CFTR is essential for sperm fertilizing capacity and is correlated with sperm quality in humans

Chu-Yan Li1,3†, Ling-Ying Jiang2†, Wen-Ying Chen1†, Kun Li1, Hui-Qiang Sheng4, Ya Ni1, Jian-Xin Lu3, Wan-Xiang Xu5, Song-Ying Zhang2,6, and Qi-Xian Shi1,6

1Unit of Reproductive Physiology, Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang 310013, China 2Assisted Reproductive Unit, Department of Obstetrics and Gynecology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310016, China 3Department of Laboratory Medicine, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China 4The Sperm Bank of Zhejiang Province, Hangzhou, Zhejiang 310012, China 5Shanghai Institute of Planned Parenthood Research, Shanghai 200032, China

† CY, Li, LY, Jiang and WY, Chen, contributed equally to this work.

BACKGROUND: Our previous studies have demonstrated the cystic fibrosis transmembrane conductance regulator (CFTR) is important for capacitation and male fertility in mouse and guinea pig spermatozoa. However, the exact function of CFTR on human sperm fertilizing capacity, and correlation with sperm quality has not been established. The present study may shed light on some unexplained male infertility, and on a possible new method for diagnosis of male infertility and strategy for male contraception.

METHODS: To assess the effect of CFTR on human sperm fertilizing capacity, we examined sperm capacitation and the acrosome reaction using chlortetracycline staining, analyzed sperm hyperactivation by computer-assisted semen analysis (CASA), measured intracellular cAMP levels using ELA and evaluated sperm penetration of zona-free hamster eggs assay in fertile men. The percentage of spermatozoa expressing CFTR from fertile, healthy and infertile men (mainly teratospermic, asthenoteratospermic, asthenospermic and oligospermic) was conducted by indirect immunofluorescence staining.

RESULTS: Progesterone significantly facilitated human sperm capacitation and ZP3 triggered the acrosome reaction, both were significantly inhibited by CFTR inhibitor-172 (CFTRinh-172; 10 nM–1 μM) in a dose-dependent manner. The presence of 100 nM CFTRinh-172 markedly depressed intracellular cAMP levels, sperm hyperactivation and sperm penetration of zona-free hamster eggs. In addition, the percentage of spermatozoa expressing CFTR in the fertile men was significantly higher than healthy and infertile men categories (P < 0.01).

CONCLUSIONS: CFTR is essential for human sperm fertilizing capacity and the impairment of CFTR expression in spermatozoa is correlated with a reduction of sperm quality. These results suggest that defective expression of CFTR in human sperm may lead to the reduction of sperm fertilizing capacity.

Key words: CFTR / human sperm / fertilizing capacity / sperm quality

Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is known to act as a cAMP-activated Cl⁻ channel, mutations of which are responsible for cystic fibrosis (CF), a common hereditary disease characterized by defective Cl⁻ and HCO₃⁻ transport with clinical manifestations of progressive lung disease, pancreatic insufficiency and infertility in both sexes (Quinton, 1990, 1999; Rowe et al., 2005; Chan et al., 2006). Almost 97% CF male patients are infertile due to congenital bilateral absence of the vas deferens (CBAVD) with resultant obstructive azoospermia (Wong, 1998; Cuppens and Cassiman, 2004). Other causes of azoospermia include isolated anomalies of the seminal vesicles, congenital unilateral absence of the vas deferens (Meschede et al., 1997) and Young’s syndrome (Le Lannou et al., 1995). The anatomical defect may be traced back to the embryonic stage, indicating a possible requirement of CFTR or CFTR-mediated anion secretion for normal development of the male genital tract. The absence of sperm in the ejaculatory duct of CF males has led us to consider possible involvement of CFTR in...
normal human sperm function. Moreover when spermatozoa from CF patients with CBAVD are used for intracytoplasmic sperm injection, fertilization rates are not reduced, suggesting a specific defect in zona pellucida penetration or membrane fusion capacity in these spermatozoa (Silber et al., 1994). Meanwhile, it is unclear whether CFTR mutations cause a reduction of sperm quality. Some investigators have provided evidence for this (van der Ven et al., 1996; Jakubiczka et al., 1999; Dohle et al., 2002), although others have argued against it (Jakubiczka et al., 1999; Mak et al., 2000; Stuhrmann and Dork, 2000; Ravnik-Glavac et al., 2001). Testicular biopsies of some CF men have revealed a decreased number of mature spermatozids in the seminiferous epithelium of the testes, and many of the testicular spermatozoa are malformed. Importantly, van der Ven et al. (1996) reported that 17.5% healthy men with infertility due to the reduction of sperm quality and 14.3% men with azoospermia have at least one mutation in the CFTR gene. These observations strongly suggest the involvement of CFTR in sperm matogenesis, although Mak et al. (2000) did not detect the CFTR mutations in infertile men with primary testicular failure.

To date, the literature suggests that CFTR gene mutations in healthy men may cause a reduction of sperm quality, and this prompted us to hypothesize that CFTR plays an important role in human sperm fertilizing capacity and is correlated with sperm quality. It has been reported that CFTR is expressed in germ cells at specific stages of spermatogenesis (Trezise et al., 1993) and that functional CFTR protein is present in the sertoli cells (Boockfor et al., 1998), as well as in the epithelial cell of the epididymis in rats (Leung et al., 1996; Wong, 1998; Gong et al., 2000, 2001). Our previous studies demonstrated that CFTR is involved in the secretion and transport of HCO\(^3\) in the epithelium of mouse uterine endometrium, defects of which lead to a reduction of sperm capacitation and fertilizing capacity (Wang et al., 2003). We have also demonstrated, using CF heterozygote mouse spermatozoa, that impaired CFTR function may result in the reduction of sperm fertilizing capacity (Xu et al., 2007). Hernandez-Gonzalez et al. (2007) showed that CFTR is involved in mouse sperm capacitation by regulating Na\(^+\) channel activity (EnaC). Recently, Chen et al. (2009) reported that a functional interaction between CFTR and SLC26A3 (a Cl\(^-/\)HCO\(^3\) exchanger) lead to sperm capacitation, and the inhibition of the anion exchange reduced sperm capacitation in guinea pig, Chen et al. (2004) also reported that Cl\(^-\) is required for guinea pig sperm capacitation, hyperactivation and fertilization. Moreover, Wertheimer et al. (2008) demonstrated that Cl\(^-\) is essential for mouse sperm capacitation and capacitation-associated events, such as the increase in tyrosine phosphorylation, the increase cAMP levels, hyperactivation and fertilization. Although we, and other groups, have shown a correlation between CFTR and capacitation in mouse and guinea pig spermatozoa, the exact function of CFTR on human sperm fertilizing capacity has not been established. Although we have demonstrated the expression and localization of the CFTR protein in human ejaculatory spermatozoa, the percentage of spermatozoa expressing CFTR in fertile versus infertile men categories is not established. Therefore, further investigations are needed to elucidate the exact role of CFTR in human male reproduction beyond spermatogenesis.

Mammalian spermatozoa must undergo a preparation period in the female reproductive tract, or be cultured in an appropriate artificial medium, to acquire fertilizing potential (Chang, 1951, Austin, 1952; Yanagimachi, 1994a, b). This phenomenon is termed sperm capacitation. Therefore, the broad definition of capacitation, i.e. acquisition of fertilizing potential, becomes more focused on the potential for undergoing the acrosome reaction. Thus, sperm capacitation is a prerequisite for the acrosome reaction, which is an exocytotic event releasing hydrolytic enzymes from the acrosome to enable spermatozoa to penetrate the egg investments and the plasma membrane, in response to natural agonists, progesterone and the zona pellucida (Wassarman, 1990; Roldan et al., 1994; Yanagimachi, 1994a, b). Therefore, sperm capacitation and the acrosome reaction can be used to assess sperm fertilizing capacity. For many years, one of the practical approaches to assessing capacitation and the acrosome reaction has been to measure the ability of spermatozoa to undergo an induced acrosome reaction, even if capacitation and the acrosome reaction are two separate events. This approach exploits the operational definition of capacitation (Florman et al., 1992; Yanagimachi, 1994a, b; Visconti et al., 1998): measuring the increase in the number of spermatozoa ‘acrosome-reacted’ in response to an acrosome reaction inducer. The method, in general, appears highly reliable when the natural acrosome reaction inducers, zona pellucida or progesterone, are used. Sperm penetration of zona-free hamster eggs assay is another reliable method of assessing sperm fertilizing capacity (Yanagimachi et al., 1976; Atikken et al., 1983a, b).

The aims of the present study were to investigate whether: (i) CFTR is involved in human sperm fertilizing capacity; (ii) the percentage of spermatozoa expressing CFTR in human is correlated with sperm quality.

### Materials and Methods

#### Reagents

CFTRinh-172 was purchased from Calbiochem Inc (La Jolla, CA, USA; catalog no. 219670). A mouse monoclonal anti-human CFTR antibody was acquired from Abcam (Cambridge, UK; catalog no. ab2784). Progesterone, dimethylsulfoxide (DMSO), chlorotetracycline (CTC), L-cysteine, BSA (Fraction V), Hochest 33258 and Hepes were obtained from Sigma (St. Louis, MO, USA). To prepare CTC, 10 ml of chilled buffer of Tris (20 mM) and NaCl (130 mM) (TN) was added to 8.8 mg L-cysteine followed by 2.6 mg CTC. The CTC solution was vortexed for 30 s, adjusted to pH 7.8 with 1 M NaOH and placed on ice. This solution was freshly prepared before use. CFTRinh-172 and progesterone were dissolved in DMSO so that the final concentration of DMSO in each solution was below 0.05%

#### Media

Human Tube Fluid (HTF) with or without Hepes was used in the study. 100 ml HTF medium consisted of 526.1 mg NaCl, 37.0 mg KCl, 26.5 mg CaCl\(_2\) (2H\(_2\)O), 24.9 mg MgSO\(_4\)(7H\(_2\)O), 210.5 mg NaHCO\(_3\), 110.2 mg glucose, 10 mM sodium lactate, 2.97 mg sodium pyruvate, 15.92 mg KH\(_2\)PO\(_4\), 476.6 mg Hepes, 400 mg bovine serum albumin (fraction V) and 6 mg penicillin. 476.6 mg Hepes in this medium was replaced by 58.5 mg NaCl to yield Hepes-free HTF medium. This medium had a
final osmolality of 305–310 mOsm/kg and pH 7.5 at room temperature (25°C).

### Study subjects and sample preparation

Semen samples were obtained from fertile men, whose partners had conceived spontaneously within 1–2 years and proven fertilizing ability in vitro, healthy men, whose partners had not conceived spontaneously within 1–2 years, and infertile men. Proven fertilizing ability was assessed using the sperm penetration of zona-free hamster eggs assay, of which spermatozoa (SPA ≥ 70%) were used in the study. Semen samples of both healthy and infertile men were obtained from the Reproductive Center of Sir Run Run Shaw Hospital, Zhejiang University. An informed consent was obtained from each participant. Each subject completed an extensive questionnaire regarding his age, social status, working conditions, diet, smoking habits and medical history, including history of testicular dysfunction, urogenital abnormality, mumps, surgical operation and varicocele which all were excluded.

Semen samples were collected by masturbation into a sterile open end plastic container. Sperm morphology analysis was performed using a modified Papanicolaou staining method. For the function tests, spermatozoa were washed through a Percoll discontinuous gradient (45–90%) in HTF medium containing 4 mg BSA/ml by centrifugation at 600 g for 15 min. To determine the percentage of spermatozoa expressing CFTR, spermatozoa were washed through PBS at 500 g for 5 min.

### Evaluation of sperm capacitation and the acrosome reaction

Sperm capacitation and the acrosome reaction were assessed by CTC staining as described by Lee et al. (1987) and DasGupta et al. (1993). This conventional method for assessing sperm capacitation and the acrosome reaction is based on alteration in staining patterns of the sperm head during the process. (i) To examine the effect of CFTR on human sperm capacitation, washed spermatozoa were preincubated with or without various concentrations of CFTRinh-172 (10 nM–1 μM) and 2.5 μM progesterone, except for negative control, for 5 h under capacitating conditions. Spermatozoa suspensions were washed with fresh HTF medium and then stimulated with 15 μM progesterone, except for negative control, for 15 min before evaluation of sperm acrosomal status. (ii) To assess the effect of CFTRinh-172 on human sperm acrosome reaction, spermatozoa were preincubated for 5 h in HTF medium CFTRinh-172-free under capacitating conditions and then washed with fresh HTF medium. The washed spermatozoa were exposed to CFTRinh-172 at 10 nM–1 μM for 30 min and then stimulated with 20 μg/ml rhuZP3a for 15 min before assessment of the acrosome reaction. Samples were then immediately diluted by the addition of an equivalent amount of 8% glutaraldehyde and mixed gently for 5 min at 37°C. Spermatozoa were stained with CTC for 10 min at 37°C and then stained with Hoechst 33258 solution (final concentration of Hoechst was 1 μg/ml). Samples were incubated in the dark for 2 min and immediately examined using phase-contrast and fluorescence microscopy (1000×). For CTC staining, a mercury excitation beam passed through a 402-nm filter and fluorescence emission with a DM 455 dichroic mirror (Nikon BV-3 filter) was used. For Hoechst 33258 staining, a 330-nm filter and fluorescence emission via a DM 400 dichroic mirror (Nikon UV-2A filter) was used.

A minimum of 200 spermatozoa were counted per sample. The three patterns of spermatozoa CTC staining were: the ‘F’ pattern, uncapacitated; the ‘B’ pattern, capacitated with acrosome-intact; and the ‘AR’ pattern, capacitated and acrosome-reacted. Sperm capacitation was evaluated by the incidence of ‘B’ pattern and ‘AR’ pattern with progesterone induction. The sum of ‘B’ pattern plus ‘AR’ pattern is designated as sperm capacitation. The sperm acrosome reaction was assessed by the incidence of ‘AR’ pattern using 20 μg/ml rhuZP3a.

### Measurement of intracellular cAMP in spermatozoa using EIA

Measurement of intracellular cAMP in spermatozoa was conducted using EIA according to Chen et al. (2009). Briefly, spermatozoa were incubated for 5 h with or without 100 nM CFTRinh-172 in HTF medium under capacitating conditions. Spermatozoa suspension (50 μl; 10⁶ cells) was 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 were then lyophilized and assayed cAMP levels according to the manufacturer’s protocol.

### Assessment of sperm hyperactivated motility by computer-assisted semen analysis

Sperm hyperactivated motility was analyzed by a computer-assisted semen analysis (CASA) system, Hamilton-Thorne Research Motility Analyzer (HTM-IVOS S/N with software Version 12.3N, Beverly, MA, USA), as previously described by Mortimer (2000). After incubation of 0 h, and 5 h, a 2 μl aliquot of each sample was pipetted into a prewarmed microcell slide chamber with a depth of 20 μm. 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 with a 20 objective. For each sample, 10 randomly-selected fields containing more than 200 motile tracks were examined at 60 Hz. Curvilinear velocity (VCL), straight line velocity (VSL), lateral head displacement (ALH), average path velocity (VAP), linearity of progression (LIN, VSL/VCL multiplied by 100) and percentages of motility (motile) were recorded. Spermatozoa were designated as hyperactive if they had a VCL ≥ 150 μm/s, ALH ≥ 7.0 μm/s and LIN ≤ 50%.

### Evaluation of sperm penetration of zona-free hamster eggs assay

Hamster oocytes were obtained by ovulation induction, as previously described (Shi et al. 1989). Sperm fertilizing ability was evaluated by sperm penetration of zona-free hamster eggs assay (SPA) according to Yangmachi et al. (1976); Atken et al. (1983a) and Shi et al. (1991). Washed spermatozoa were preincubated in Hepes-free HTF medium with or without 100 nM CFTRinh-172 for 5 h under capacitating conditions. Zona-free hamster eggs in Hepes-free HTF medium were introduced separately into spermatozoa suspensions (50 μl; 50 μl each) and co-incubated for 3 h. After 3 h of incubation, eggs were rinsed three times with Hepes-free HTF medium, transferred to a slide and compressed carefully under a cover glass supported at its corners with drops of paraffin wax-Vaseline mixture (1:9, V/V). Eggs were examined with a phase-contrast microscope (400×) for the presence of a decondensed spermatozoa head with its tail within the ooplasm as an evidence of fertilization. The penetration rate of eggs by spermatozoa was taken as the spermatozoa fertilization capacity.

### Immunofluorescent staining

Indirect immunofluorescence studies of CFTR protein localization were performed as described previously by Xu et al. (2007). Briefly, spermatozoa were washed by centrifugation through PBS and fixed in 4% paraformaldehyde overnight at 4°C, and then were smeared on slides previously coated with poly-l-lysine and air-dried. Spermatozoa were permeabilized with 0.1% Triton X-100/PBS for 10 min at room temperature. After washing with PBS three times, the slides were blocked with 10%
normal goat serum at room temperature for 1 h. Spermatozoa were incubated with primary anti-CFTR antibody (dilution: 1:500) at 4°C overnight. Slides were washed three times in PBS and incubated with secondary antibody (anti-mouse IgG+IgM-FITC, Abcam, Cambridge, UK, ab47830, 1:500) in dark room for 1 h at room temperature. Unbound antibody was removed by washing with PBS three times for 5 min each and then counterstained with Hoechst 33258 (final concentration of Hoechst 1 μg/ml) for 2 min. The slides were stored in the dark before examination. At least 200 spermatozoa were counted in each sample to quantify the percentage of spermatozoa expressing CFTR protein.

Statistical analysis
Results are expressed as 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

Semen parameters of fertile, healthy and infertile men
Semen samples were obtained from fertile men ($n = 15$, 26–38 years). Semen of healthy men ($n = 30$, 24–36 years of age) had normal characteristics according to the WHO (1999) guidelines and standard andrology criteria, but whose partners had not conceived spontaneously within 1–2 years. Semen parameters of healthy men in the study were: sperm concentration $100.8 ± 49.1 \times 10^6$/ml, sperm motility (a + b%) 71.5 ± 10.8% and normal morphologic spermatozoa 19.2 ± 4.1%. Semen samples analysis of infertile men ($n = 161$, 25–44 years of age) with reduced sperm quality including volume, liquefaction time, viscosity, pH, odor, aggregation and presence of epithelial cells and leukocytes, were carried out according to the WHO guidelines (1999). Semen profiles of teratospermic: normal morphologic spermatozoa 6.1 ± 4.1%, asthenotaratospermic: sperm motility (a + b%) 32.1 ± 10.2% and normal morphologic spermatozoa 4.4 ± 4.0%, asthenospermic: sperm motility (a + b%) 31.78 ± 9.9%, oligospermic: sperm concentration 11.2 ± 4.3 millions/ml. These data are shown in Fig. 1.

Inhibition of progesterone-induced sperm capacitation by CFTRinh-172
The three sequential and distinct patterns of CTC staining on human spermatozoa were observed as follows: the first stage was marked by the CTC fluorescence pattern designated as the ‘F’ pattern with uniform fluorescence in the head, which was characteristic of uncapacitated, acrosome-intact sperm. The second stage was designated as the ‘B’ pattern with a fluorescence-free band in the post-head region, which was characteristic of capacitated, acrosome-intact sperm. The third stage designated as the ‘AR’ pattern with dull or absent fluorescence, was characteristic of acrosome-reacted sperm. Bright fluorescence in the midpiece was seen on all sperm samples. Typical examples of these spermatozoa were shown in Fig. 2. Progesterone promoted the ‘F’ pattern to ‘B’ pattern transition, followed by an increase in the ‘AR’ pattern, reaching a maximum value (‘B + AR’ patterns: 61.0%) (Fig. 3B) after 5 h of incubation, indicating progesterone (2.5 μM) facilitated markedly human sperm capacitation.

However, this stimulatory effect was inhibited significantly by CFTRinh-172 in a dose-dependent manner with concentrations of 10 nM–1 μM, as compared with positive control, $P < 0.01$ (Fig. 3B).

Inhibition of intracellular cAMP levels by CFTRinh-172
The mean level of cAMP in spermatozoa incubated in HTF medium under capacitating conditions for 5 h was dramatically elevated as compared with that observed before treatment. However, this elevation of intracellular cAMP was completely abolished when spermatozoa were incubated in the presence of 100 nM CFTRinh-172 for the same period of time ($P < 0.01$) (Fig. 4).

Inhibition of spermatozoa hyperactivated motility by CFTRinh-172
CASA analysis revealed that after incubation of 5 h, the percentage of hyperactivated spermatozoa significantly increased as compared with spermatozoa at 0 h ($P < 0.01$). In contrast, when spermatozoa were incubated in HTF with 100 nM CFTRinh-172 for 5 h under capacitating conditions, the percentage of hyperactivated spermatozoa was significantly decreased as compared with the positive control ($P < 0.01$).
conditions, sperm motility was not affected (Fig. 5A), but the percentage of hyperactivated spermatozoa was significantly lower compared with untreated samples ($P < 0.01$) (Fig. 5B).

Inhibition of the rhuZP3a-induced acrosome reaction by CFTRinh-172

The acrosome reaction induced by rhuZP3a (20 μg/ml) was markedly inhibited by CFTRinh-172 in a dose-dependent manner, as compared with positive control ($P < 0.01$) (Fig. 6).

Inhibition of sperm penetration of zona-free hamster eggs by CFTRinh-172

Besides sperm capacitation and the acrosome reaction, sperm penetration of zona-free hamster eggs assay (SPA) was used to assess the human sperm fertilizing capacity, in particular the ability of sperm-oocyte fusion. The presence of a decondensed sperm head with its tail within the ooplasm is taken as an evidence of sperm-oocyte fusion (Fig. 7). The percentage of spermatozoa penetration of zona-free hamster eggs in the presence of 100 nM CFTRinh-172 significantly decreased (32.5%, as compared with negative control of 80.2% and positive control of 87.4%, $P < 0.01$) (Table I).

Localization and expression of CFTR in human spermatozoa

As shown in Fig. 8A, CFTR was localized to the equatorial segment of human sperm head. However, the percentage of spermatozoa expressing CFTR varied in different subjects (Fig. 8Aa–f). The percentage of spermatozoa expressing CFTR in fertile men (87.9 ± 3.9%) was significantly higher than in healthy men (56.5 ± 20.8%) with...
normal sperm parameters but impaired fertility capacity (as compared with fertile men, \(P < 0.01\)) (Fig. 8B). Infertile men categories (mainly, teratospermic, asthenoteratospermic, asthenospermic and oligospermic) had significantly lower levels as compared with fertile men, \(P < 0.01\) (Fig. 8B).

**Discussion**

It is well established that progesterone stimulates sperm capacitation in animal and human (Foresta et al., 1993; DasGupta et al., 1994; Kay et al., 1994; Revelli et al., 1994; Roldan et al., 1994; Barboni et al., 1995; De Lamirande et al., 1998). In this study, we demonstrated that progesterone significantly stimulated human sperm capacitation, however, this stimulatory effect was markedly inhibited by CFTRinh-172 in a dose-dependent manner (Fig. 3B). Capacitation is also associated with sperm hyperactivated motility, which is considered to be an inviolable adjunct to capacitation (Suarez, 1996; Yanagimachi, 1994a, b). CFTRinh-172 also significantly inhibited sperm hyperactivated motility, but did not affect sperm motility (Fig. 5). This further supports the hypothesis that CFTR is involved in the regulation of sperm capacitation. These results are in agreement with those reported in mouse sperm (Hernandez-Gonzalez et al., 2007; Xu et al., 2007). It is well known that HCO\(_3\) is essential for sperm capacitation (Boatman and Robbins, 1991; Shi and Roldan, 1995; Visconti et al., 1995a, b), as prevention of HCO\(_3\)-induced intracellular alkalization results in failure of capacitation (Boatman, 1997; Visconti et al., 1999). Recently, we demonstrated that CFTR is involved in HCO\(_3\) secretion and transport in mouse uterine endometrium epithelial cells, and impaired HCO\(_3\) secretion in the epithelial cells of uterine endometrium in mouse also reduces capacitation and fertilizing capacity in co-cultured spermatozoa (Wang et al., 2003). The question arises, how does HCO\(_3\) gain access to the interior of the sperm cell? Several transport mechanisms have been proposed. It has been suggested that a Cl\(^-\)/HCO\(_3\) exchanger exists in sperm (Okamura et al., 1988; Visconti et al., 1990; Spira and Breitbart, 1992), which is involved in mediating the HCO\(_3\) effects on sperm functions. Evidence supporting this came from experiments in which inhibitors of this anion exchanger, 4,4\(_0\)-diisothiocyanatostibene-2,2\(_0\)-disulfonic acid (DIDS) and 4-acetomido-4\(_0\)-isothiocyanatostilbene-2,2\(_0\)-disulfonic acid (SITS), blocked the HCO\(_3\) effect. In contrast, Tajima et al. (1987) and Tajima and Okamura (1990) demonstrated that DIDS enhances HCO\(_3\)-induced activation of porcine sperm, which is reasonable because the anion exchanger blockers inhibit the efflux of endogenous HCO\(_3\) derived from metabolic CO\(_2\), so that HCO\(_3\) accumulates intracellularly and stimulates the soluble adenyl cyclase (sAC). However, the non-specific action of DIDS and SITS, apart from that on the anion exchanger, has made the interpretation of the results inconclusive. For example, possible inhibition of the H\(^+\)-pump activity (Chanson and Taiz, 1985) may in turn influence the intracellular pH (pHi) which is known to play an important role in sperm capacitation. Recently,
Table 1 Inhibition of human sperm penetration of zona-free hamster eggs by CFTRinh-172

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of eggs examined</th>
<th>Number of eggs penetrated</th>
<th>Rate of penetration (%)</th>
<th>Index of penetration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone (P4) (15 μM)</td>
<td>111</td>
<td>97</td>
<td>87.4</td>
<td>2.69</td>
</tr>
<tr>
<td>Control</td>
<td>86</td>
<td>69</td>
<td>80.2</td>
<td>3.24</td>
</tr>
<tr>
<td>CFTRinh-172 (100 nM)</td>
<td>123</td>
<td>40</td>
<td>32.5*</td>
<td>0.87*</td>
</tr>
</tbody>
</table>

*The penetration rate and index of spermatozoa into the zona-free hamster eggs in the presence of 100 nM CFTRinh-172 significantly decrease as compared with the control (P < 0.01, n = 5).

Figure 8 (A) Localization and expression of CFTR in human sperm.

The CFTR is localized on the equatorial segment of sperm head. The expression of CFTR in fertile (a), healthy (b) and infertile men categories, mainly teratospermic (c); asthenoteratospermic (d); asthenospermic (e) and oligospermic (f). (B) The percentage of spermatozoa expressing CFTR protein from the fertile, healthy and infertile men, mainly teratospermic, asthenoteratospermic, asthenospermic and oligospermic. A total of at least 200 spermatozoa were counted to assess the percentage of spermatozoa expressing CFTR protein in fertile, healthy and infertile categories. The results are expressed as the mean ± SEM. a compared with b, c, d and e, P < 0.01; b compared with c, d, e, P < 0.01; c compared with d, e, P < 0.01; d compared with e, P < 0.05.
another possible HCO$_3^-$ entry pathway has also been proposed: the Na$^+$/HCO$_3^-$ co-transporter is present in mouse sperm and is coupled to events regulating capacitation, indicating its possible involvement in transporting HCO$_3^-$ across the sperm membrane (Demarco et al., 2003). This regulatory effect of HCO$_3^-$ on sperm capacitation is mediated by sAC (Chen et al., 2000; Cann, 2004). HCO$_3^-$ activates the sAC in the sperm cytoplasm, which increases intracellular cAMP levels and protein tyrosine phosphorylation and thereby triggers a signaling cascade and subsequent sperm capacitation (Visconti et al., 1995a, b; Chen et al., 2000, 2009; Chan et al., 2006; Hernandez-Gonzalez et al., 2007; Xu et al., 2007). Most recently studies from our group, and others, have shown that a crucial role for CFTR in the cAMP-activation of anion channels conducting HCO$_3^-$ and Cl$^-$, which are essential for sperm capacitation and capacitation-associated events in guinea pig and mouse (Chen et al., 2004, 2009; Shi et al., 2006; Hernandez-Gonzalez et al., 2007; Xu et al., 2007; Wertheimer et al., 2008). In the present study, CFTRinh-172 significantly prevented the increase in intracellular cAMP levels of human spermatozoa (Fig. 4), suggesting that HCO$_3^-$ induced sAC activation, appears to be blocked by inhibition of CFTR. In addition, the operation of anion exchanger (SLC26A3) requires the involvement of CFTR as a Cl$^-$ channel to provide a recycling pathway for Cl$^-$. Without proper CFTR function, the entry of HCO$_3^-$ would be disrupted due to lack of Cl$^-$ exchange, resulting in capacitation failure (Chen et al., 2009).

Moreover, CFTRinh-172 significantly blocked the acrosome reaction induced by rhuZP3a in a concentration-dependent fashion, indicating that CFTR may also be involved in the acrosome reaction (Fig. 6). This finding is in contrast to the report by Hernandez-Gonzalez et al. (2007) that showed diphénylamine-2-carboxylic acid (DPC), an inhibitor of CFTR, inhibited mouse sperm capacitation, rather than the zona pellucida-induced acrosome reaction. The reason for the apparent conflict may be due, at least in part, to the fact that the different studies used different CFTR inhibitors, and spermatozoa were exposed to inhibitors for the different times during the acrosome reaction. In this study incubated human spermatozoa were exposed to CFTRinh-172 for 30 min, whereas capacitated mouse spermatozoa were exposed to DPC for only 5 min.

Sperm fertility capacity is assessed not only by sperm capacitation and the acrosome reaction, but also by sperm penetration of zona-free hamster eggs assay (Yanagimachi et al., 1976; Aitken, 1983a, b; Koulisher and Debruy, 1983; Shi et al., 1990, 1991). This study showed that CFTRinh-172 at 100 nM significantly inhibited sperm penetration of zona-free hamster eggs and thus decreased the sperm-egg fusion (Table I). Assessment of the sperm acrosome reaction induced by ZP3 has been used to detect specific defects of sperm fertilizing ability (Liu and Baker, 1994). The acrosome reaction has at least two functions, i.e. it renders spermatozoa capable of penetrating through the zona pellucida of the oocyte and fusing with the oocyte membrane. The acrosome reaction is triggered by a engagement of a ligand, ZP3, on the oocyte. As a sperm receptor, ZP3 involves carbohydrate-mediated gamete recognition and adhesion (Mortillo and Wassarman, 1991). ZP3 also serves as a tool for diagnosis of male infertility but the supply of human ZP3 is very limited. However, bacteria-expressed recombinant ZP3 is biologically active in the stimulation of sperm motility, induction of sperm acrosome reaction, stimulation of Ca$^{2+}$ influx and promotion of sperm-oocyte fusion (Brewis et al., 1996; Dong et al., 2001; Bray et al., 2002; Caballero-Campo et al., 2006; Ni et al., 2007). In this study, we used recombinant rhuZP3a, and provide further evidence of its use in measuring the acrosome reaction. Our results show that CFTRinh-172 strongly inhibited the sperm acrosome reaction induced by rhuZP3a and sperm-oocyte fusion, suggesting that CFTR protein may be an integral part of both these processes. The CFTR protein is localized in the equatorial segment of human spermatozoa, which acts as a site of sperm-oocyte fusion (Mortillo and Wassarman, 1991; Yanagimachi, 1994a, b; Primakoff and Myles, 2002). Therefore, we suggest that the CFTR protein serves as a mediator of sperm-oocyte fusion. These results are in agreement with those of Silber et al. (1994) who suggest that spermatozoa of CF patients may have a specific defect in the sperm-zona pellucida penetration. The present study demonstrated that CFTRinh-172 not only prevented the acrosome reaction in response to ZP3 but also impaired the sperm-oocyte fusion. To our knowledge, this is the first report of the involvement of CFTR in human sperm capacitation, the acrosome reaction and sperm-oocyte fusion in vitro.

Although previous studies have demonstrated the expression of CFTR protein in mature human sperm, it is still unclear whether the percentage of spermatozoa expressing CFTR in human sperm is correlated with sperm quality. In the present study, we found that the percentage of spermatozoa expressing CFTR protein in the fertile, healthy and infertile men spermatozoa differed significantly. The highest level of spermatozoa expressing CFTR was found in fertile men (87.9%), followed by healthy men (56.5%) with normal sperm parameters and finally infertile men, mainly teratospermia (47.0%), asthenoteratospermia (40.2%), asthenospermia (28.8%) and oligospermia (18.7%), of which oligospermia was the lowest, indicating the percentage of sperm expressing CFTR protein was correlated with sperm quality. Furthermore, this finding also supports the hypothesis that CFTR is correlated with primary spermatogenesis in the testis (van der Ven et al., 1996; Vong, 1998; Gong et al., 2001). For scientific research and clinical work, the method used to assess male fertility capacity is the semen frequent analysis, which includes semen volume, sperm concentration, motility, viability, morphology, pH, viscosity, liquefaction time and agglutination. Although these conventional analysis methods can reveal some aspects of sperm function, they do not necessarily accurately reflect male fertility capacity (Bostofte et al., 1984; Shi et al., 1990). The results in the present study provide important evidence that the percentage of sperm expressing CFTR protein may be an additional indicator of male fertility capacity. The present study also strongly supports the involvement of the CFTR gene mutations in the reduction of sperm quality and subsequently the decrease in sperm fertilizing capacity (van der Ven et al., 1996; Jakubcza et al., 1999; Dohle et al., 2002).

In conclusion, our study indicates that CFTR is necessary for human sperm capacitation, the acrosome reaction and of sperm-oocyte fusion, and the impairment of sperm expressing CFTR protein is correlated with a reduction of sperm quality. The defective expression of CFTR in sperm may lead to a reduction in sperm fertilizing capacity. This work may provide a biological rationale for poor sperm quality in men harboring the CFTR mutations. The results not only help to explain some unexplained male infertility but also shed light on a possible new method for diagnosis of male infertility and strategy for male contraception.
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