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ORIGINAL ARTICLE *Andrology*

Inhibition of sperm capacitation and fertilizing capacity by adjudin is mediated by chloride and its channels in humans

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STUDY QUESTION: Does adjudin disrupt chloride ion (Cl^-) ion transport function in human sperm and impede sperm capacitation and fertilizing ability *in vitro*?

SUMMARY ANSWER: In this study the results indicate that adjudin is a potent blocker of Cl^- channels: disrupting Cl^- ion transport function results in a decline in sperm capacitation and fertilizing ability in humans *in vitro*.

WHAT IS KNOWN ALREADY: Although our previous studies have demonstrated that adjudin exerts its effect by disrupting sertoli-germ cell adhesion junctions, most notably apical ectoplasmic specialization by targeting testin and actin filament bundles that disrupts the actin-based cytoskeleton in sertoli cells, it remains unclear whether adjudin impedes Cl^- ion transport function in the human sperm.

STUDY DESIGIN, SIZE AND DURATION: Semen samples were obtained from 45 fertile men (aged 25–32). Spermatozoa were isolated from the semen in the human tube fluid (HTF) medium by centrifugation through a discontinuous Percoll gradient, and incubated with adjudin at 10 nM–10 μM and/or other reagents under capacitating conditions for 0–5 h.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We evaluated the effect of adjudin and different reagents on sperm functions with which they were incubated at 37°C. Sperm motility and hyperactivation were analyzed by a computer-assisted sperm analysis (CASA) system. Sperm capacitation and the acrosome reaction were assessed by chlortetracycline fluorescence staining. Sperm fertilizing ability was evaluated by sperm penetration of zona-free hamster egg assay, and cellular cAMP levels in spermatozoa were quantified by the EIA kit. The proteins tyrosine, serine and threonine phosphorylation in the presence or absence of adjudin were analyzed by means of a immunodetection of spermatozoa, especially, compared the effect of adjudin on sperm hyperactivation and capacitation in the complete HTF medium with the Cl^--deficient HTF medium as well as the various Cl^- channel blockers.

MAIN RESULTS AND THE ROLE OF CHANCE: Adjudin significantly inhibited sperm hyperactivation but not sperm motility. Adjudin-induced inhibition of sperm capacitation was reversible, and it was found to block the rhuZP$_3$β- and progesterone-induced acrosome reaction in a dose-dependent manner. Adjudin also blocked sperm penetration of zona-free hamster eggs, and significantly inhibited both forskolin-activated transmembrane adenylyl cyclase and soluble adenylyl cyclase activities leading to a significant decline in the cellular cAMP levels in human spermatozoa. Adjudin failed to reduce sperm protein tyrosine phosphorylation but it did prevent sperm serine and threonine protein phosphorylation. Interestingly, adjudin was found to exert its inhibitory effects on sperm capacitation and capacitation-associated events *only* in the complete Cl^--HTF medium but not Cl^--deficient medium, illustrating the likely involvement of Cl^-. Adjudin inhibits the fertility capacity of human sperm is mediated by disrupting chloride ion and its transport function.

LIMITATIONS, REASONS FOR CAUTION: This study has examined the effect of adjudin only on human sperm capacitation and fertilizing ability *in vitro* and thus has some limitations. Further investigations *in vivo* are needed to confirm adjudin is a potent male contraceptive.

[†]These authors contributed equally to this work.

WIDER IMPLICATIONS OF THE FINDINGS: Our studies demonstrated that adjudin inhibition of capacitation is reversible and its toxicity is low, opening the door for the examination of adjudin as a mediator of male fertility control. Adjudin may be a safe, efficient and reversible male antifertility agent and applicable to initial clinical trials of adjudin as a male antifertility agent in humans.

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Key words: adjudin / human sperm / fertilizing capacity / chloride

Introduction

Earlier studies have shown that adjudin [1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, formerly called AF-2364], an analog of lonidamine [1-(2,4-dichlorobenzyl) 1H-indazole-3-caboxylic acid], is a potential male contraceptive (Cheng *et al.*, 2011). Adjudin exerts its effect by disrupting Sertoli-germ cell adhesion junctions, most notably apical ectoplasmic specialization (apical ES, a testis-specific anchoring junction type), by targeting testin and actin filament bundles that disrupt the actin-based cytoskeleton in Sertoli cells (Mruk and Cheng, 2011), causing the release of spermatids prematurely from the seminiferous epithelium, thereby resulting in rat infertility (Cheng *et al.*, 2001). However, the effects of adjudin on sperm fertilizing ability *in vitro*, in particular humans have yet to be evaluated.

It is known that ejaculated human spermatozoa must spend a period of time in the female genital tract or in appropriate artificial medium *in vitro* in order to gain the capacity to fertilize an egg. This phenomenon is termed capacitation (Yanagimachi, 1994; Visconti *et al.*, 1998, 2011; Florman and Ducibella, 2006). Capacitated spermatozoa acquire the ability to bind specifically to the zona pellucida (ZP) of the ovum and undergo the acrosome reaction (Liu and Baker, 1994). The acrosome reaction is an exocytosis event induced by progesterone or the ZP (Roldan *et al.*, 1994; Yanagimachi, 1994). The acrosome reaction is crucial for successful sperm binding and penetration of the ZP, and is followed by plasma membrane fusion with the egg (Florman and Storey, 1982; Wassarman, 1987). The development of *in vitro* procedures for assessing the fertilizing capacity of human spermatozoa has been greatly enhanced due to wide-spread application of IVF for therapy of infertility (Yanagimachi *et al.*, 1976; Steptoe and Edwards, 1978; Aitken *et al.*, 1983; Trounson and Webb, 1984).

It was reported that AF-2785 [1-(2,4-dichlorobenzyl)-*1H*-indazole-3-acrylic acid], another analog of lonidamine, and a potent blocker of CFTR (cystic fibrosis transmembrane conductance regulator), plays an important role in male reproduction by impeding the secretion of chloride ions from the epithelial cells in the rat epididymis (Gong *et al.*, 2000, 2002; Gong and Wong, 2000). It remains to be determined if adjudin inhibits the CFTR function leading to the reduction in sperm-fertilizing ability. CFTR acts as a cAMP-activated chloride (Cl^-) channel, which plays an important role in male reproductive function (Wong, 1998). Besides CFTR Cl^- channels in the sperm, four structural Cl^- channel families have been identified to date: (i) Cl^--selective ion channels (Estevez *et al.*, 2003); (ii) the $GABA_A$ receptor/Cl^- channels (Shi and Roldan, 1995); (iii) Ca^{2+}-activated Cl^- channels (Orta *et al.*, 2012) and (iv) $Na^+/K^+/Cl^-$ co-transporter channels (Wertheimer *et al.*, 2008). As with other Cl^- channels in the sperm, CFTR is regulated by pharmacological agents, such as forskolin and bicarbonate, which may activate separately the transmembrane adenylyl cyclases (tmACs) and soluble adenylyl cyclase (sAC) activities, leading to an increase in the intracellular cAMP levels and subsequent sperm capacitation and the acrosome reaction (Leclerc and Kopf, 1995; Scholich *et al.*, 1997; Buck *et al.*, 1999; Chen *et al.*, 2000; Baxendale and Fraser, 2003). There is evidence for tmAC activity in the head region of capacitated mouse sperm in bicarbonate free-human tube fluid (HTF) medium (Liguori *et al.*, 2004). Recent studies have shown that CFTRinh-172, a specific CFTR channel protein blocker (Muanprasat *et al.*, 2004), completely blocked sperm capacitation and male fertility (Xu *et al.*, 2007; Chen *et al.*, 2009; Li *et al.*, 2010). Since adjudin is an analog of lonidamine, the question arises whether adjudin inhibits human sperm fertilizing capacity *in vitro*, which is mediated via changes in Cl^- transport. We thus sought to assess the effects of adjudin on human sperm fertilizing ability at a non-toxic dose, and to delineate its possible mechanism of action *in vitro*.

Materials and Methods

Reagents

Adjudin was provided by Prof. C. Yan Cheng. CFTRinh-172 was purchased from Calbiochem, Inc. (La Jolla, CA, USA; catalog no. 219670). Chlortetracycline (CTC), L-cysteine, BSA (Fraction V, fatty acid free), Hoechst 33258, sodium pyruvate, sodium L-lactate, progesterone, forskolin, pentoxifylline, dibutyryl cyclic AMP (dbcAMP), paraformaldehyde, hyaluronidase, DPC (diphenylamine-2-carboxylate), DIDS (4′4,diisothiocyano-stilbene-2′,2,-disulfonic acid), gluconate, ethylene glycol tetraacetic acid, ATP, 3-isobutyl-1-methyl xanthine, bicuculline and pronase were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethyl sulfoxide was obtained from Merck (Darmstadt, Germany). Percoll™ was purchased from GE Healthcare BioSciences AB (Uppsala, Sweden). Anti-phosphotyrosine (PY) monoclonal antibody, rabbit anti-phosphoserine (PS) antibody and rabbit anti-phosphothreonine (PT) antibody were ordered from Invitrogen (Camarillo, USA). β-tubulin antibody (ab6046) was obtained from Abcam, Inc. (MA, USA). Recombinant human ZP3β (rhuZP3β) was a gift from Prof. Xu, Wan Xiang, Shanghai Institute of Planned Parenthood Research. Chemical reagents of analytical grade were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). The cAMP enzyme immunoassay (EIA) kit was purchased

from GE Healthcare (Little Chalfont, Buckinghamshire, UK, Catalog no. RPN 225).

Media

The media used throughout this study were HTF medium and BWW (Biggers–Whitten–Whittingham) medium. HTF (100 ml) medium consisted of 90.0 mM NaCl, 4.96 mM KCl, 1.80 mM $CaCl_2$ $2H_2O$, 6.98 mM $MgCl_2$, 25.05 mM $NaHCO_3$, 10.0 mM glucose, 10.0 mM sodium lactate, 0.27 mM sodium pyruvate, 1.17 mM KH_2PO_4, 2.0 mM HEPES, 400 mg BSA (fraction V, fatty acid-free) and 6 mg penicillin G (hereafter referred to as complete medium). For the HEPES-free HTF medium, an additional 58.5 mg NaCl was used per 100 ml medium in order to obtain a final osmolality of 300–305 mOsm/kg and pH 7.5 at room temperature (25°C). The complete Cl^--HTF medium contained 110.2 ± 5.5 mM Cl^-; it was quantified by using a chloride-selective electrode (Roche Co., USA). All the Cl^-, including NaCl, KCl, $MgCl_2$ and $CaCl_2$, in the HTF medium was replaced with gluconic salt to yield Cl^--deficient HTF medium (Cl^--DF HTF). Cl^--DF HTF medium contained 26.0 ± 0.4 μM Cl^- as measured previously described by Chen *et al*. (2009). Preparation of the BWW medium was according to Biggers *et al*. (1971).

Collection and preparation of spermatozoa

Semen samples were obtained from 45 fertile men (aged 25–32), who were examined according to the World Health Organization (1999) standard andrology criteria, with the approval of the Human Medical Ethics Committee of the Zhejiang Academy of Medical Sciences. Only samples with motility ≥85%, viability ≥90% and sperm concentration $\geq 40 \times 10^6$ cells/ml were used. The sperm were isolated from the semen in the HTF medium by centrifugation at 600*g* for 15 min through a discontinuous Percoll gradient (45–90%) containing 4 mg/ml BSA. The sperm were washed and preincubated in the HTF medium with 2.5 μM progesterone and various concentrations of adjudin (10 nM–10 μM) except the negative control. Sperm were incubated for 5 h in a humidified CO_2 incubator at 37°C under 5% CO_2 /95% air (V/V). Sperm concentration was adjusted to $20–30 \times 10^6$ cells/ml. Sperm viability at this stage was 90–95%, as estimated using a trypan blue exclusion test.

Analysis of sperm motility and hyperactivation by CASA

Sperm motility and hyperactivation were analyzed by a CASA system, namely the Hamilton Throrne Research Motility Analyzer (HTM-IVOS with software Version 12.3N, Beverly, MA, USA), as previously described by Mortimer *et al*. (1998); Mortimer (2000) and Li *et al*. (2010). After incubation for 1 , 3 and 5 , a 5 μl aliquot of sperm suspension of each sample was pipetted into a microcell slide chamber with a depth of 20 μm, prewarmed to 37°C (Leja Products B.V., GN Nieuw Vennep, The Netherlands). The slide was then placed onto a warm stage (37°C) attached to an Olympus BH-2 compound microscope (Olympus Optical Co. Ltd, Tokyo, Japan) and analyzed under pseudo-dark-field illumination. For each sample, 10 randomly selected fields containing >200 moving sperm cell tracks were examined at 60 Hz. Curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), linearity % (LIN, VSL/VCL multiplied by 100) and the percentage of motility were recorded. Spermatozoa were designated as hyperactive motility if they had a VCL ≥150 μM/s, ALH ≥7.0 μM and LIN ≤50%.

Evaluation of capacitation and the acrosome reaction monitored by CTC fluorescent staining

Sperm capacitation and the acrosome reaction in human spermatozoa were assessed by CTC fluorescence staining as previously described by DasGupta *et al*. (1993), Fraser *et al*. (1995), Shi *et al*. (1997) and Li *et al*. (2010). This conventional method for assessing sperm capacitation and the acrosome reaction is based on the alteration in staining patterns of the sperm head during capacitation process. To examine the effect of adjudin on human sperm capacitation, washed sperm were pre-incubated for 5 h with or without various concentrations of adjudin (10 nM–10 μM) and 2.5 μM progesterone, except the negative control, under capacitating conditions. Sperm suspensions were washed with the fresh HTF medium and then stimulated with 15 μM P_4, except the negative control, for 15 min before the evaluation of sperm acrosomal status. The degree of spermatozoa capacitation was evaluated by the incidence of including 'B' pattern or 'B' plus 'AR' patterns by the spontaneous or the inducers with progesterone and/or pentoxifylline. According to DasGupta *et al*. (1993), Rathi *et al*. (2001), Shi *et al*. (1997) and Li *et al*. (2010), the sum of 'B' pattern or 'AR' patterns or the sum of 'B'+'AR' patterns is taken as an indicator of sperm capacitation *in vitro*. To assess the effect of adjudin on human acrosome reaction, sperm were preincubated for 5 h in the adjudin-free HTF medium under capacitating conditions and then washed with the fresh HTF medium. The washed spermatozoa were exposed to adjudin at 10 nM–10 μM for 15 min and then stimulated for 15 min with 20 μg/ml rhuZP3β or 15 μM progesterone, except the negative control, before the assessment of the acrosome reaction (Ni *et al*., 2007; Li *et al*., 2010). Samples were then immediately fixed by adding an equivalent amount of 8% glutaraldehyde and mixed gently for 5 min at 37°C. Sperm were stained with CTC for 10 min at 37°C and then stained with a vital dye, Hoechst 33258 (final concentration at 1 μg/ml), for 2 min in the dark at 37°C to discriminate between the living (negative staining) and dead cells (positive staining). Only the samples with Hoechst 33258-negative staining, i.e. living spermatozoa, were counted to assess capacitation and the acrosome reaction by acrosomal staining status. Samples were immediately examined at ×1000 using a fluorescence and phase-contrast microscope. An excitation beam that passed through a 380 nm and DM 420 nm fluorescence emission (Nikon 80i, Tokyo, Japan) was used in each sample. A total of 200 cells were assessed by two individuals.

Evaluation of human sperm penetration of zona-free hamster egg assay (SPA)

Hamster oocytes were obtained by superovulation as previously described (Shi *et al*., 1991; Li et al., 2010). Sperm fertilizing ability was evaluated by SPA as earlier described (Yanagimachi *et al*., 1976; Aitken *et al*., 1983; Shi *et al*., 1989, 1991). To determine whether adjudin prevents the fusion of sperm with zona-free hamster egg, washed spermatozoa were preincubated with or without adjudin 10 μM in the HEPES-free HTF medium for 5 h under capacitating conditions. Zona-free hamster eggs in the HEPES-free HTF medium were introduced separately into sperm suspensions (50:50 μl, eggs: spermatozoa ≥1.5–2.0:1000) and co-incubated for 3 h. After co-incubation, eggs were rinsed three times with HEPES-free HTF medium, transferred to a slide and compressed carefully under a coverglass supported at its corners with drops of paraffin wax–vaseline mixture (1:9, V/V). To rule out a possible direct role of adjudin on zona-free hamster egg, these eggs were preincubated for 0.5 h with or without 10 μM adjudin under capacitating conditions. Thereafter the eggs were washed two times to remove adjudin and then co-incubated for 3 h with capacitated spermatozoa that had been preincubated in the adjudin-

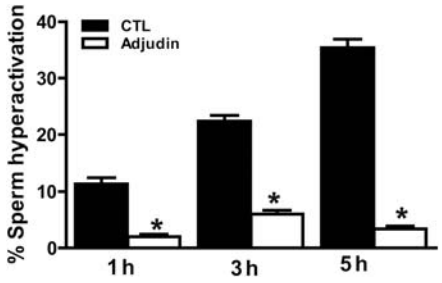

Figure 1 Adjudin inhibits human spermatozoa hyperactivated motility. Sperm were incubated separately in the HTF medium for 1, 3 and 5 h with or without adjudin under capacitating conditions. Sperm motility and hyperactivation were assessed by using CASA. The results are expressed as the mean ± SEM ($n = 8$). *$P < 0.001$, when compared with control (adjudin-free). CTL, control; CASA, computer assisted sperm analysis.

free HTF medium for 5 h under capacitating conditions. The eggs were examined by phase-contrast microscopy (×400) for the presence of a decondensed sperm head with its tail within the ooplasm as evidence of fertilization. The penetration rate of eggs by spermatozoa is taken as the sperm fertilization capacity (Shi *et al*., 1989; Li *et al*., 2010).

Extraction of spermatozoa and measurement of cAMP levels

Cellular cAMP levels in spermatozoa were measured by the EIA kit as previously described (Xu *et al*., 2007; Chen *et al*., 2009). Briefly, spermatozoa were separately incubated in the HTF medium for 0 and 5 h with or without 10 μM forskolin in the presence or absence of 10 μM adjudin in the complete HTF medium under capacitating conditions. At the end of the incubation, sperm were centrifuged for 20 min (150*g*) at room temperature. Sperm suspensions (50 μl containing ~2×10^6 cells) were separately added to an equal volume of the same medium. Incubations were terminated by the addition of five volumes of ice-cold 100 mM HCl in 100% ethanol. Samples were kept on ice for 30 min and then were lyophilized and assayed for cAMP levels.

Immunodetection of protein tyrosine, serine and threonine phosphorylation

Spermatozoa were incubated separately at 5 min and 5 h under capacitating conditions, washed twice with PBS and protein extracted in SDS lysis buffer. The proteins were separated by SDS-PAGE (10% acrylamide gel) and subsequently transferred to Immobilon-P (Millipore Corporation, Bedford, MA, USA) membrane. The membrane was blocked with 3% BSA. Immunodetection of phosphorylated proteins were carried out using PY, PS or PT antibodies as described previously (Visconti *et al*., 1995a,b; Naz, 1999; Chen *et al*., 2009). Proteins were detected using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, USA) according to the manufacturer's instructions. After the phosphorylated proteins were detected, the same membrane was stripped. β-tubulin antibody (1 μg/ml) was used to re-immunoblot. The molecular weights of the detected proteins were deduced in comparison with a prestained protein ladder (Fermentas, Thermo Fisher Scientific, Inc., Burlington, USA). The gray intensity was quantified by Image J software (http://imagej.nih.gov/ij).

Effect of adjudin on capacitation elicited by forskolin, pentoxifylline or progesterone

Forskolin is an activator of transmembrane adenylate cyclases (tmACs) and directly stimulates the enzyme activity, and in turn increases intracellular cAMP levels leading to sperm capacitation (Leclerc and Kopf, 1995; Scholich *et al*., 1997; Fraser *et al*., 2003; Insel and Ostrom, 2003). Pentoxifylline is an inhibitor of phosphodiesterase, which suppresses cAMP degradation thereby increasing cAMP production that leads to sperm capacitation (Dasgupta *et al*., 1993). To examine whether adjudin inhibits forskolin- and pentoxifylline- or progesterone-elicited sperm capacitation, washed sperm were preincubated with or without 10 μM forskolin and 3.6 μM pentoxifylline or 2.5 μM P_4 and/or 10 μM adjudin, 1 μM CFTRinh-172 or 10 μM bicuculline (an antagonist of $GABA_A$ receptor/Cl^- channel) for 5 h under capacitating conditions and sperm capacitation was evaluated using CTC fluorescent staining.

The role of adjudin or different Cl^- channel blockers on sperm capacitation

One of the upstream targets of cAMP is the CFTR Cl^- which acts as a cAMP-activated Cl^- channel and is present both in mature mouse and human sperm (Hernandez-Gonzalez *et al*., 2007; Xu *et al*., 2007; Li *et al*., 2010). Thus, the question arises as to whether adjudin inhibition of human sperm capacitation disrupts the major conductance of CFTR Cl^-, which may also play a role leading to the reduction in sperm capacitation. Therefore, (i) to investigate whether the Cl^- ion and its channels are involved in adjudin inhibition of sperm capacitation, washed sperm were preincubated for 5 h in the complete Cl^--HTF or Cl^--DF HTF medium in the presence of 10 μM adjudin and/or 2.5 μM progesterone, except the negative control, under capacitating conditions and the percentage of sperm capacitation was evaluated as mentioned above. (ii) To further explore whether the requirement for Cl^- is mediated by cAMP when sperm capacitation was inhibited by adjudin, sperm were incubated for 1–5 h under capacitating conditions in the HTF medium with or without Cl^- and in the presence or absence of cAMP agonist, dbcAMP (1 mM) or/and adjudin (10 μM) and then evaluated for sperm capacitation as described above. (iii) To further determine whether adjudin and various structural Cl^- channels blockers exert their effects on sperm capacitation with different sensitivities, sperm were incubated separately for 5 h with or without the various transport blockers of Cl^- channels and adjudin in the complete HTF medium. Among them, CFTR-172 (1 μM), adjudin (10 μM), DPC (10 μM), DIDS (10 μM) and bicuculline (10 μM) were tested to understand if sperm capacitation is affected differently by various Cl^- transport blockers.

Statistical analysis

Results are expressed as the means ± SEM. For statistical analysis, two-tail Student's *t*-tests were used. For three or more groups, data were analyzed by one-way ANOVA and Dunnett's post-hoc test. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Effects of adjudin on human sperm motility and hyperactivation

CASA analysis revealed that the percentages of sperm hyperactivation were increased significantly after incubation of 3 and 5 h versus 1 h ($P < 0.01$). ALH, and VCL were, in particular, greatly increased ($P < 0.05$), whereas LIN significantly decreased ($P < 0.01$), indicating

Table I Effect of adjudin on human sperm motility and hyperactivated motion parameters in the HTF medium.

Treatment	Motility (%)	VCL (μm/s)	VSL (μm/s)	ALH (μm)	LIN (%)	VAP (μm/s)
Control	75.0 ± 7.8	154.9 ± 14.1	50.3 ± 14.3	7.8 ± 0.2	32.5 ± 4.5	75.1 ± 11.1
Adjudin (10 μM)	72.0 ± 10.5	116.9 ± 14.2*	52.8 ± 12.6	4.8 ± 1.2*	52.4 ± 4.4*	60.0 ± 10.4*

Sperm were incubated in the HTF medium for 5 h with or without 10 μM adjudin under capacitating conditions and then washed with the fresh HTF. Sperm hyperactivated motility motion and motility were analyzed by CASA. The results are expressed as the mean ± SEM of eight different experiments ($n = 8$). CASA, computer-assisted sperm analyzer; VCL, curvilinear velocity; VSL, straight line velocity; ALH, amplitude of lateral head displacement; VAP, average path velocity; LIN, linearity (VSL/VCL by multiple 100).
*When compared with control, $P < 0.01$.

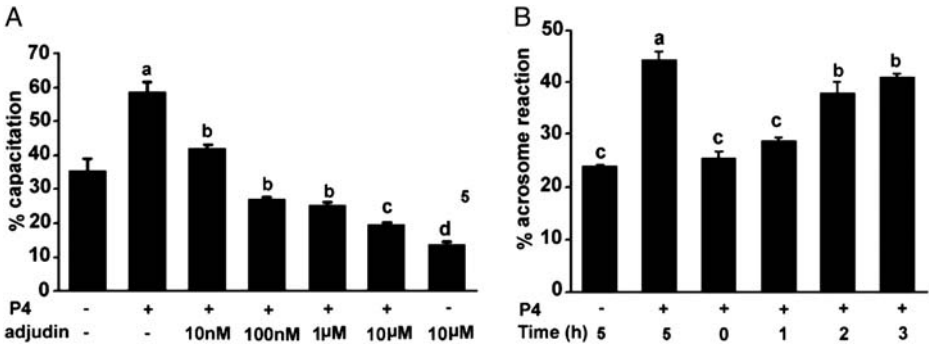

Figure 2 (**A**). Inhibition of progesterone-elicited capacitation in human sperm by adjudin. Sperm were incubated with adjudin alone and/or in combination with progesterone (P4) 2.5 μM and different concentrations of adjudin (10 nM–10 μM) in the HTF medium for 5 h at 37°C. The percentages of sperm capacitation ('B'+'AR' patterns) are expressed as the mean ± SEM ($n = 3$). a, compared with control (progesterone- and adjudin-free), $P < 0.001$; b versus a, c versus b and d versus c, $P < 0.05$ to < 0.001. (**B**) Adjudin inhibition of capacitation in human sperm is reversible. Sperm were preincubated for 5 h in HTF with 10 μM adjudin under capacitating conditions, and then spermatozoa were washed twice with the fresh HTF medium to remove adjudin and re-incubated thereafter in adjudin-free HTF for 1–3 h. The results ('AR' pattern alone) are expressed as the mean ± SEM ($n = 3$). Identical letters indicate that there is no significant difference. b when compared with a (P_4 alone), $P > 0.05$. a versus c, $P < 0.01$; b versus c, $P < 0.01$.

that spermatozoa express strongly hyperactivated motility. In contrast, when sperm were incubated in the HTF medium with 10 μM adjudin for 3 and 5 h under the same conditions, the percentages of sperm hyperactivation were decreased markedly compared with the adjudin-free samples, $P < 0.01$ (Fig. 1), but it did not affect sperm motility (Table I), indicating that adjudin has no cytotoxicity at concentrations up to 10 μM, consistent with a recent report in rat (Su *et al*., 2011).

Inhibition of progesterone-induced sperm capacitation by adjudin

The three sequential and distinct patterns of CTC fluorescence staining on human spermatozoa were observed (shown in the Supplementary data, Fig. S1) as earlier reported (Li *et al*., 2010). Sperm capacitation was increased significantly in the HTF medium with 2.5 μM progesterone for incubation up to 5 h, which promoted the transition from F pattern to B pattern ('B' pattern from 2.3 ± 1.2% at 0 h up to 15.6 ± 2.5% at 5 h), followed by an increase in 'AR' patterns. The sum of 'B' + 'AR' patterns were increased from 21.3 ± 3% up to 35.0 ± 6% (Fig. 2A). These results showed that progesterone facilitated the 'F' pattern to 'B' pattern transition, followed by an increase in the 'AR' patterns, reaching a maximum value (the sum of 'B + AR' patterns: 58.5%) when compared with control ($P < 0.001$) after 5 h of incubation. These results demonstrated that progesterone significantly facilitated human sperm capacitation. However, this stimulatory effect was abolished by adjudin in a dose-dependent manner with concentrations of 10 nM–10 μM versus control ($P < 0.01$–$P < 0.001$), showing that adjudin significantly inhibits spontaneous- (Fig. 2a) and progesterone-induced sperm capacitation.

Inhibition of human sperm capacitation by adjudin is reversible

Capacitation appears to be a reversible event (Shi and Friend, 1983; Yanagimachi,1994; Ni *et al*., 2009). Thus, a question arises as to whether the adjudin-induced inhibition of capacitation is reversible. To address the question, sperm were preincubated with 10 μM adjudin for 5 h in the HTF medium under capacitating conditions and washed twice with the fresh HTF medium to remove adjudin. Sperm were re-incubated for 1 to 3 h in the adjudin-free HTF medium under the same conditions and the cells were then challenged with 15 μM progesterone for 15 min prior to assessing the sperm acrosomal status. The percentages of 'AR' patterns progressively

increased with the extension of incubation time (from 1 to 3 h) and were comparable with those of progesterone alone (positive control, $P > 0.05$) after an incubation period of 3 h. These results illustrated that capacitation was recovered in these spermatozoa, because only capacitated spermatozoa are able to undergo the acrosome reaction, indicating that the adjudin-inhibited capacitation is reversible (Fig. 2B) and it is unlikely that its capacitation-blocking effect is the result of cytotoxicity.

Adjudin inhibits sperm acrosome reaction induced by rhuZP3β or progesterone

To explore whether adjudin has an effect on capacitated spermatozoa, we examined the effect of adjudin on sperm acrosome reaction induced by rhuZP3β or progesterone. The results illustrated that sperm acrosome reactions induced by rhuZP$_3$β (35.5 ± 3.2%) (Fig. 3A) and by progesterone (35.2 ± 3.8%) (Fig. 3B) were increased markedly in the adjudin-free HTF medium versus negative control ($P < 0.01$). However, this stimulatory effect on the acrosome reaction induced by rhuZP$_3$β or progesterone was blocked completely by adjudin in a dose-dependent manner at 10 nM–10 μM, versus positive control (adjudin-free, $P < 0.05$–$P < 0.001$). The acrosome reactions induced by rhZP3β (12.1 ± 1.8%) and by progesterone (12.8 ± 2.2%) were inhibited completely by adjudin at 10 μM versus positive control (rhZP$_3$ β- or progesterone-induced acrosome reaction) ($P < 0.001$).

Inhibition of sperm–egg fusion in zona-free hamster egg by adjudin

The percentage of human spermatozoa penetration of zona-free hamster eggs in the presence of 10 μM adjudin was reduced significantly (27.9%, versus the control 70.8%, $P < 0.001$; Table II). Despite their repeated contact, only a few spermatozoa in the presence of adjudin attached to the ova surface when compared with control (shown in Supplementary data, Fig. S2). To evaluate if adjudin directly affects the fertilizing ability of zona-free hamster eggs, we pre-incubated the eggs for 0.5 h in the BWW medium containing 10 μM adjudin, which is the time consistent with the duration of fertilization *in vivo*. Thereafter eggs were washed twice with the fresh BWW to remove adjudin. Similarly, when spermatozoa were preincubated for 5 h in the HTF medium without adjudin, and then mixed with the ova and further co-incubated for 3 h under the same conditions, the fertilizing capacity of the zona-free hamster eggs was not affected significantly which was similar to control ($P > 0.05$, data not shown), indicating that adjudin did not directly affect the fertilizing ability of zona-free hamster eggs.

Inhibition of intracellular cAMP levels in spermatozoa by adjudin

To further examine whether adjudin inhibition of capacitation is mediated by intervention upstream of the cAMP signaling pathway, sperm were incubated for 5 h with or without 10 μM adjudin in the complete Cl^--HTF medium under capacitating conditions. The results showed that the mean levels of intracellular cAMP $[cAMP]_i$ in spermatozoa incubated in the HTF medium for 5 h were increased significantly. Forskolin prominently elevated the $[cAMP]_i$ levels (increased by 30%) in spermatozoa versus control at 5 h ($P < 0.01$). However, this stimulating effect on $[cAMP]_i$ levels was abolished completely (decreased by 60%) when sperm were incubated in the presence of 10 μM adjudin for the same period of time (Fig. 4) versus forskolin alone ($P < 0.001$). These results suggest that adjudin inhibits the forskolin-induced the increase in $[cAMP]_i$ levels that is through the activation of tmACs activity. However, the possibility that HCO_3^- in the medium may directly activate the sperm sAC activity leading to the increase in $[cAMP]_i$ levels should not be ruled out. To examine if adjudin inhibits sAC activity, the $[cAMP]_i$ levels of spermatozoa in the presence of HCO_3^- were measured. The results showed that adjudin also significantly inhibited $[cAMP]_i$ levels activated by HCO_3^- (Fig. 4,

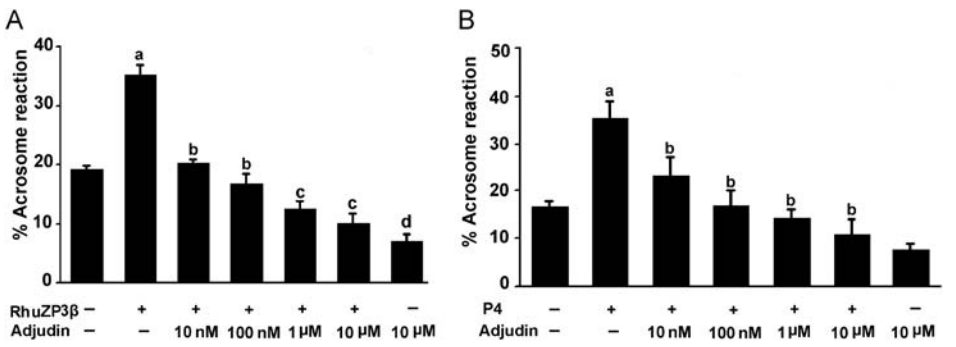

Figure 3 (**A**) Inhibition of rhuZP3β-triggered the acrosome reaction in human sperm by adjudin. Sperm were capacitated in the HTF medium for 5 h, washed, resuspended in the fresh HTF medium and exposed to different concentrations of adjudin (10 nM–10 μM) for 15 min and then the acrosome reactions were triggered with rhuZP3β (20 μg/ml) for 15 min. Results are expressed as the mean ± SEM ($n = 3$). a when compared with control, $P < 0.001$; b compared with a (rhuZP3β alone), $P < 0.01$ to < 0.001. c versus b, $P < 0.05$; d versus c, $P < 0.05$. (**B**) Inhibition of progesterone-induced acrosome reaction in human sperm by adjudin. Sperm were incubated in the HTF medium under capacitating conditions for 5 h to induce capacitation, washed, resuspended in the HTF containing different concentrations of adjudin for 15 min and then elicited with 15 μM P_4 for 15 min before evaluation of the acrosome reaction. Results are expressed as the mean ± SEM ($n = 4$). a when compared with control, $P < 0.001$; b when compared with progesterone, $P < 0.01$ to < 0.001.

Table II Inhibition of human sperm penetration of zona-free hamster eggs by adjudin.

Treatment	Number of eggs examined	Number of eggs penetrated	Penetration index	Percentage of penetration
Control	65	46	2.8	70.8
Adjudin (10μM)	86	24	1.2	27.9*
Progesterone (P4, 15 μM)	44	36	3.0	81.0

Sperm were preincubated with or without adjudin 10 μM in HTF for 5 h, washed, added separately to BWW medium containing 15–20 zone-free hamster oocytes and then co-incubated for 3 h at 37°C under 5% CO_2 in air. Results are expressed as mean ± SEM. These data were represented in five different experiments ($n = 5$); adjudin significantly different from controls used. Paired samples test (*$P < 0.001$).

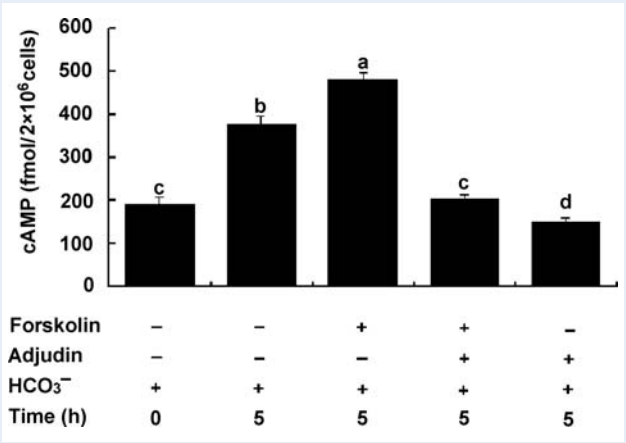

Figure 4 Inhibition of forskolin- and HCO_3^- stimulated increase in the intracellular cAMP levels of human spermatozoa by adjudin. Sperm were separately incubated for 0 and 5 h in the HTF medium (containing 25 mM HCO_3^-) with or without 3.6 μM forskolin and/or 10 μM adjudin. The results are expressed as the means ± SEM (fmol/2 × 10^6 cells; $n = 5$). The forskolin treatment (**a**) when compared with controls (**b**), (**c**) and (**d**) (HCO_3^- plus adjudin) after incubation of 0 h and 5 h. Adjudin significantly inhibits the increase in $[cAMP]_i$ level activated by both forskolin and HCO_3^- (a versus c, $P < 0.001$; a versus b, $P < 0.05$; b versus c, $P < 0.01$) or only HCO_3^- (d versus b, $P < 0.01$).

d versus b, $P < 0.001$). Thus, adjudin inhibited $[cAMP]_i$ levels, which was through both tmAC and sAC signaling pathways.

Effect of adjudin on protein tyrosine, serine and threonine phosphorylation

To further investigate whether adjudin affects protein tyrosine, serine and threonine phosphorylation during human sperm capacitation, protein tyrosine, serine and threonine phosphorylation and their signal intensities were analyzed quantitatively by western blotting. The results illustrated that protein tyrosine was phosphorylated increasingly during capacitation at multiple molecular regions, including 55, 72, 100, 120, 190 and 210 kDa. However, adjudin at 10 and 100 μM was not capable of inhibiting this increase in capacitation-dependent protein tyrosine phosphorylation, which was similar to that of control as shown in Fig. 5A and D ($P > 0.05$). However, adjudin at 10 and 100 μM was capable of inhibiting the increase in serine phosphorylation at 70 kDa protein (Fig. 5B and E, $P < 0.05$), and in threonine phosphorylation of 60, 72, 95, 110 and 130 kDa proteins (in Fig. 5C and F), compared with the control, $P < 0.05$. The results imply adjudin inhibits capacitation via the signal pathway of serine and threonine phosphorylation, but not tyrosine phosphorylation.

Forskolin and pentoxifylline-induced capacitation is inhibited by adjudin

Forskolin and pentoxifylline or progesterone alone were found to stimulate significantly sperm capacitation ('B' + 'AR' patterns) versus control ($P < 0.001$). However, this stimulatory effect was abolished entirely by adjudin (Fig. 6A) or by CFTRinh-172 (Fig. 6B) versus control ($P < 0.001$). Bicuculline was found to prohibit progesterone-induced capacitation, but it did not inhibit forskolin and pentoxifylline-induced capacitation (Fig. 6C). These findings suggest that adjudin inhibition of forskolin and pentoxifylline-induced capacitation may be mediated by disrupting the cAMP-activated Cl^- channel (i.e. CFTR Cl^- Channel), while its inhibition of progesterone-induced capacitation may be via the $GABA_A$ receptor/Cl^- channel, respectively.

Inhibition of capacitation-associated Cl^- channels transport by adjudin

CFTR acts as a upstream regulator of the intracellular cAMP production in sperm, which plays a role in modulating the exchanger for the Cl^-/HCO_3^- (Chen *et al.*, 2009) and Na^+/HCO_3^- co-transporter (Demarco *et al.*, 2003). The results showed that adjudin significantly blocked sperm capacitation in the complete Cl^--HTF medium versus control ($P < 0.001$), but it did not exert its inhibiting effect to sperm capacitation in the Cl^--DF HTF medium versus control in the Cl^--DF HTF medium ($P > 0.05$) (Fig. 7A), suggesting that adjudin inhibits sperm capacitation via Cl^- and its channels. Addition of 1 mM dbcAMP to a sperm suspension in Cl^--DF HTF medium completely overcame adjudin-induced inhibition of sperm hyperactivation (Fig. 7B) and capacitation (Fig. 7C) in the absence of Cl^-. These results suggest that adjudin inhibits sperm hyperactivation and capacitation through the prevention of Cl^- and its channels, which exert their effects upstream of the cAMP signaling pathway.

To further determine whether adjudin and various structural Cl^- channel blockers exert their effects on sperm capacitation with different sensitivities, sperm were incubated with various structural blockers of Cl^- channels versus adjudin in the complete HTF medium. The

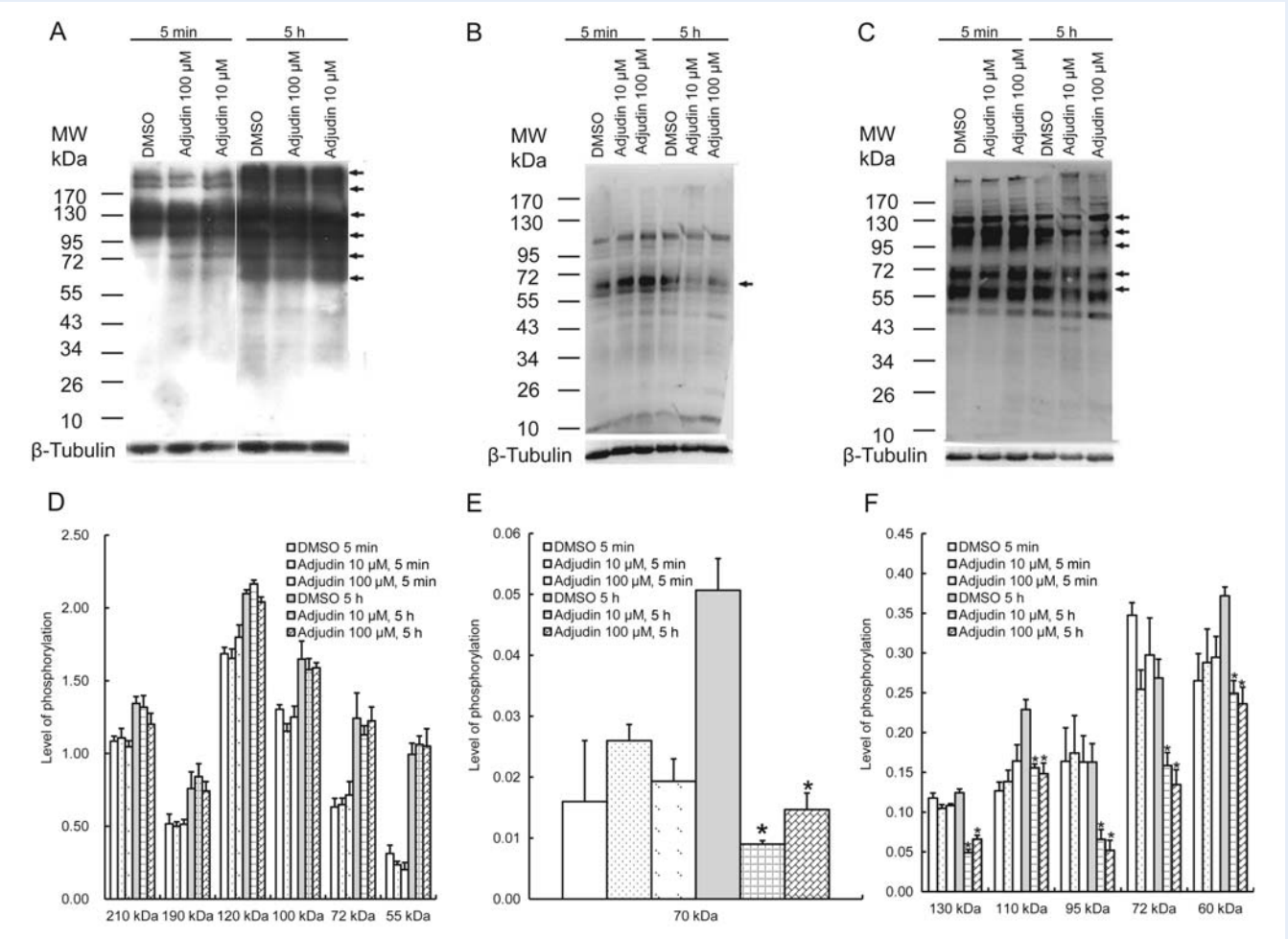

Figure 5 Effect of adjudin on human sperm protein phosphorylation during capacitation. The western blotting and the quantitative analysis of signal intensity of protein phosphorylation were performed as described in the section Materials and methods. (**A**) tyrosine, (**B**) serine and (**C**) threonine phosphorylation. Each experiment was repeated at least three times. The bands indicated by arrows were further analyzed by densitometry. Their phosphorylated levels (**D**–**F**) were expressed by the ratio of the gray intensity of bands compared with loading controls, β-tubulin (mean ± SEM, $n = 3$). The results indicated that 10 and 100 μM adjudin were not capable of inhibiting the increase in protein tyrosine phosphorylation compared with the control, $P > 0.05$, but did inhibit serine phosphorylation at 70 kDa protein, and threonine phosphorylation at 60, 72, 95, 110 and 130 kDa proteins, when compared with the control, $^*P < 0.05$.

results showed that these Cl^- transport blockers significantly prevented sperm capacitation, suggesting different Cl^- transport channels are present in the sperm. Among them, CFTRinh-172 seems to be the most potent, followed by adjudin. DPC (CFTR antagonist) and DIDS (Cl^- selective transport inhibitor) displayed lower sensitivity to inhibition of sperm capacitation: the sensitivity of the former was higher than that of the later. In addition, bicuculline markedly inhibited capacitation caused by progesterone, but it did not prevent capacitation elicited by forskolin/pentoxifylline, suggesting that three structural Cl^- channel families present in sperm cells are mediated via different Cl^- transporters (Fig. 8). The order of sperm capacitation with sensitivity to different Cl^- channel transport inhibitors was CFTRinh-172 > adjudin > DPC > DIDS > bicuculline. The results suggest that adjudin inhibition of sperm hyperactivation and capacitation may be mediated by disrupting different Cl^- channels *per se* and/or Cl^- transport across the channels.

Discussion

Herein, we demonstrate for the first time that adjudin significantly inhibits human sperm hyperactivation, capacitation, the acrosome reaction, intracellular cAMP levels and protein serine/threonine residues phosphorylation; the net result of these effects blocks the sperm–egg fusion. The inhibitory effects of adjudin on sperm capacitation and the acrosome reaction were found to be dose dependent. More importantly, adjudin significantly inhibited sperm hyperactivation and fertilizing capacity, however, it did not affect sperm motility at a concentration of 10 μM (Table I). Since sperm cells are sensitive to various toxic compounds (Jin *et al*., 2008), sperm motility is a reliable indicator for evaluating toxic compounds. It is more important that adjudin did not affect spermatozoa motility at a concentration of 10 μM (Table I). The result of this study is in agreement with Mruk *et al*. (2006) who reported that the toxicity of adjudin is low in rats.

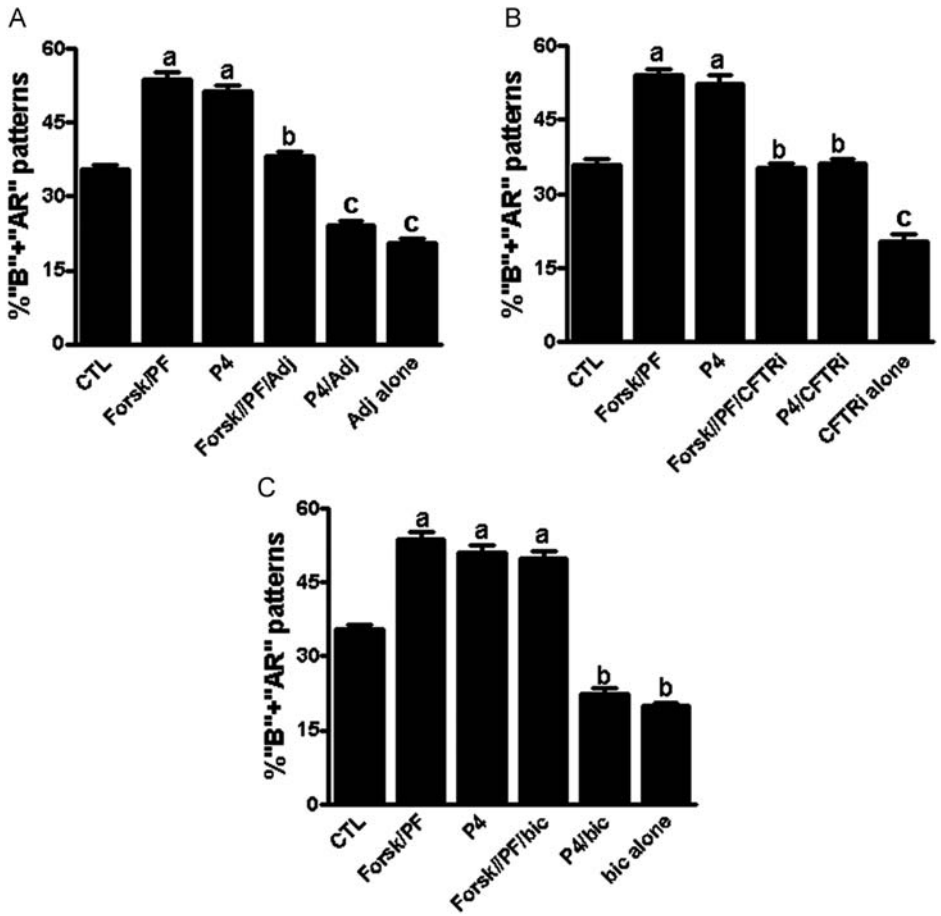

Figure 6 Inhibition by adjudin (**A**), CFTRinh-172 (**B**) and bicuculline (**C**) on a combination of forskolin- and pentoxifylline- or progesterone-induced capacitation in human sperm. Sperm were preincubated with or without a combination of forskolin (10 μM) and pentoxifylline (3.6 μM) or progesterone (15 μM) alone for 5 h in the presence of or in the absence of 10 μM adjudin (A), 1 μM CFTRinh-172 (B) or 10 μM bicuculline (C) in the HTF medium under capacitating conditions, samples were washed and then incubated for 30 min before evaluation of sperm capacitation ('B' + 'AR' patterns). The results are expressed as the mean ± SEM of five different experiments ($n = 5$). For each group, letters above bars indicate significant differences. a, when compared with CTL, $P < 0.001$; b versus a, $P < 0.01$; c versus b, $P < 0.01$; a versus c, $P < 0.001$. CTL, control; CFTR, cystic fibrosis transmembrane conductance regulator; P4, progesterone.

Furthermore, the cytotoxicity of adjudin is far lower than gossypol, which is an extensively studied potential male contraceptive (Shi *et al.*, 1987). Earlier studies have shown that sperm capacitation is also associated with sperm hyperactivated motility, with both events occurring simultaneously and dependent on exogenous calcium influx (Yanagimachi, 1994; Suarez and Pacey, 2006). Inhibition of sperm hyperactivation expression suggests that adjudin is also linked to capacitation. However, capacitation is a reversible event, but the acrosome reaction is not (Shi and Friend, 1983; Yanagimachi, 1994), and these two events are sequential but separate processes (Florman *et al.*, 1992; Yanagimachi, 1994). We have shown herein that the inhibition of adjudin on capacitation is reversible. It seems to be similar to what Grima et al reported , who proposed adjudin inhibition of spermatogenesis in rat was reversible (Grima et al., 2001). It is noted that only capacitated spermatozoa are capable of undergoing an acrosome reaction in response to progesterone and ZP (Roldan *et al.*, 1994), achieving sperm–egg fusion to complete fertilization. Our observations illustrated that adjudin not only significantly inhibited the acrosome reaction induced by progesterone, but also markedly prevented the acrosome reaction triggered by rhuZP3β, thereby blocking the sperm–oocyte fusion. In short, adjudin is capable of blocking sperm hyperactivated motility, capacitation, the acrosome reaction, $[cAMP]_i$ increase, protein serine and threonine phosphorylation and sperm–ovum fusion.

It is known that multiple steps in the fertilization process are controlled by sperm capacitation (Yanagimachi, 1994; Fraser *et al.*, 2003; Florman *et al.*, 2007; Visconti *et al*, 2011). Meanwhile, sperm capacitation is known to associate with ion channels and a cAMP/PKA-dependent phosphorylation increase (Wertheimer *et al.*, 2008; Visconti *et al.*, 2011). However, the mechanism by which adjudin prevents sperm capacitation is unknown. Previous studies have demonstrated that AF-2785, an analog of lonidamine, is a potent blocker of CFTR (Gong and Wong, 2000; Gong *et al.*, 2002). Since adjudin shares similar structural features with

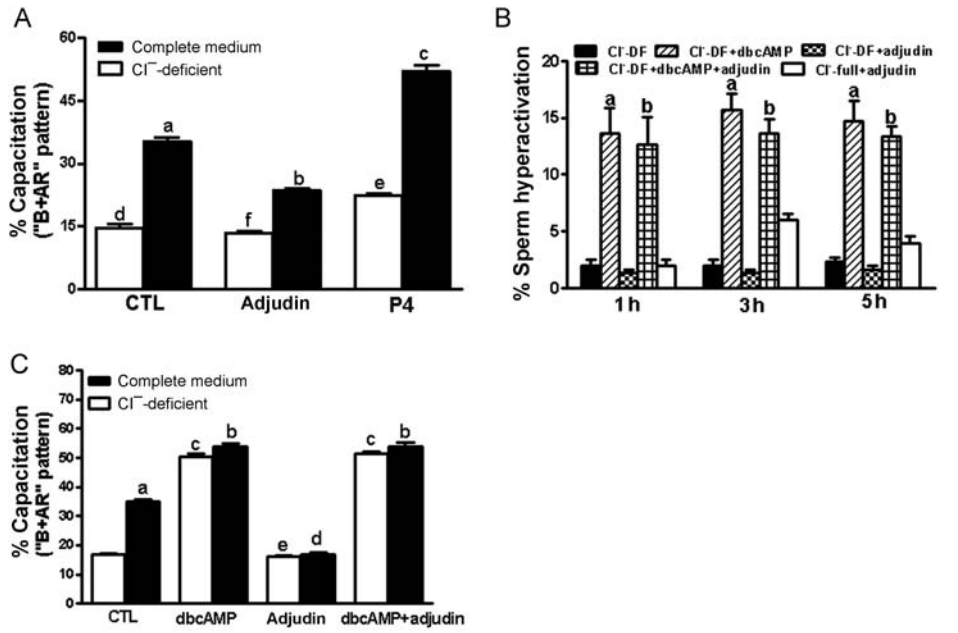

Figure 7 (**A**) Effect of adjudin on human sperm capacitation in the complete Cl^--HTF medium or the Cl^--DF HTF medium. Sperm were separately incubated for 5 h in the complete Cl^--HTF or Cl^--DF HTF medium with or without 10 μM adjudin under capacitating conditions. The results are expressed as the mean ± SEM ($n = 5$). a compared with Cl^--DF HTF medium (d), $P < 0.001$; b versus f, $P < 0.01$; c versus e, $P < 0.001$. e versus d, $P > 0.05$. (**B**) dbcAMP rescues the inhibition of human sperm hyperactivation induced by adjudin. Sperm were separately incubated for 1–5 h in the complete Cl^--HTF- and/or Cl^--DF HTF medium without or with adjudin (10 μM) in the presence of or the absence of 1 mM dbcAMP. Sperm hyperactivation was evaluated by CASA. The results are expressed as the mean ± SEM ($n = 5$). a versus b, $P > 0.05$; a/b when compared with Cl^--DF HTF or Cl^--full HTF alone or plus adjudin, $P < 0.001$. (**C**) dbcAMP rescues the inhibition of human sperm capacitation induced by adjudin. Sperm were separately incubated for 5 h in the complete Cl^--HTF medium with or without 10 μM adjudin or in the presence or absence of 1 mM dbcAMP. Sperm capacitation ('B' + 'AR' patterns) was evaluated by CTC staining. The results are expressed as the mean ± SEM ($n = 5$). There is no difference in sperm capacitation when treated with dbcAMP in the full HTF or DF-Cl HTF medium in the presence or absence of adjudin. b versus c, $P > 0.05$; e versus d, $P > 0.05$, except for a versus CTL, $P < 0.001$. CTL, control; dbcAMP, dibutyryl cAMP; P4, progesterone.

AF-2785, it is possible that it blocks the function of CFTR Cl^- transport in sperm, thereby leading to a failure in sperm capacitation. It is noted that CFTR is a cAMP-activated Cl^- channel whose primary role is involved in the transport of HCO_3^- and Cl^- across the plasma membrane (Reddy *et al.*, 1999; Wang *et al.*, 2003). Furthermore, it has been demonstrated that CFTR Cl^- is present in the equatorial segment of the mouse, guinea pig and human sperm head (Xu *et al.*, 2007; Chen *et al.*, 2009; Li *et al.*, 2010) and in the middle piece of mouse sperm tails (Hernandez-Gonzalez *et al.*, 2007; Wertheimer *et al.*, 2008; Chavez *et al.*, 2012). Previous studies have shown that both Cl^- and HCO_3^- are essential for sperm capacitation in the mouse, guinea pig, rat and human (DasGupta *et al.*, 1993; Okamura *et al.*, 1993; Chen *et al.*, 2004, 2009; Wertheimer *et al.*, 2008; Jin *et al.*, 2009). We demonstrated herein that adjudin significantly blocked sperm hyperactivation and capacitation in the complete Cl^--HTF medium but not in Cl^--DF HTF medium, suggesting adjudin inhibits sperm hyperactivation and capacitation via CFTR Cl^- and other Cl^- transporters, perhaps by blockade of Cl^-/HCO_3^- exchanger through Cl^- channels (Chen *et al.*, 2009), Na^+/HCO_3^- co-transporter (Demarco *et al.*, 2003), $Na^+/K^+/Cl^-$ co-trasporters (Wertheimer *et al.*, 2008). This postulate is based on findings in which the addition of dbcAMP to spermatozoa incubated in the Cl^--free medium was shown to rescue sperm hyperactivation and capacitation (Fig. 7B and C), indicating that Cl^- is necessary for sperm capacitation and its function is upstream of the cAMP/PKA signaling pathway. It seems to be very similar to what Wertheimer *et al.* reported in mouse sperm (Wertheimer *et al.*, 2008). CFTRinh-172 and DPC are not able to inhibit the increase in tyrosine phosphorylation (Hernandez-Gonzalez *et al.*, 2007; Wertheimer *et al.*, 2008). Here we show the same lack of effect on capacitation-dependent tyrosine phosphorylation. However, our previous observations and the present studies have demonstrated that CFTRinh-172 and DPC are capable of inhibiting sperm capacitation and fertilization in mouse and human sperm *in vitro* (Xu *et al.*, 2007; Chen *et al.*, 2009; Li *et al.*, 2010) which are different from the results reported by Hernandez-Gonzalez *et al.* (2007) and Wertheimer *et al.* (2008). Their results indicated that CfTRinh-172 and DPC are not able to inhibit sperm hyperactivation and fertilization *in vitro*. At present the reason for these different results is still unknown. It is possible that the source of reagents used is different.

It is established that cAMP is downstream of the Cl^- effect in capacitation and the cAMP/PKA signaling pathway is necessary for sperm capacitation (Florman *et al.*, 2007; Wertheimer *et al.*, 2008; Chen

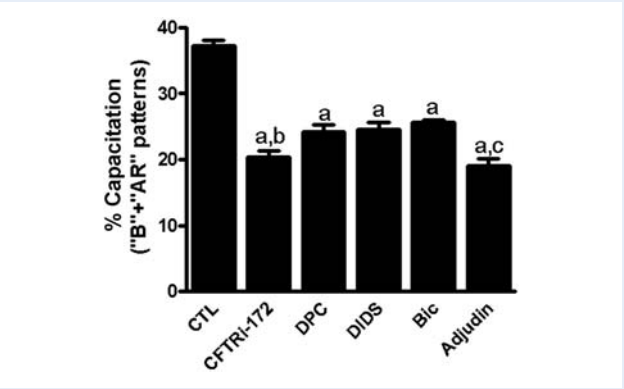

Figure 8 Inhibition of human sperm capacitation by different blockers of Cl^- channels. Sperm were preincubated with or without different blockers of Cl^- channels, CFTRinh-172 (1 μM), adjudin (10 μM), DPC (10 μM), DIDs (10 μM) or/and bicuculline (Bic, 10 μM) for 5 h in the complete HTF medium under capacitating conditions. The results of capacitation ('B' + 'AR' patterns) are expressed as the mean ± SEM ($n = 5$). a compared with control, $P < 0.01$; b compared with a, $P > 0.05$; c compared with a, $P < 0.05$. Bic, bicuculline; CFTR, cystic fibrosis transmembrane conductance regulator; DID, 4′4,diisothiocyanostilbene-2,2-disulfonic acid; DPC, diphenylamine-2-carboxylate.

et al., 2009). The dbcAMP is able to rescue sperm hyperactivation and capacitation in the Cl^--DF medium indicating that cAMP is involved in the Cl^- requirement for sperm hyperactivation and Cl^- exerts its effects upstream of the cAMP/PKA signaling pathway (Wertheimer *et al.*, 2008; Chen *et al.*, 2009).

Numerous proteins undergo phosphorylation at tyrosine, serine and threonine residuals by the activation of cAMP/PKA, and protein phosphorylation depends upon the balance action of protein kinases and protein phosphatase during capacitation (Signorelli *et al.*, 2012). In the present study, adjudin is not capable of inhibiting the increase in tyrosine phosphorylation, but it is able to block the increase in serine/threonine phosphorylation. It suggests that there may be two signaling pathways that regulate protein phosphorylation at tyrosine and serine/threonine residues in human sperm during capacitation (Naz, 1999; Hess *et al.*, 2005; Visconti *et al.*, 2011). The reasonable understanding may be that adjudin affects the PKA pathway, which phosphorylates several proteins at serine and threonine residues, but not activate or balance the kinases and phosphatases downstream of its signal pathway, leading to the phosphorylation of tyrosine residues (Signorelli *et al.*, 2012).

Another possible mechanism is that adjudin interferes upstream of signaling pathways by mediating the effects of cAMP. It is known that the G protein-regulated tmACs are unaffected by HCO_3^-. Even when sub-maximally stimulated by HCO_3^-, the tmACs activity is insensitive to HCO_3^- (Baxendale and Fraser, 2003; Fraser *et al.*,2003). Although a purified, engineered tmACs V type (tmACV), a soluble form of mACs, is fully responsive to forskolin (Scholich, *et al.*, 1997; Insel and Ostrom, 2003), it is completely insensitive to HCO_3^-. Therefore, HCO_3^- stimulation is not a general feature of all ACs, mammalian cells and spermatozoa possesses two independent regulated cAMP signal transduction systems: G protein-tmACs and bicarbonate- and calcium-regulated sACs (Leclerc and Kopf, 1995; Scholich *et al.*, 1997; Baxendale and Fraser, 2003; Liguori *et al.*, 2004). In this study our results showed that forskolin significantly promoted the increase in $[cAMP]_i$ levels, which may be acting on tmACs activity and subsequent increasing in $[cAMP]_i$ levels leading to capacitation. HCO_3^- present in the HTF medium directly activates sAC activity to induce an increase in $[cAMP]_i$ levels, accompanying capacitation. There is evidence that CFTR is present in the sperm which plays a critical role for regulation of the Cl^-/HCO_3^- exchanger and CFTR interaction, leading to the entry of HCO_3^- into sperm (Hernandez-Gonzalez *et al.*, 2007; Xu *et al.*, 2007; Chen *et al.*, 2009; Chavez *et al.*, 2012) and in this sense, HCO_3^- may directly activate sperm sAC activity. Therefore, there are two signaling pathways that regulate ACs activity leading to capacitation.

In short, it is likely that adjudin inhibits sperm capacitation and capacitation-associated events, such as hyperactivation, capacitation, the acrosome reaction and sperm–egg fusion via: (i) obstructing Cl^- transport function in the sperm by impeding Cl^- transporters and (ii) inhibiting tmACs and sAC activities, in turn, leading to a decline in $[cAMP]_i$ levels and of serine/threonine protein phosphorylation. Thus, besides serving as a potential male contraceptive, adjudin can also be used in females as a potential contraceptive by blocking the fertilizing capacity of sperm in humans.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

Q.X.S. and C.Y.C. were involved in substantial contributions to conception and design. K.L., Y.H., Y.N., W.Y.C. were involved in acquisition of data. Q.X.S., K.L., W.Y.C., J.X.L. were involved in analysis and interpretation of data. Q.X.S., K.L. were involved in drafting the article. C.Y.C., Q.X.S. and R.S.G. were involved in revising it critically for important intellectual content. C.Y.C., Q.X.S. were involved in final approval of the version to be published.

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Conflict of interest

None declared.

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