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# Low Level of Cystic Fibrosis Transmembrane Conductance Regulator Is Associated with Human Sperm Autophagy and Vitality

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## Abstract

Low sperm motility is one of the main causes of male infertility. Cystic fibrosis transmembrane conductance regulator (CFTR, an anion channel protein) is related to the progressive motility of sperm. CFTR disruptor CFTRinh-172 or forskolin (FSK) in this study were used to treat human sperm separately, and the rates of sperm autophagy and progressive motility, mitochondrial membrane potential (MMP) and ATP concentration, and the expression levels of related factors were detected to explore their relationship. It was showed that sperms treated with CFTRinh-172 or FSK reduced the levels of cAMP, CFTR and PKA, but increased sperm autophagy rate, expression levels of AMPK and LC3B. However, reactive oxygen species content had no significant difference. It was indicated that low level of CFTR performed with cAMP and its downstream effectors such as PKA and AMPK to regulate mitochondrial structure and function, leading to increased autophagy rate and reduced vitality of sperm.

## Keywords

Autophagy, CFTR, Sperm, Vitality

## 1. Introduction

Autophagy is a critical cellular biological phenomenon regulated through complex signal networks, and participates in various physiological or pathological

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processes such as cell vitality, neurodegeneration, infectious diseases, cancer, etc. [1]. Low sperm motility is one of the main causes of male infertility [2], and higher levels of autophagy-related proteins (such as microtubule-associated proteins 1A/1B light chain 3B, LC3B protein) have a protective effect on sperm activity [3].

Cystic fibrosis transmembrane conductance regulator (CFTR) is an adenosine triphosphate (ATP)-gated anion channel protein that depends on the second messenger cyclic adenosine monophosphate (cAMP) in cells [4]. It can be detected in various organelles such as cell membranes and mitochondria. It is highly expressed at varied developmental stages of sperm, mostly at the site of fertilization band [5]. CFTR may affect mitochondrial membrane potential (MMP), oxidative stress and energy metabolism by regulating sperm ion balance. Abnormal MMP can promote the production of excessive reactive oxygen species (ROS) by mitochondria, leading to free radical chain reactions, and further damage mitochondrial structure and function. So, ATP synthesis is hindered, and cell vitality and function get disrupted.

CFTR also interacts with other downstream effectors of cAMP such as protein kinase A (PKA) and adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK). PKA enhances the activity of CFTR by binding to its regulatory domain and phosphorylating CFTR, while AMPK binds to the nuclear binding domain of CFTR and reduces its activity [6]. Inhibition of CFTR reduces human sperm PKA activity [7] and increases AMPK activity [8], thereby possibly affecting acrosomal enzyme activity, tyrosine phosphorylation and acrosome reaction. PKA and AMPK also interact with autophagy to regulate cell constructions and functions [9].

Chemical reagents have been developed to regulate CFTR. CFTRinh-172 is a gold standard for studying CFTR function because of its specificity, reversibility and low toxicity on inhibiting the activity of CFTR [10]. Forskolin (FSK) is a natural diterpenoid antioxidant with high safety. FSK directly activates adenylate cyclase (AC) to catalyze ATP into cAMP, hence increasing the level of cellular cAMP and phosphorylating CFTR [11].

The present study used CFTR disruptors to incubate human sperm and detected CFTR upstream and downstream molecules, MMP and ATP levels, so as to explore the biological process of CFTR affecting sperm motility through the autophagy pathway. This study would provide novel scientific basis for the potential intervention measures to improve human sperm motility.

## 2. Materials and Methods

The normal semen samples and clinical data of this study were collected from men who underwent pre-marital health check-up at the Center of Reproductive Medicine, Affiliated Hospital of Guilin Medical University from June 2021 to December 2022. According to "WHO Laboratory Manual for the Examination and Processing of Human Semen" (5$^{th}$ Edition), the normal semen quality stan-

dards are: 1) semen volume ≥ 1.5 mL; 2) semen pH value: 7.2 - 7.8; 3) sperm concentration ≥ $15 \times 10^6$/mL; 4) total sperm motility [PR + non-progressive rate (NP)] ≥ 40% and progressive rate of sperm motility (PR) ≥ 32%; 5) liquefaction time < 30 min; 6) color: gray-white or light yellow.

Inclusion criteria: 1) males aged 18 - 45 years old; 2) no serious exposure to important environmental factors (such as smoking, pesticides and other chemicals); 3) no obvious abnormality in mental and psychological conditions. Exclusion criteria: a) insufficient semen volume; b) with a history of severe reproductive system trauma; c) with diseases such as malignant tumors, hemophilia, and severe mental illnesses; d) with severe necrospermia, azoospermia, or cryptospermia; e) long-term use of drugs that may affect sperm function.

This study was approved by the Medical Ethics Committee of the hospital. Written informed consents were given by the research subjects.

### 2.1. On-Site Investigation and Semen Sample Analysis

Clinical data of the subjects were collected on site using a questionnaire, including medical history, family history, body mass index, smoking and drinking habits, chemical exposure, mental and psychological conditions, etc.

Visitors collected the semen samples using masturbation. After complete liquefaction, the sample was analyzed by Computer-aided sperm analysis system (CASA, ZJ-3000E, Xuzhou, China) on the semen quality including sperm vitality (indicated by PR). The remaining semen after the analysis was washed and purified with human fallopian tube fluid (HTF) according to the SWIM-UP method. The sperm concentration was adjusted to $5 \times 10^6$ cells/mL. The CFTRinh-172 (GlpBio, USA) group was divided into 5 dose groups, *i.e.*, 0 μM (control), 100 μM, 500 μM, 1000 μM, and 5000 μM; similarly, the FSK (MCE，USA) group was divided into 0 nM (control), 20 nM, 50 nM, 100 nM, and 500 nM. PR was detected at the 20$^{th}$, 40$^{th}$, 60$^{th}$, and 80$^{th}$ minutes after the reagent administration in each group. All samples were tested three times and the average of PR was obtained according to the "WHO Laboratory Manual for the Examination and Processing of Human Semen" (5$^{th}$ Edition) for the quality control of the experiments.

The optimal exposure dose and duration of the reagents for the following experiments were determined based on the vitality of the sperms.

### 2.2. Assay of Sperm Autophagy Level with MDC Method

According to the instructions of the cell autophagy staining test kit (Monodansylcadaverine, MDC method, Solarbio, China), the semen sample was washed and re-suspended, and the cell concentration was adjusted to $1 \times 10^6$ cells/mL. 10 μL MDC Stain was added and gently mixed for staining at room temperature in darkness for 15 - 45 minutes, then it was centrifuged at ×800 g for 5 minutes. The cells were collected and re-suspended, and the droplet was smeared on the glass slide and covered. Then cell autophagy was observed with a fluorescence

microscope (Leica, Germany) with excitation filter wavelength of 355 nm and blocking filter wavelength of 512 nm within 1 hour after staining. The autophagy rate (%) was calculated.

### 2.3. Detection of Sperm ATP Level by Spectrophotometry

Semen sample was washed with HTF preheated to 37˚C, then incubated at 37˚C in 5% $CO_2$ incubator. After centrifugal purification, the sperm concentration was adjusted to $1 \times 10^6$ cells/mL and lysed. According to the instructions of the enhanced ATP detection test kit (Beyotime, China), ATP concentration (nmol/μL) in the sample was detected using a full-wavelength scanning multifunctional microplate reader (BioTek, USA) at the excitation wavelength of 535 nm and the emission wavelength of 587 nm.

### 2.4. Measurement of Sperm ROS Level with Fluorescent Method

ROS were detected using the fluorescent probe DCFH-DA of the Reactive Oxygen Species Assay Kit (Yeasen, China). According to the instructions, the probe was loaded, incubated and washed in turn. The fluorescence microscope was separately set with excitation/emission at 485 nm and 535 nm for ROS detection using the FITC channel. The ROS level (%) was then computed.

### 2.5. Analysis of Sperm MMP Level with JC-1 Method

According to the guide of the mitochondrial membrane potential detection test kit (Byotime, China), the suspension of sperm sample was transferred into a test tube and JC-1 staining working solution was added and mixed. The mixture was incubated in a light-shielded constant temperature water bath at 37˚C for 20 minutes and then centrifuged. The sediment was retained and washed with JC-1 staining buffer (1×). The sperm cells were re-suspended in JC-1 staining buffer (1×) and centrifuged again. Then 1× JC-1 staining working solution was added and placed in a fluorescence microplate reader. The excitation light and emission light were respectively set at 525 nm and 590 nm to detect JC-1 polymer (%).

### 2.6. Detection of the Levels of Sperm cAMP, CFTR, AMPK, PKA and LC3B

The cell concentration was adjusted to $1 \times 10^6$ cells/mL with PBS (pH 7.2 - 7.4). After repeated freezing and thawing, the intracellular components were released and centrifuged twice, and the supernatant was collected. According to the manual of the human cAMP monophosphate ELISA kit (Jiangsu Jingmei, China), samples were incubated and dyed. The concentration of cAMP (nmol/L) was detected using a full-wavelength scanning multifunctional microplate reader and calculated.

Similar operations were performed on sperms for the assessment of the levels of CFTR, AMPK, PKA and (microtubule-associated proteins 1A/1B light chain

3B) LC3B with ELISA according to the operation instructions of the kits.

### 2.7. Statistical Analyses

All data were statistically analyzed using SPSS19.0 (SPSS Inc., Chicago, IL, USA). The general clinical data were compared using t-test or chi-square test to check the differences between the two groups. The effect of the reagents on sperm vitality was analyzed by two-way repeated measures ANOVA to investigate both main effects and interaction effects. The levels of sperm ROS, MMP, cAMP, CFTR, PKA, AMPK and LC3B before and after treatment in each group were compared and analyzed using paired sample t-test. Because the data of sperm autophagy levels and ATP within the group were not normally distributed, they were analyzed by Wilcoxon signed-rank test for paired samples. Multiple factor linear regression analysis was applied on the association of PR and the chemicals under the adjustment of the bias. p was set at <0.05, double sided.

## 3. Results

### 3.1. General Clinical Condition of the Subjects

A total of 202 subjects was qualified and included in this study. The subjects were all mentally and neurologically health, without any major diseases or family health history, or exposure of severely harmful chemicals. The semen samples were randomly divided into two treatment categories, *i.e.*, CFTRinh-172 treatment or FSK treatment. There was no significant difference in age, abstinence days, sperm concentration, total sperm motility, percentage of progressive sperm (PR, %), body mass index, smoking rate, and drinking rate between the two groups (Table 1).

### 3.2. Impact of Reagents on PR of Sperms

After incubation with CFTRinh-172 or FSK for 60 minutes, the PR of the exposure groups was significantly lower than that of the control groups, and there

Table 1. General and clinical conditions of the subjects (SD ± SEM or n, %).

| | CFTRinh-172 treated semen (n = 101) | FSK treated semen (n = 101) |
|---|---|---|
| Age (years) | 33.54 ± 4.41 | 33.32 ± 5.0 |
| Body mass index (kg/m$^2$) | 22.57 ± 1.61 | 22.38 ± 1.27 |
| Smoking (n, %) | 33 (32.67) | 41 (40.59) |
| Drinking (n, %) | 43 (42.57) | 45(44.55) |
| Abstinence days (days) | 4.30 ± 1.41 | 5.02 ± 3.84 |
| Sperm concentration (×10$^6$cells/mL) | 98.93 ± 16.54 | 106.74 ± 18.19 |
| Total sperm motility (%) | 76.48 ± 9.84 | 75.97 ± 10.53 |
| PR of sperm motility (%) | 58.72 ± 12.44 | 57.02 ± 12.92 |

were also differences among time points. The multiple factor linear regression analysis found that the longer exposure time of CFTRinh-172, the lower PR ($\beta$ = −0.053, P < 0.05); and the higher concentration of CFTRinh-172, the lower PR ($\beta$ = −0.002, P < 0.001) (Figure 1(a)). Similar results were observed on the regressions of FSK, *i.e.*, the longer exposure time of FSK, the lower PR ($\beta$ = -0.261, P < 0.001); and the higher concentration of FSK, the lower PR ($\beta$ = −0.010, P < 0.05) (Figure 1(b)). The results suggest that CFTRinh-172 or FSK had a significant dose- and time-dependent inhibitory effect on PR of sperm motility.

The optimal conditions for samples treatment were determined as 5000 μM CFTRinh-172 or 50 nM FSK for 60 minutes, based on the moderate PR rates.

### 3.3. Effect of Reagents on Sperm Autophagy Levels

Observed and counted under a positive fluorescence microscope, the sperm autophagy rate was found to be increased significantly after 60 minutes of treatment with 5000 μM CFTRinh-172 (Figure 2(a)) or 50 nM FSK (Figure 2(b)) compared with the control groups.

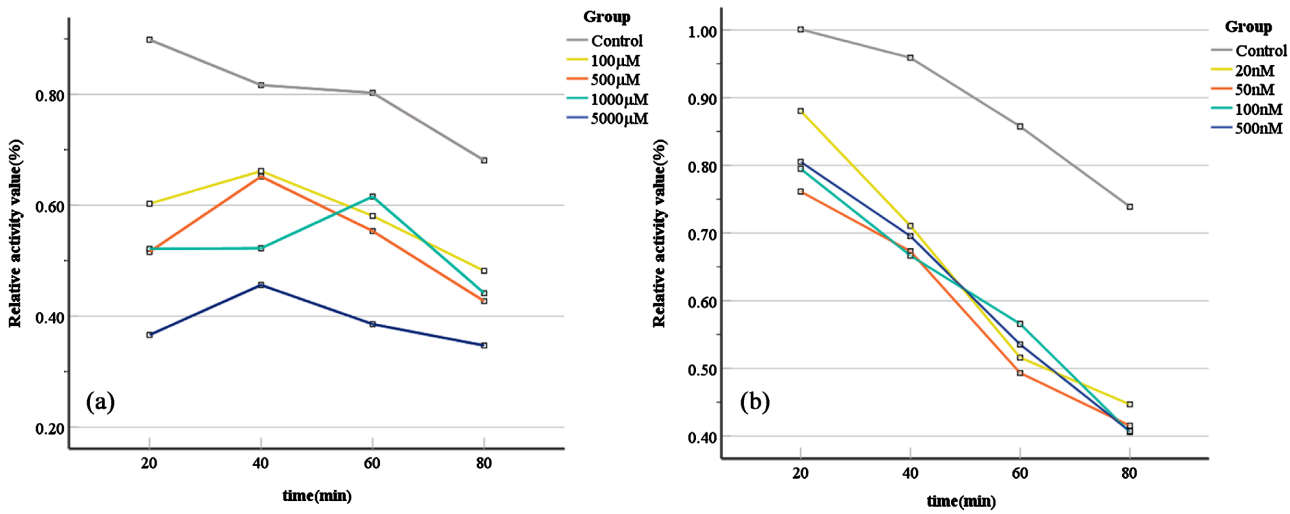


Figure 1. Incubation with CFTRinh-172 (a) or FSK (b) decreased the rates of progressive sperm in a way associated with exposure concentrations and duration (n = 15).

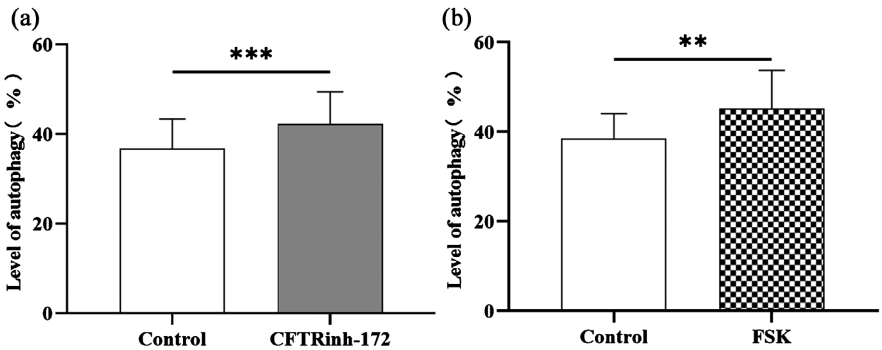


Figure 2. Incubation with CFTRinh-172 ((a), ***p < 0.001) or FSK ((b), n = 20, **p < 0.01) for 60 minutes increased autophagy rates of sperm.

### 3.4. Influence of Reagents on Sperm ATP Levels

After incubation with 5000 μM CFTRinh-172 (Figure 3(a)) or 50 nM FSK (Figure 3(b)) for 60 minutes respectively, the sperm ATP production was significantly reduced compared with the control groups.

### 3.5. Impact of Reagents on Sperm ROS Levels

After treatment with 5000 μM CFTRinh-172 (Figure 4(a)) or 50 nM FSK (Figure 4(b)) for 60 minutes respectively, there was no significant difference in sperm ROS content compared with the control groups.

### 3.6. Effect of Reagents on Sperm MMP

After incubation with 5000 μM CFTRinh-172 (Figure 5(a)) or 50 nM FSK (Figure 5(b)) for 60 minutes respectively, the sperm levels significantly decreased compared with the control groups.

### 3.7. Impact of Reagents on Sperm cAMP, CFTR, AMPK, PKA and LC3B

The incubation of sperms with 5000 μM CFTRinh-172 or 50 nM FSK for 60 minutes respectively significantly reduced the levels of cAMP (Figure 6(a), Figure 6(b)), CFTR (Figure 6(c), Figure 6(d)) and PKA (Figure 6(e), Figure 6(f)) of

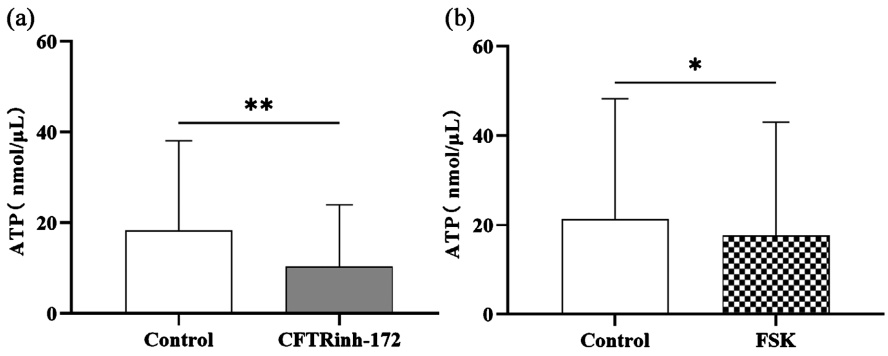


Figure 3. Incubation with CFTRinh-172 ((a), n = 20, **p < 0.01) or FSK (b), n = 20, *p < 0.05) for 60 minutes decreased ATP concentrations of sperm.

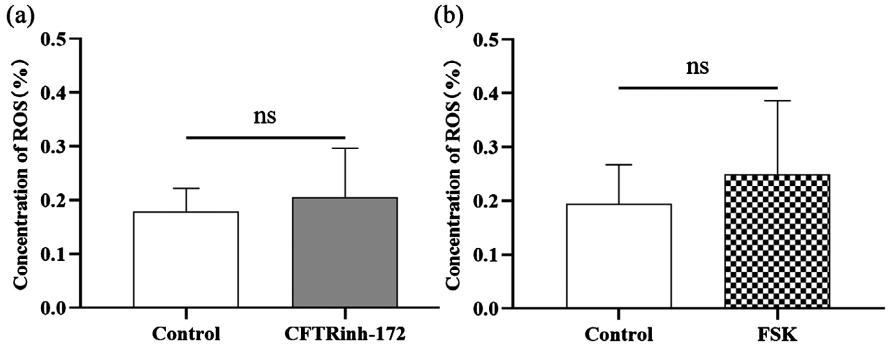


Figure 4. Incubation with CFTRinh-172 ((a), n = 15, p > 0.05) or FSK ((b), n = 15, p > 0.05) for 60 minutes showed no significant effect on ROS levels of sperm.

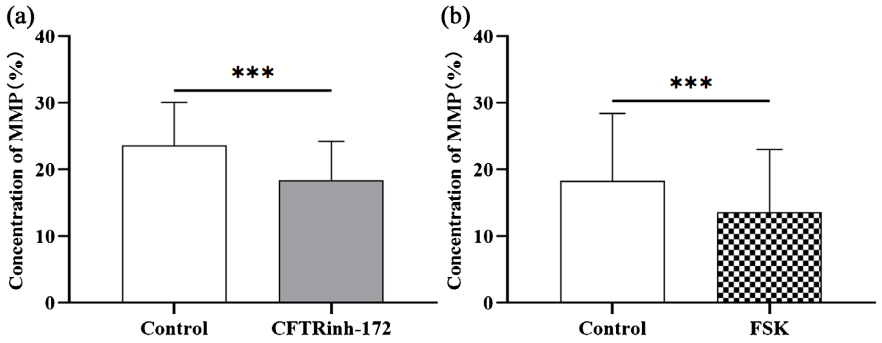


**Figure 5.** Incubation with CFTRinh-172 ((a), n = 20, ***p < 0.01) or FSK ((b), n = 20, ***p < 0.001) for 60 minutes decreased MMP levels of sperm.

the sperms, but increased the levels of AMPK (**Figure 6(g)**, **Figure 6(h)**) and LC3B (**Figure 6(i)**, **Figure 6(j)**), compared with their control groups.

## 4. Discussion

This study found that incubated with CFTR disruptor, CFTRinh-172 or FSK, human sperm showed reduced ATP level, mitochondrial membrane potential, cAMP content and CFTR and PKA protein expression levels, while increased the degree of autophagy, AMPK and LC3B protein expression levels, and no significant difference of ROS content. The results suggested that CFTR might work with cAMP and its downstream effectors such as PKA and AMPK to regulate MMP and energy metabolism, affecting sperm autophagy and vitality. This study provided novel insight into the mechanism of CFTR affecting sperm vitality through autophagy.

### 4.1. CFTR and Autophagy of Human Sperm

Studies found that CFTR level in sperm was positively correlated with sperm forward motility [12] [13], the expression rate of CFTR in infertile patients (including teratozospermia, asthenozoospermia, oligozoospermia) was significantly lower than that in normal male fertility (35.89% and 88.13%, respectively) [14]. In patients with asthenozoospermia, low CFTR expression level is adverse to progressive motility of sperm, sperm capacitation rate, and acrosome reaction rate (OR values are 3.3, 2.7, 3.1 respectively, p values are all less than 0.01) [12]. This study set 5 concentration gradients for CFTR disruptors, CFTRinh-172 and FSK, respectively, and detected sperm vitality at varied time points. It was found that the inhibitory effect of CFTRinh-172 or FSK on sperm vitality showed significant correlations with dose or duration of exposure.

Sperm eliminates unnecessary cytoplasmic components through autophagy to promote flagellum formation and facilitate sperm movement [7]. The level of CFTR in cells is closely related to autophagy and is crucial to keep normal function for cells [15] [16]. The results of this study suggested that the decreased CFTR levels in normal human sperm might promote autophagy rate and damage sperm vitality. However, some studies showed that natural antioxidant

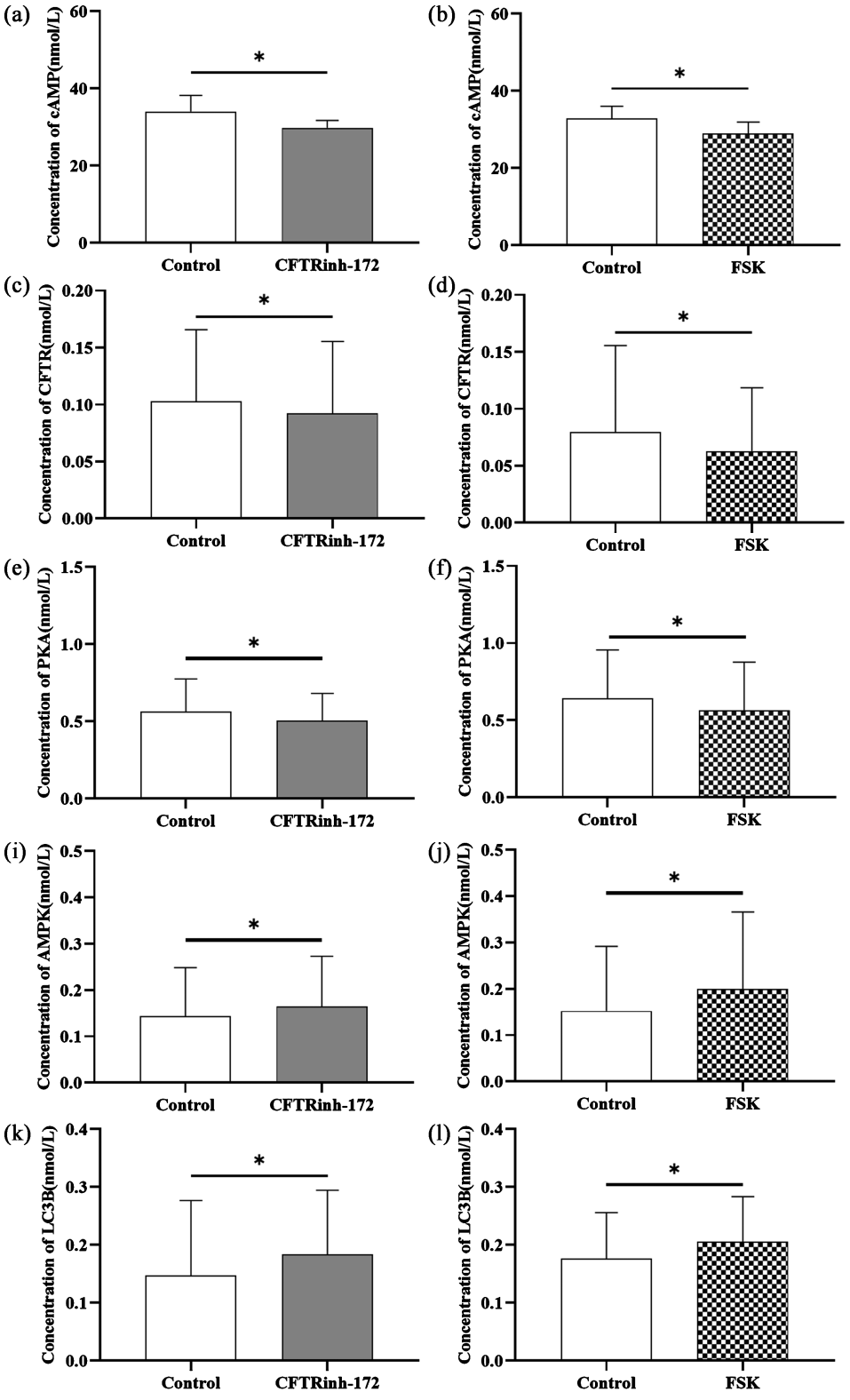


**Figure 6.** Impact of drugs on sperm cAMP, CFTR, AMPK, PKA and LC3B (n = 6, *p < 0.05). Incubation with CFTRinh-172 for 60 minutes down-regulated levels of cAMP (a), CFTR (c), PKA (e) of the sperms, while up-regulated that of AMPK (g) and LC3B (i). Likewise, incubation with FSK for 60 minutes decreased the levels of cAMP (b), CFTR (d), PKA (f), while increased that of AMPK (h) and LC3B (j) of sperm.

epigallocatechin gallate (EGCG) restored CFTR function in type II CFTR mutation patients [17] or mice [18], promoting cell autophagy. The varied effects of CFTR on cells possible related to 1) type and health condition of cells and 2) quantity and activity of CFTR.

This study found that, compared with the control groups, the sperm motility decreased significantly after incubation with CFTRinh-172 or FSK for 60 minutes. The inhibitory effect of CFTRinh-172 on sperm motility showed correlation with dose or exposure duration, which was in line with expectations. FSK promoted sperm autophagy, which was consistent with similar research results [17] [18]. However, the ROS level of the FSK group did not change significantly, and the sperm motility decreased, which was unexpected. Even after we made multiple and large adjustments on FSK concentration (10 nM - 50 μM) and action time (5 - 150 minutes) during the pilot experiments, the trend of the results remained unchanged. This was possibly related to its diverse mechanisms. FSK promoted autophagy of various tumor cells through the PI3K/AKT/mTOR signaling pathway [19], which plays a key role in sperm energy metabolism, cell growth, proliferation and differentiation processes [20]. Similarly, antioxidants such as molecular hydrogen regulate the balance between autophagy and oxidative stress through PINK1 (PTEN-induced putative kinase 1)/Parkin, p53, mTOR, PI3K/Akt/Gsk3$\beta$, Fork-head-box O1 (FoxO1) and other pathways to affect cell vitality. This suggests that it is worthy of further exploring the effect of antioxidant FSK on sperm autophagy and vitality [21].

### 4.2. CFTR and Mitochondrial Function of Sperms

CFTR induced changes in intracellular chloride anion (Cl–) concentration affect diverse cellular functions such as gene and protein expression and activities, post-translational modifications of proteins, cellular volume, cell cycle, cell proliferation and differentiation, membrane potential, reactive oxygen species levels, and intracellular/extracellular pH. Cl– also modulates functions in different organelles, including endosomes, phagosomes, lysosomes, endoplasmic reticulum, and mitochondria. Mitochondria are the only organelles retained by mature sperm, which continuously produce ROS to maintain normal physiological activities of sperm such as acrosome reaction and sperm capacitation [22]. However, excessive ROS over the antioxidant capacity could impair the mitochondrial membrane of sperm and reduce the MMP [23]. In the present study, ROS level of sperm increased slightly after incubation with 5000 μM CFTRinh-172 or 50 nM FSK for 60 minutes, respectively, but there was no significant difference compared with their control groups, and MMP decreased in both treatment groups. The MMP was positively correlated with sperm motility. The decrease of MMP inhibited ATP synthesis and sperm motility [24]. This study found that ATP level of sperm after 60 minutes of treatment with 5000 μM CFTRinh-172 or 50 nM FSK, respectively, was significantly lower than that of their control groups, indicating that CFTR may interfere with MMP and cause energy meta-

bolism disorders.

### 4.3. Synergy of CFTR and cAMP Downstream Effectors on Sperm Autophagy

Sperm motility is closely related to transmembrane signaling of cells, among which the cAMP/PKA pathway is the critical one. CFTR and PKA and AMPK are all downstream effectors of cAMP. PKA and AMPK are generally paired enzymes to regulate energy metabolism and maintain redox balance by sensing the AMP/ATP ratio. Under the condition of sufficient ATP, PKA acts with pyruvate synergistically to maintain normal cell structure and function; otherwise, PKA inhibits the generation of cAMP from ATP de-phosphorylation, and releases cytochrome C from mitochondria, and activates AMPK [25]. CFTR also interacts with other downstream effectors of cAMP such as PKA and AMPK. The combination of PKA and CFTR enhances CFTR activity, while the combination of AMPK and CFTR reduces CFTR activity [6]. Inhibition of CFTR reduces human sperm PKA activity [7] and increases AMPK activity [8]. The content of LC3B is proportional to the degree of autophagy [3]. This study found that treatment with 5000 μM CFTRinh-172 or 50 nM FSK for 60 minutes, respectively, reduced the levels of cAMP, CFTR and PKA proteins in sperm compared with their control groups, while the levels of AMPK and LC3B proteins increased. The decrease of MMP inhibits ATP synthesis and directly causes the decrease of cAMP level [24]. This consistency of the levels of CFTR, autophagy, ATP and MMP suggested that CFTR interacts synergistically with cAMP, PKA and AMPK in cells to regulate sperm autophagy.

### 4.4. Limitations

Firstly, because the subjects were only from men undergoing pre-marital health check-ups instead of male adults of community, then some bias such as health condition and semen quality possibly were introduced, which might affect the generalizability of the results. Secondly, the sample volume was limited, preventing the use of multiple intervention agents. Thirdly, CFTR plays broad functions via complex mechanisms, but only few proteins were analyzed on the expression levels in the present study. Therefore, the roles and mechanisms of CFTR in sperm autophagy and vitality were not fully revealed. We will further explore these aspects including intracellular chloride anion (Cl–) concentration, post-translational modifications of proteins, cell cycle, and intracellular/extracellular pH using transgenic and gene knockout techniques, various omics techniques in a larger population sample in future research.

## 5. Conclusion

Low level of CFTR performed with cAMP and its downstream effectors such as PKA and AMPK to regulate MMP and energy metabolism, leading to increased autophagy rate and reduced motility of sperm. The findings would provide a

novel theoretical basis for the diagnosis and treatment of low vitality of human sperm.

## Author Contributions

Yonghua He and Chaoyan Ou designed the experiments and wrote the manuscript. Jie Hu, Han Liu, Liangzhao Liu and Linfeng Mo carried out the experiments and analyzed the data. Xuming Liang supervised and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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## Ethical Considerations & Disclosure(s)

This study obtained the approval of the Medical Ethics Committee of Guilin Medical College Affiliated Hospital and the informed consent of the research subjects. The study was conducted in accordance with the local legislation and institutional requirements.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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