

Figure 3: Effect of anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® on progesterone-, zona pellucida-, and calcium ionophore-induced acrosome reaction. The abscissa represent pretreatment by anti-IP3R1/BioPORTER® or not and the inducer used for each group was referenced by "+" below. The rates of ZP and calcium ionophore-induced acrosome reactions were similar ($P = 0.205$), and both rates were significantly higher than those induced by progesterone (*vs* ZP: $P = 0.026$; *vs* A23187: $P = 0.001$). Pretreatment with anti-IP3R1/BioPORTER® significantly reduced the acrosome reaction, regardless of mode of induction by progesterone, ZP, or calcium ionophore (Group progesterone, Group ZP, and Group calcium ionophore: $P < 0.001$). The data are expressed as the mean ± s.d., *$P < 0.05$, ***$P < 0.001$, $n = 4$. ZP: zona pellucida; IP3R1: inositol 1,4,5-trisphosphate type-1 receptor; s.d.: standard deviation.

calcium influx and led to the inhibition of the acrosome reaction. Hence, it also reduced the calcium curve and acrosome reaction induced by calcium ionophores (**Figure 2** and **3**).

Effects with or without calcium in the culture medium on the inositol 1,4,5-trisphosphate/BioPORTER®-induced acrosome reaction

When spermatozoa were treated in the regular culture medium containing calcium, IP3/BioPORTER® induced an acrosome reaction ($P < 0.001$). IP3/BioPORTER® also induced an acrosome reaction in calcium-deficient culture medium ($P = 0.018$). However, the acrosome reaction induced by IP3/BioPORTER® was significantly increased when the culture medium contained calcium ($P < 0.001$) (**Figure 4**). No differences in sperm viability were found among the groups. As a negative control, transfer of β-galactosidase into sperm by BioPORTER® had no effect on the acrosome reaction ($P = 1.000$). Meanwhile, to eliminate the possible impact of the transport tool, PBS/BioPORTER® was used which had no effect on the acrosome reaction ($P = 0.606$) (**Figure 4**). In addition, the slight calcium influx due to liquid addition was similar to that observed in the control group (the area under the curve was used for statistical analysis: $P = 0.285$) (**Supplementary Figure 3b**).

DISCUSSION

In this study, we first demonstrated the effectiveness of BioPORTER® in spermatozoa and used it to provide direct evidence of IP3-gated calcium channel involvement in the acrosome reaction in human spermatozoa. Through treatment of spermatozoa with IP3/BioPORTER® in the

presence or absence of calcium in the culture medium, we found that opening of the IP3-gated calcium channel led to extracellular calcium influx. This particular extracellular calcium influx may be the main process of the final step of the acrosome reaction signaling pathway.

Before fertilization, spermatozoa must undergo two sequential processes. The first is capacitation, which involves complex changes to prepare for the acrosome reaction. In previous reports, capacitation requires overnight incubation with EBSS/3% BSA[10,41–44] and a 3-h incubation with human tubal fluid or Ham's F-10/3.5% human serum albumin.[40] Considering sperm survivability and the availability of experimental materials, we explored the effect of different incubation times with EBSS/3% BSA on capacitation. After a 3-h incubation, the mean percentage of capacitated spermatozoa was 57.8 % and was close to that of the reported data, which were determined after overnight incubation (63.4%).[10] These findings showed that spermatozoa could achieve optimal capacitation with a 3-h incubation of EBSS/3% BSA.

In the second step, the spermatozoa undergo the acrosome reaction, which is the main preparatory process for fertilization. Although the acrosome reaction includes a complex signal transduction process, all other forms of stimulus-activated exocytosis and secretion of the acrosomal contents are ultimately mediated by elevation in calcium levels, regardless of the activator, progesterone, or ZP. Moreover, the elevation of calcium level consists of two components: a fast and transient influx followed by a slower and sustained influx.[45–50] The initial calcium influx activates phospholipase C and generates IP3 and diacylglycerol. This influx then activates IP3R and protein kinase C, which open the calcium channels at different sites. The depletion of calcium in the acrosome activates an SOC channel, resulting in a very fast and sustained increase in cytosolic calcium, leading to the acrosome reaction. This model is assumed by many researchers to be the final step of the progesterone- and ZP-induced acrosome reaction.[2,13,22,23] There is

some indirect evidence, such as the identification of a nonmitochondrial calcium storage pool in human spermatozoa and the stimulatory effect of thapsigargin (an inhibitor of the pump of the intracellular calcium storage pool) on the acrosome reaction, for this hypothesis.[23] Direct evidence has also been provided by several permeable specific inhibitors.[26–28] Evidence of IP3R involvement in the acrosome reaction of human spermatozoa has also been reported.[29] IP3R1 antibody is a potential inhibitor that provides more evidence for the acrosome reaction signaling pathway. In addition, more reliable evidence may be obtained if we could stimulate IP3R directly by IP3. Unfortunately, both molecules have low cell membrane penetrability. As mentioned above, BioPORTER® is reported to be an effective macromolecule delivery tool in many cell lines.[36,37] However, in spermatozoa, there have been no related reports on this topic. In this study, we are the first to indicate that BioPORTER® is an effective tool to deliver molecules with low cell membrane penetration into spermatozoa. It is also a mild transfer reagent that has no impact on the survival of spermatozoa.

In this study, we used three inducers. The calcium ionophore A23187 increased cytosolic calcium directly, as reflected by the calcium curve, which quickly approached the maximum fluorescence intensity and then remained constant. For progesterone, the maximum fluorescence intensity was approached more slowly, and the fluorescence subsequently remained at a steady state, which is very similar to the curve of A23187 induction. A 2016 study indicated that both A23187 and progesterone utilize the signaling module cAMP/Epac/Rap1/PLCε/IP3 after the initial cytosolic calcium level is elevated.[51] The rise time of the calcium signals is much slower than described in the previous report, which assessed the responses of human sperm to progesterone by loading indo-1/AM using flow cytometry.[52] This difference may be caused by the lower final concentration of progesterone (3 μmol l^{-1} *vs* 20 μmol l^{-1}) and lower temperature (25°C *vs* 37°C) during the flow cytometry analysis. The curve presented a slow upward trend and did not reach a plateau in the limited recording time for ZP. This result suggests that the ZP-induced initiatory cytosolic calcium elevation may involve a series of transduction events and open calcium ion channels in sequence. ZP-induced calcium influx did not create a plateau on the curve in the limited recorded time, thus indicating that slow transduction events may be involved. We can assume that the fluorescence intensity of the ZP group will reach a peak after some time, and the maximum fluorescence intensity may be close to that in the calcium ionophore group. The acrosome reaction rates induced by ZP and the calcium ionophore were similar, and both rates were significantly higher than that induced by progesterone. Regardless of whether induction was by progesterone, ZP, or A23187, anti-IP3R1/BioPORTER® pretreatment reduced calcium influx.

According to the literature, at least two steps of calcium influx are involved in the acrosome reaction.[2,22,53] The first one is triggered by the calcium ionophore and progesterone directly. For ZP, this step was very slow because it requires a series of sequential events. The calcium influx in this step mainly activates IP3R, which causes the depletion of calcium in the acrosome. Then, the SOC channel located in the sperm outer membrane is activated, resulting in a second calcium influx. This fast and sustained calcium influx is the key to triggering the acrosome reaction. Anti-IP3R1 suppresses the second calcium influx and leads to the inhibition of the acrosome reaction. Hence, it could also reduce the calcium time course and acrosome reaction induced by calcium ionophores.

IP3/BioPORTER® induced an acrosome reaction in calcium-containing culture medium, and the reaction rate was similar to that induced by ZP and the calcium ionophore. This treatment can also induce acrosome reactions in calcium-deficient culture medium,
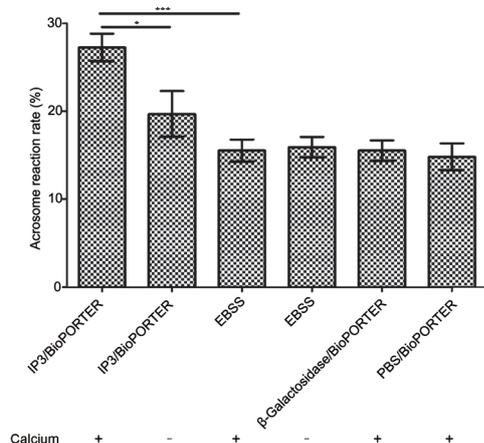

Figure 4: Effects on inositol 1,4,5-trisphosphate/BioPORTER®-induced acrosome reaction with or without calcium in the culture medium. IP3/BioPORTER® induced acrosome reactions in the regular culture medium containing 2.4 mmol l^{-1} calcium chloride ($P < 0.001$). It also induced an acrosome reaction in calcium-deficient culture medium ($P = 0.018$). However, the acrosome reaction induced by IP3/BioPORTER® was significantly increased when the culture medium contained calcium ($P < 0.001$). As a negative control, transfer of β-galactosidase into spermatozoa by BioPORTER® had no effect on the acrosome reaction ($P = 1.000$). PBS/BioPORTER® was also used to eliminate the possible impact of the transport tool and had no effect on the acrosome reaction ($P = 0.606$). The data are expressed as the mean ± s.d., $^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$. IP3: inositol 1,4,5-trisphosphate; PBS: phosphate-buffered saline; s.d.: standard deviation.

which may be caused by a small amount of calcium introduced into the medium by other reagents during the preparation. However, the acrosome reaction induced by IP3/BioPORTER® was significantly increased when the culture medium contained calcium. This finding indicates that the opening of the IP3-gated calcium channel will lead to extracellular calcium influx. This particular extracellular calcium influx is most likely the main process of the final step of the acrosome reaction signaling pathway.

In conclusion, BioPORTER® is a mild and effective permeabilization tool to introduce molecules with low membrane permeability into spermatozoa. Using this tool, we transferred IP3R1 antibody into sperm and determined that it could suppress the calcium influx and acrosome reaction induced by progesterone, ZP, and a calcium ionophore. This finding indicates that the IP3R1 antibody is a valid IP3R inhibitor and provides evidence of IP3-gated calcium channel involvement in the acrosome reaction in human spermatozoa. Moreover, we proved that the transfer of IP3 into sperm can induce an acrosome reaction, which provides more reliable evidence for this pathway. In addition, by treating spermatozoa with IP3/ BioPORTER® in the presence or absence of calcium culture medium, we showed that opening of the IP3-gated calcium channel will lead to extracellular calcium influx. This particular extracellular calcium influx may be the major process of the final step of the acrosome reaction signaling pathway. BioPORTER® is a useful permeabilization tool to introduce molecules with low membrane permeability into spermatozoa and can be applied to more in-depth research on sperm function in future work.

AUTHOR CONTRIBUTIONS

YYL designed and performed the experiments, analyzed and interpreted the data, and wrote the article. YPJ and LYD analyzed the data and engaged in critical discussions. KML designed and supervised the experiments and engaged in critical discussions. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on *Asian Journal of Andrology* website.

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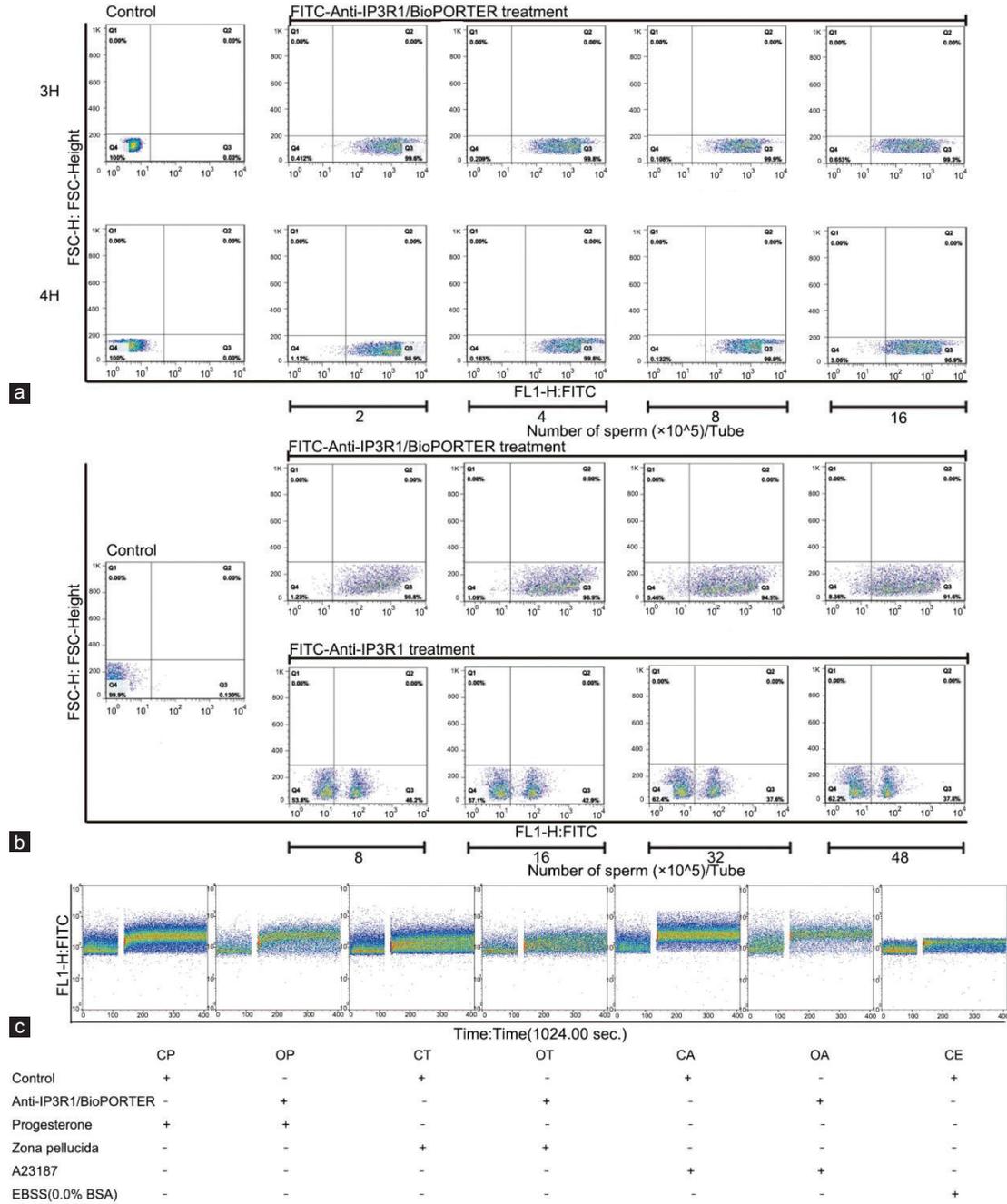
**Supplementary Figure 1:** The related flow cytometry pseudocolor graphs of **Figures 1** and **2**. (**a**) BioPORTER® transfer efficiency of anti-IP3R1 at different treatment times. (**b**) BioPORTER® transfer efficiency of anti-IP3R1 at different concentrations. (**c**) Effect of anti-IP3R1/BioPORTER® on progesterone-, zona pellucida-, and calcium ionophore-induced calciuminflux. IP3R1: inositol 1,4,5-trisphosphate type-1 receptor.
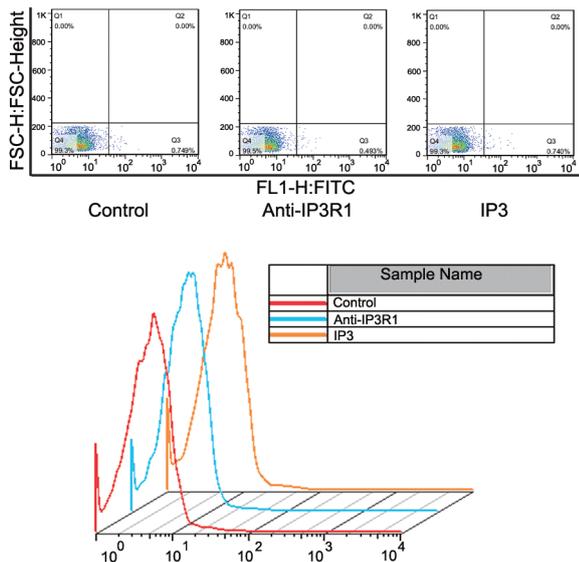
Supplementary Figure 2: Additional negative control in the BioPORTER® transfer efficiency experiment. No positive FITC fluorescence was recorded after non FITC conjugated anti-inositol 1,4,5-trisphosphate type-1 receptor and inositol 1,4,5-trisphosphate without BioPORTER treatments. FITC: fluorescein isothiocyanate.
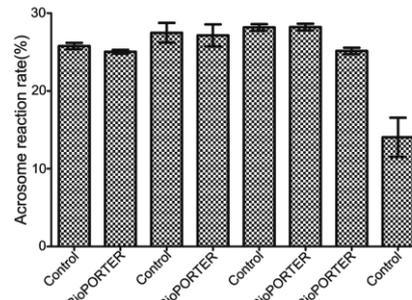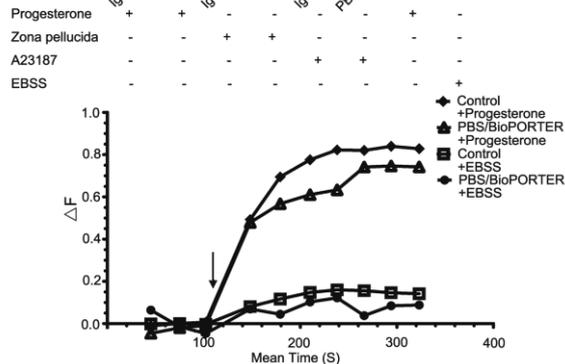
Supplementary Figure 3: Negative controls for anti-inositol 1,4,5-trisphosphate type-1 receptor/BioPORTER® and inositol 1,4,5-trisphosphate/BioPORTER® treatment. (**a**) Effect of IgG/BioPORTER® and PBS/BioPORTER® on acrosome reaction. Pretreatment with IgG/BioPORTER® had no effect on the acrosome reaction induced by progesterone, ZP, or calcium ionophore (Group progesterone: $P = 0.102$; Group ZP: $P = 0.109$; Group calcium ionophore: $P = 1.000$). Moreover, PBS/BioPORTER® was used to eliminate the possible impact of the transport tool and had no effect on the acrosome reaction induced by progesterone ($P = 0.285$). The data are expressed as the mean ± s.d., $n = 3$. (**b**) The effect of PBS/BioPORTER® on calcium influx. PBS/BioPORTER® did not affect the calcium influx induced by progesterone and the slight calcium influx due to liquid addition was similar to that observed in the control group (the area under the curve was used for statistical analysis: group progesterone: $P = 0.109$; Group EBSS: $P = 0.285$). Black arrows indicate when progesterone and EBSS were added. The data are expressed as the mean, $n = 3$. s.d.: standard deviation; PBS: phosphate-buffered saline; ZP: zona pellucida.