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Original Article

AF-2364 is a prospective spermicide candidate

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Abstract

Inhibition of sperm motility has recently become a promising target for male contraceptive development. AF-2364, an analogue of Lonidamine (LND), had a contraceptive effect when orally administered to adult Sprague–Dawley rats. LND can also target mitochondria to inhibit oxygen consumption and block energy metabolism in tumour cells. However, there are no reports of the effects of AF-2364 on human sperm function. Herein we describe the action of AF-2364 on human sperm *in vitro*, as well as the mechanisms involved. AF-2364 specifically blocked human sperm motility *in vitro*. Further experiments revealed that AF-2364 can target sperm mitochondrial permeability transition (MPT) pores to induce the loss of sperm mitochondrial membrane potential ($\Delta\Psi m$) and decrease ATP generation; however, no significant changes in the cytoskeletal network or the human sperm proteome were detected after exposure to AF-2364. Incubation of AF-2364 with other human or mouse cell lines indicated that the spermicidal effect at the lower concentration was specific. In summary, the spermicidal effect of AF-2364 involves direct action on sperm MPT pores, and this compound should be further investigated as a new spermicide candidate.

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Keywords: AF-2364, mitochondria permeability transition pore, sperm motility, spermicide

1 Introduction

Several recent studies have focused on inhibition of sperm motility as a promising target for male contraceptive development [1]. The major energy source for activated sperm motility is the mitochondrial sheath located in the mid-piece of the flagellum, which produces ATP through the tricarboxylic acid cycle. There is a long-standing debate on whether glycolysis or oxidative phosphorylation (OXPHOS) is the major biochemical pathway supplying the energy needed to mediate sperm motility [2]. Mitochondria clearly contribute to sperm motility, as normal mitochondrial structure is vital for motility [3–5], mitochondria are required for sperm function, and mitochondrial activity correlates with sperm motility [6–8]. These studies suggest that the mitochondrial membrane potential ($\Delta\Psi m$), used as a mitochondrial function marker, is associated with

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sperm motility and even *in vitro* fertilization rate [9–12]. Therefore, any chemical agent that can target sperm mitochondria and regulate the mitochondrial ΔΨm might inhibit sperm motility and can be developed as a contraceptive candidate.

AF-2364 or Adjudin™, also known as 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, is a less toxic but functionally similar analogue of Lonidamine (LND). AF-2364 has been shown to disrupt adherens junctions (AJs) between Sertoli cells and round and elongated spermatids in the adult rat and has been tested as a potential male contraceptive [13, 14]. In 2006, AF-2364 was conjugated to a recombinant follicle-stimulating hormone mutant protein, which served as its 'carrier' to Sertoli cells in the testis. This study is now moving towards human trials, though some major issues still need to be resolved [15, 16]. Recent research revealed a similar AF-2364-induced inhibition of spermatogenesis in the rabbit [17]. However, no published studies have determined the effects of AF-2364 on human sperm function or fertility in general. As LND can inhibit oxygen consumption and block energy metabolism in tumour cells by targeting the mitochondria permeability transition (PT) pore and causing the loss of ΔΨm [18, 19], we wondered whether AF-2364 would target human sperm mitochondria to influence sperm function and thus warrant further development into a spermicide candidate.

In this work, AF-2364 had a spermicidal effect when applied to human sperm *in vitro*. Subsequent experiments attempted to identify the mechanisms underlying this effect. We determined that AF-2364 blocks energy metabolism by targeting the human sperm mitochondrial PT (MPT) pore and decreasing the mitochondrial ΔΨm. The specificity of the spermicidal effect was confirmed by incubating AF-2364 with other human and mouse cell types. We also evaluated whether *in vitro* exposure to AF-2364 affected the cytoskeleton and used two-dimensional electrophoresis (2-DE) to assess induction of other molecular pathways. Our results indicate that the mitochondrion is the main target of AF-2364 in human sperm.

2 Materials and methods

2.1 Sperm preparation

This study was conducted at the Laboratory of Reproductive Medicine, Nanjing Medical University (Nanjing, China), and the Department of Reproductive Medicine, The First Affiliated Hospital with Nanjing Medical University (Nanjing, China). Sperm samples were collected, with informed consent, in sterile containers after 3–4 days of sexual abstinence. Semen samples were allowed to liquefy for 30 min at 37ºC. Sperm parameters (volume, sperm concentration, percentage of motile sperm and motion characteristics) were evaluated according to the World Health Organization (WHO) semen analysis manual (1999). Samples were then washed in Biggers, Whitten and Whittingham medium (BWW) with 45% and 90% Percoll gradients and finally resuspended in 1 × BWW at a final concentration of 50×10^6 cells mL^{-1}.

2.2 Incubation of sperm with AF-2364 and analysis of sperm motility

AF-2364 (a generous gift from Professor C. Yan Cheng, Center for Biomedical Research, Population Council, New York, NY, USA) was resolved in ethanol as a stock solution at 3 mmol L^{-1}. After sperm preparation, the resuspended sperm were evenly divided and exposed to 24, 48, 72 or 96 μmol L^{-1} (concentrations A, B, C and D, respectively) AF-2364 or the same volume of ethanol for 15, 30, 60, 150 or 240 min at 37ºC with 5% CO_2. At each time point, 10 μL of sperm was used for computer-assisted semen analysis (CASA) detection (Hamilton-Thorne Research Inc., Beverly, MA, USA). Motility parameters for the experimental and control groups were measured and analyzed by paired *t*-test.

2.3 Sperm mitochondrial activity evaluation

To evaluate sperm mitochondrial activity, we measured changes in the mitochondrial ΔΨm using a unique fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1-1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1. A green fluorescent JC-1 probe exists as a monomer at low membrane potentials; however, at higher potentials, JC-1 forms yellow-fluorescent 'J-aggregates'. Briefly, after sperm samples were prepared, we incubated the resuspended cells with 72 μmol L^{-1} AF-2364 or the same volume of ethanol for 15, 30, 60, 150 or 240 min at 37ºC with 5% CO_2. Washed sperm cells from each time point were then incubated at 37ºC for 10 min with 5 μg mL^{-1} JC-1 (Beyotime Biotech, Nantong, China). Images were viewed under a fluorescence microscope (Axioskop 2 plus; Carl Zeiss, Thornwood, NY, USA) at × 200 magnification with an excitation/emission filter of 488/490 nm. The yellow/(yellow + green) fluorescence ratio and the mean densities of

the yellow fluorescent signals were calculated by flow cytometry (FACS Calibur; BD Biosciences Pharmingen; San Jose, CA, USA). For each analysis, 20 000 cells were examined and debris was ruled out based on light scatter measurements. Flow cytometry acquisition for JC-1-stained cells was performed through the FL2 channel. Data were analyzed using Cell Quest software (Becton Dickinson; Franklin Lakes, NJ, USA).

2.4. Co-incubation of sperm with AF-2364 and cyclosporin A (CsA) and sperm motility measurements

To further explore the effect of AF-2364 on sperm mitochondria, CsA, an inhibitor of the MPT, was co-incubated with the sperm and AF-2364. CsA (Sigma; St. Louis, MO, USA) was dissolved in ethanol as a stock solution at 5 mmol L^{-1}. After sample preparation, the sperm were co-incubated with 72 μmol L^{-1} AF-2364 and 5 or 10 μmol L^{-1} CsA at 37ºC with 5% CO_2. The same sperm samples were also incubated with ethanol or ethanol plus CsA as two control groups. Sperm motility parameters for each group were characterized at 15, 30, 60, 150 and 240 min with CASA detection (Hamilton-Thorne Research Inc.).

2.5 Sperm ATP analysis

After 15, 30, 60, 150 or 240 min incubation with 72 μmol L^{-1} AF-2364 or ethanol, sperm samples were washed twice and then resolved and vortexed in lysis buffer on ice. ATP was measured by luminometric methods using commercially available luciferin/luciferase reagents according to the manufacturer's instructions (ATP Assay Kit; Beyotime Biotech) on a luminometer (TD-20/20; Turner Designs; Sunnyvale, CA, USA). An average of 50×10^6 sperm was used for ATP analysis, and the ATP concentration was normalized to the standard curve.

2.6 Eosin test

Sperm eosin tests were performed after 15-min incubations with AF-2364 at A, B, C or D concentrations and after 15-, 30-, 60-, 150- or 240-min incubations with concentration C. Briefly, after the exposure, we mixed 0.1 mL sperm suspension with 0.1 mL eosin Y solution (0.5% [wt/vol] eosin in saline) and incubated the mixture at room temperature for 1 min. This mixture was then smeared on a slide, and at least 200 spermatozoa were scored on a light microscope. Spermatozoa that exhibited abnormal membrane structure and thus permitted eosin to enter the cell stained positive (orange) in the head region, while spermatozoa with normal membrane structure remained unstained.

2.7 Cell culture

A human hepatoma cell line (SMMC-7721 cells), a gastric carcinoma tumour cell line (SGC-7901) and mouse spermatocytes (GC-2) were purchased from ATCC (Manassas, VA, USA). SMMC-7721 cells and SGC-7901 cells were maintained in RPMI 1640 culture medium, while GC-2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) in a 37ºC incubator supplied with 95% room air and 5% CO_2. Calf serum (10%), 50 U mL^{-1} penicillin and 50 U mL^{-1} streptomycin were added to the culture medium. At confluence, the cells were trypsinized with 0.25% trypsin (AMRESCO; Solon, OH, USA; dissolved in PBS, pH 7.4), counted and placed in 96-well plates at a density of 5×10^4 mL^{-1} (180 μL per well) overnight (24 h).

2.8 Incubation with AF-2364 and MTT assay

The above cells were incubated with 96 μmol L^{-1} AF-2364 (experimental group), the same volume of ethanol (control group) or medium alone (blank group) for 15, 30, 60, 150 or 240 min, or extended to 8, 12 or 24 h at 37ºC with 5% CO_2. Assessment of *in vitro* cytotoxicity was done based on the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium) assay. Briefly, MTT was resolved in PBS as a stock solution of 5 mg mL^{-1}. For each treatment group, 20 μL MTT was added to each well to achieve a final concentration of 0.1 mg mL^{-1}. After a 1.5-h incubation at 37ºC, the supernatant was discarded and 150 μL DMSO was added. The 96-well plate was vibrated on a micro-vibrator for an additional 30 min and absorbance was determined using a multiplate reader (Model 680, Bio-Rad; Hercules, CA, USA) at a wavelength of 570 nm (A570). Four values were acquired for each treatment group, and at least three independent experiments were performed for each cell line.

2.9 Sperm protein extraction and two-dimensional electrophoresis (2-DE)

To further study the possible mechanism(s) by which AF-2364 inhibits human sperm motility, we used 2-DE to explore changes that occurred in the protein profile after AF-2364 treatment. We performed this experiment twice. The first time, we collected sperm samples from four individuals and treated each sample

as described above. The second time, we took sperm cells from 12 normal males and combined the samples from four individuals into one pooled sperm sample. After the sperm preparation, the separate or pooled samples were divided into half and incubated with AF-2364 or ethanol for 15 min. Therefore, there were four independent groups in the first experiment and three independent groups in the second experiment. The sperm protein extraction and 2-DE were performed as previously described by our laboratory [20, 21].

2.10 Gel image analysis and protein identification

Gels (eight in the first 2-DE experiment and six in the second) were visualized by silver staining and scanned sequentially. ImageMaster™ 2D platinum software (Version 5.0, GE Healthcare; San Francisco, CA, USA) was used to detect, quantify and match spots, as well as to perform comparative and statistical analyses on the eight simultaneously run gels from the four samples in the first 2-DE and the six gels from the second experiment. The expression level was determined using the relative volume of each spot on the gel and expressed as % Volume (% Vol = [spot volume / ∑volumes of all spots resolved in the gel]). A spot was regarded as significantly differentially expressed between groups if the average spot intensity difference was greater than 1.5-fold and the *P* value by paired *t*-test was less than 0.05. For detailed procedures, please see the reference papers published by our lab [22, 23].

2.11 Statistical analysis

All of the above experiments were repeated at least three times with different sperm samples, except the 2-DE experiments. Differences between treatments were analyzed using paired *t*-tests. All of the percentage values were subjected to arcsine square-root transformation. $P < 0.05$ was considered statistically significant. All data presented are means with the mean ± SE. We only show the significant differences between the experimental and control groups.

3 Results

3.1 AF-2364 inhibited sperm motility

CASA analysis showed that sperm motility parameters were significantly lower in the AF-2364-treated group (experimental group) compared with the ethanol-treated group (control group) at concentrations C and D. The percentage of motile spermatozoa was significantly greater in the control group ($P < 0.05$) (Figure 1A), and the percentage of rapid progressive motility sperm (A-type sperm) was much lower in the AF-2364 group ($P < 0.01$) (Figure 1B). After a 15-min incubation with 96 μmol L^{-1} (0.032 mg mL^{-1}) AF-2364, human sperm viability was inhibited by over 90%, and the percentage of A-type sperm dropped to nearly 0%. Furthermore, these sperm motility defects were time dependent. To minimize the solvent effect in the statistical analysis, we show the Δ effect (AF-2364 minus ethanol) experi-

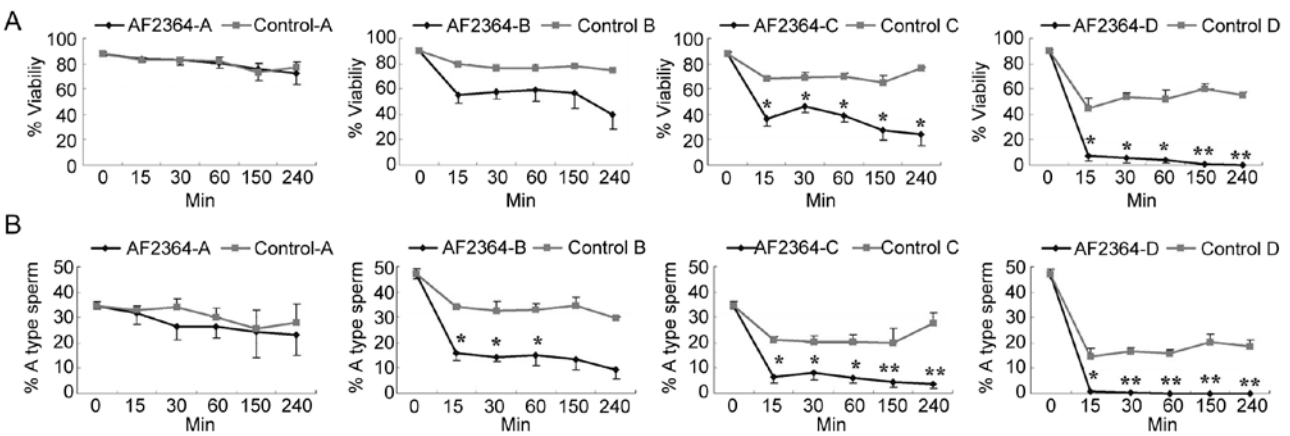

Figure 1. AF-2364-induced human sperm motility inhibition. (A): The total viability (percentage of a + b + c sperm) of human sperm incubated with 24, 48, 72 or 96 μmol L^{-1} (concentration A, B, C and D) AF-2364 or the same volume of ethanol (Control A, B, C and D) for 15, 30, 60, 150 and 240 min. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control. (B): The percentage of rapid progressive motility sperm (A type sperm) for each concentration as above. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control.

enced by the sperm samples (Supplementary Figure 1). We also recorded a sample video for each concentration, available for viewing as Supplementary data at http://reprod.njmu.edu.cn/data/index.htm. Since concentration C was the lowest effective dose, we used it as the experimental dose in subsequent experiments.

3.2 Impaired sperm mitochondrial ΔΨm

When the mitochondrial ΔΨm is intact, the JC-1 reagent aggregates inside the healthy, non-damaged mitochondria and fluoresces yellow. When the mitochondria are damaged, the ΔΨm will break down, and the JC-1 reagent will appear dispersed and fluorescent green (Figure 2). After treatment with 72 μmol L^{-1} AF-2364, the percentage of yellow sperm was significantly lower, and the mean density of the yellow fluorescent signals was decreased compared with the control group. For example, at the 15-min time point, the percentage of JC-1-positive (yellow) sperm and the mean electronic intensity decreased by 10.3% and 23.1% relative to the control group, respectively. Sperm ΔΨm was progressively lost throughout the sperm population as incubation time increased (Figures 3 and 4). To verify the flow cytometry results, we also observed the sperm under a fluorescent microscope after JC-1 staining at each incubation time point and provide images for the experimental and control groups at the 30- and 240-min time points as examples (Figure 5). To determine whether the JC-1-negative sperm were alive or dead, we combined JC-1 with the propidium iodide (PI) viability stain. These results can be seen in Supplementary Figure 2.

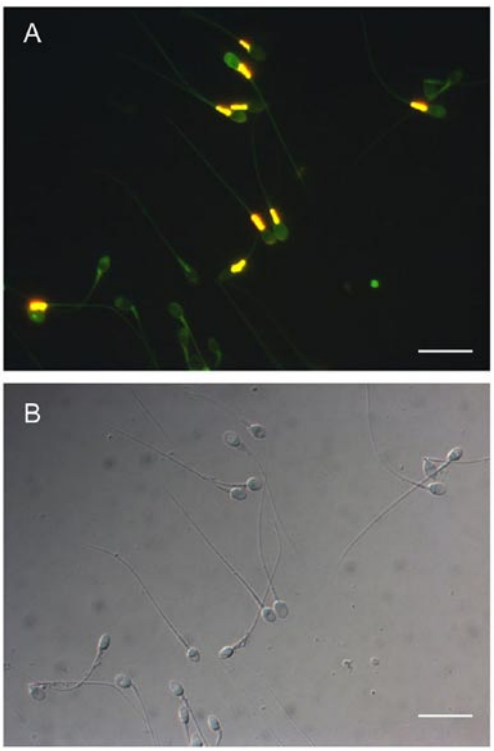

Figure 2. Representative image of nondamaged and damaged mitochondria in human sperm. (A): The nondamaged mitochondria with yellow fluoresce in the midpiece of sperm and the damaged sperm with green fluoresce. (B): Brightfield figure. Bars = 10 μm.

3.3 Partial recovery of sperm motility after co-incubation of sperm with AF-2364 and CsA

The AF-2364-induced inhibition of sperm motility was partially rescued by co-treatment with CsA. Sperm viability recovered 70.8% at 15 min and the percentage of A-type sperm recovered 50.7%, 18.2% and 18.5% at the 15-, 30- and 60-min time points, respectively, with 5 μmol L^{-1} CsA and AF-2364 ($P < 0.05$). Furthermore, when the sperm were co-incubated with AF-2364 and 10 μmol L^{-1} CsA, the protective effects were even more significant. The *P* values for viability and A-type sperm were less than 0.05 or 0.01 at each time point between the AF-2364 group and the AF + CsA group. However, there were no significant differences between the control group and the control + CsA group (Figure 6).

3.4 Decrease in sperm ATP levels without changes in sperm survival rates

After exposure to 72 μmol L^{-1} AF-2364, sperm ATP levels were significantly lower than in the control group. ATP concentrations were reduced at the 30-, 60- and 150-min time points ($P < 0.05$) (Figure 7). Eosin tests indicated that sperm survival rates were not significantly different under 72 μmol L^{-1} AF-2364 (concentration C, Figure 8B), suggesting that the ATP changes were due to the action of AF-2364 on sperm mitochondria. At the same time, we also detected a huge decrease in survival rate when sperm were treated with 96 μmol L^{-1} AF-2364 (concentration D, Figures 8A and 9).

3.5 Decreased viability rates after a long incubation with AF-2364

When SMMC-7721, SGC-7901 and GC-2 cells were incubated with the highest concentration of AF-2364 (96 μmol L^{-1}; concentration D) for the same dura-

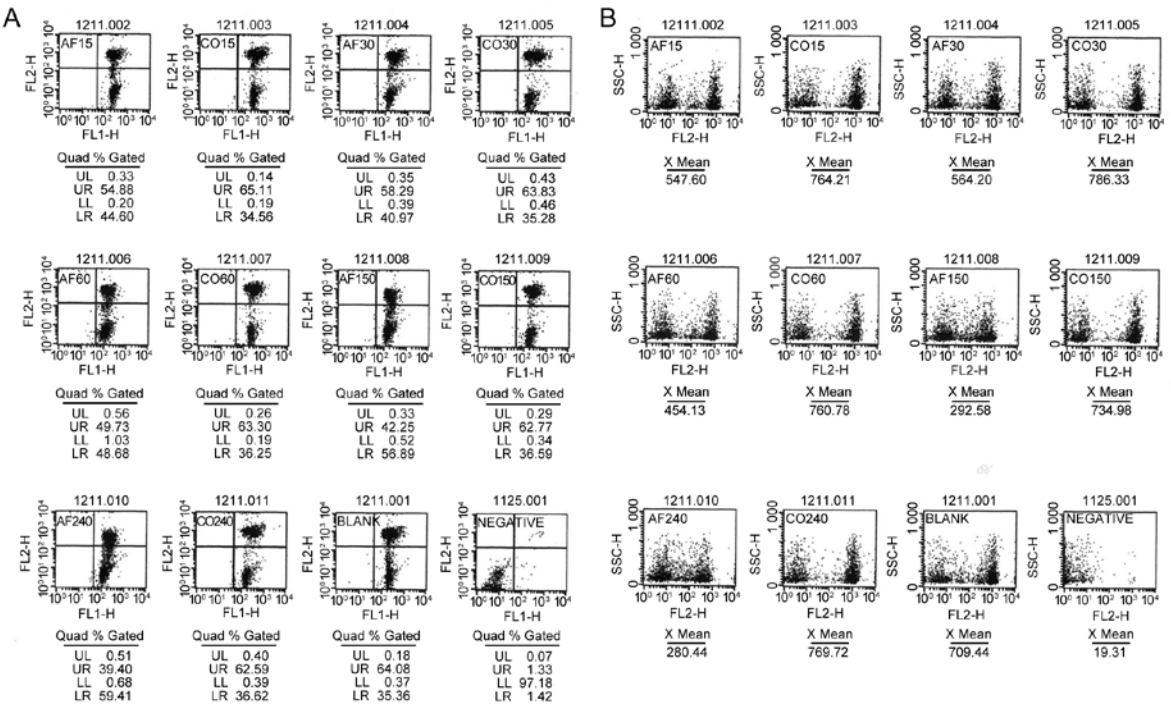

Figure 3. One of the JC-1 staining results of flow cytometry. (A): The percentage of yellow cells (UR). (B): The mean densities of the yellow fluorescent signals (X mean). AF15, 30, 60, 150, 240: sperm treated with AF-2364 at time point of 15, 30, 60, 150, and 240 min, respectively; CO15, 30, 60, 150, 240: control sperm at the same time point. BLANK: sperm stained with JC-1 directly at time 0 min. NEGATIVE: sperm with no staining.

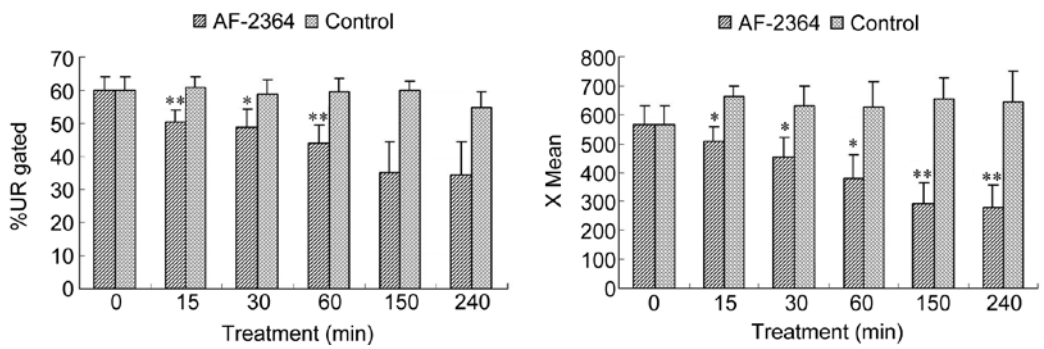

Figure 4. Flow cytometry analysis result of human sperm JC-1 staining after exposure of AF-2364. (A): Percentage of JC-1 positive sperm, showed with %UR gated. (B): The mean densities of the positive sperm fluorescent signals, showed with X mean. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control.

tions as the human sperm cells, no significant differences were observed between the experimental and control groups (Figure 10A). However, when we extended the incubation time to 8, 12 or 24 h, the survival rates were notably decreased and more severe in the experimental groups than in the control or blank groups (Figure 10B). This result suggested that the AF-2364-induced inhibition of sperm motility was sperm-specific at concentrations less than 96 μmol L^{-1} for less than 240 min.

3.6 No significant changes in human sperm proteome

In the first 2-DE, the correlation coefficient was

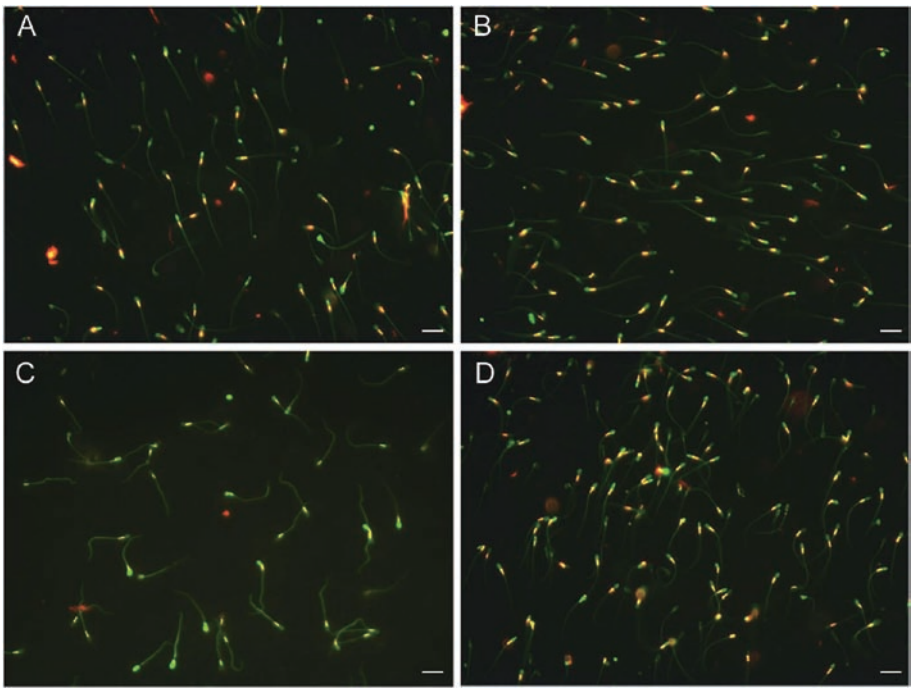

Figure 5. Fluorescent images of human sperm with JC1 staining after 30 or 240 min of incubation with 72 μmol L^{-1} AF-2364 (A, C) or the same volume of ethanol (B, D). Bars = 20 μm.

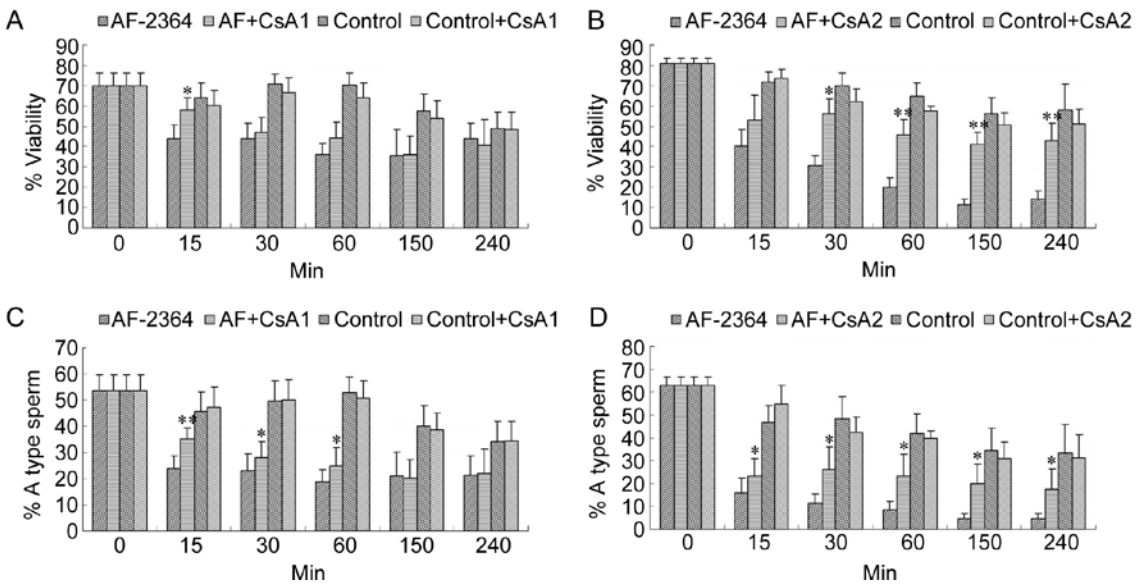

Figure 6. Protective effect of CsA to the sperm motility inhibition induced by 72 μmol L^{-1} AF-2364. (A): Coincubation of sperm with 72 μmol L^{-1} AF-2364 and 5 μmol L^{-1} CsA. (B): Coincubation of sperm with 72 μmol L^{-1} AF-2364 and 10 μmol L^{-1} CsA. AF-2364: sperm incubated with AF-2364 only; AF + CsA1: sperm treated with AF-2364 and 5 μmol L^{-1} CsA together; AF + CsA2: sperm treated with AF-2364 and 10 μmol L^{-1} CsA together; Control: sperm incubated with the same volume of ethanol only; Control + CsA: sperm incubated with the same volume of ethanol and CsA together. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences were observed between the AF-2364 and the AF + CsA groups.

over 0.88 for each gel in the AF-2364-treated group and 0.90 in the control group. In the second 2-DE, the correlation coefficient value was over 0.87 for each gel. These values indicate good reproducibility of the gels in each group, which allowed us to perform differential protein expression analysis. However, there were no significant differences between the AF-2364-treated and control groups in either experiment (Figures 11 and 12).

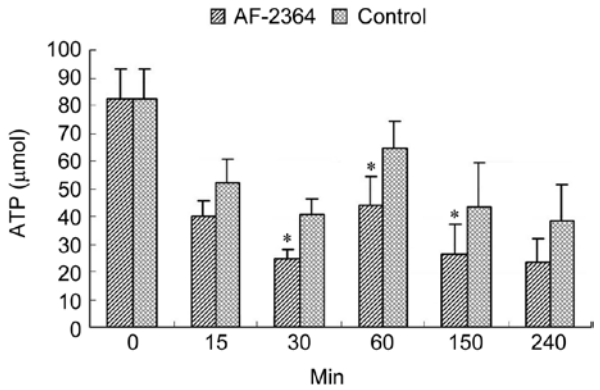

Figure 7. Analysis of sperm ATP after exposure to 72 μmol L^{-1} AF-2364 or the same volume of ethanol at different time points (control). $^{*}P < 0.05$, compared with control.

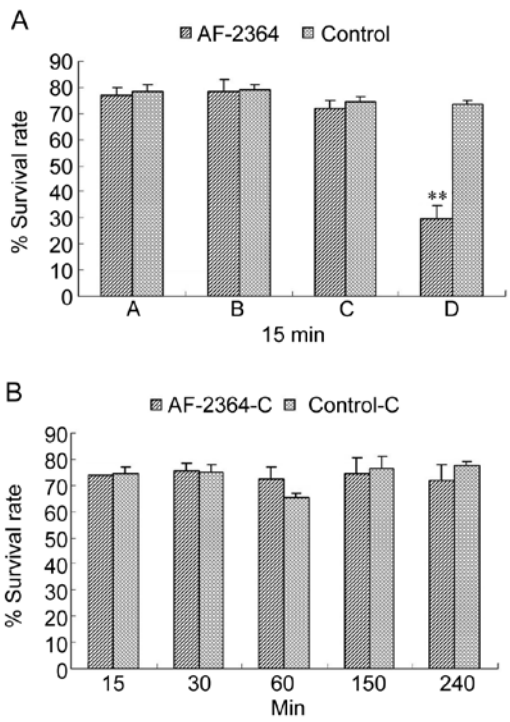

Figure 8. Results of eosin test. (A): The survival rates of sperm in AF-2364 (concentration A = 24 μmol L^{-1}, concentration B = 48 μmol L^{-1}, concentration C = 72 μmol L^{-1}, concentration D = 96 μmol L^{-1}) at 15 min. (B): The survival rates of sperm only in concentration C at 15, 30, 60, 150, 240 min, respectively. $^{**}P < 0.01$, compared with the control.

4 Discussion

4.1 AF-2364 inhibited sperm motility

Sperm structure is simple and well defined, consisting of a head and a tail. The head is needed to fuse and

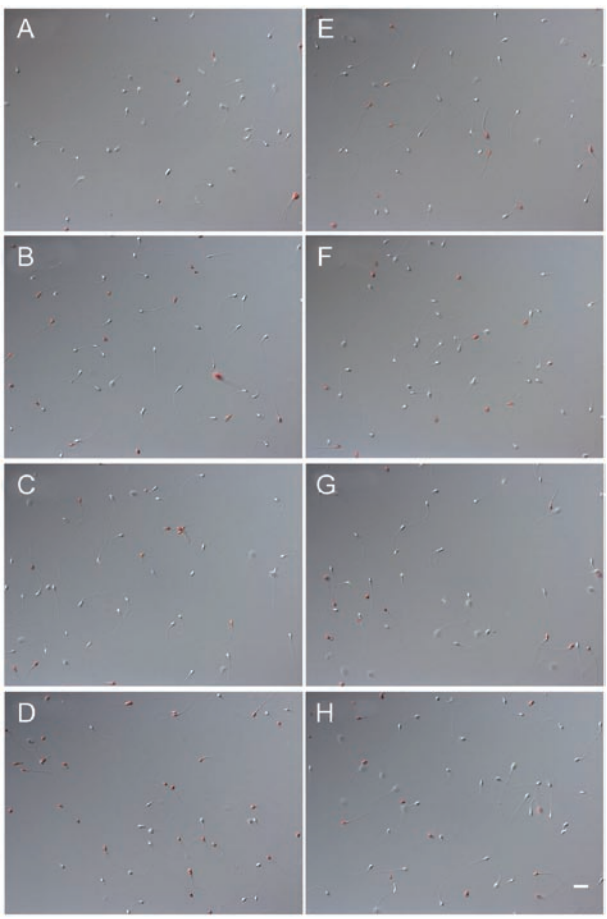

Figure 9. Representative images of eosin test in different concentrations of AF-2364 (concentration A = 24 μmol L^{-1} [A], concentration B = 48 μmol L^{-1} [B], concentration C = 72 μmol L^{-1} [C], concentration D = 96 μmol L^{-1} [D]) or the same volume of ethanol (G–H). Spermatozoa that exhibited abnormal membrane structure and that thus permitted eosin to enter the cell stained positive (orange) in the head region, while spermatozoa with normal membrane structure remained unstained. Bar = 20 μm.

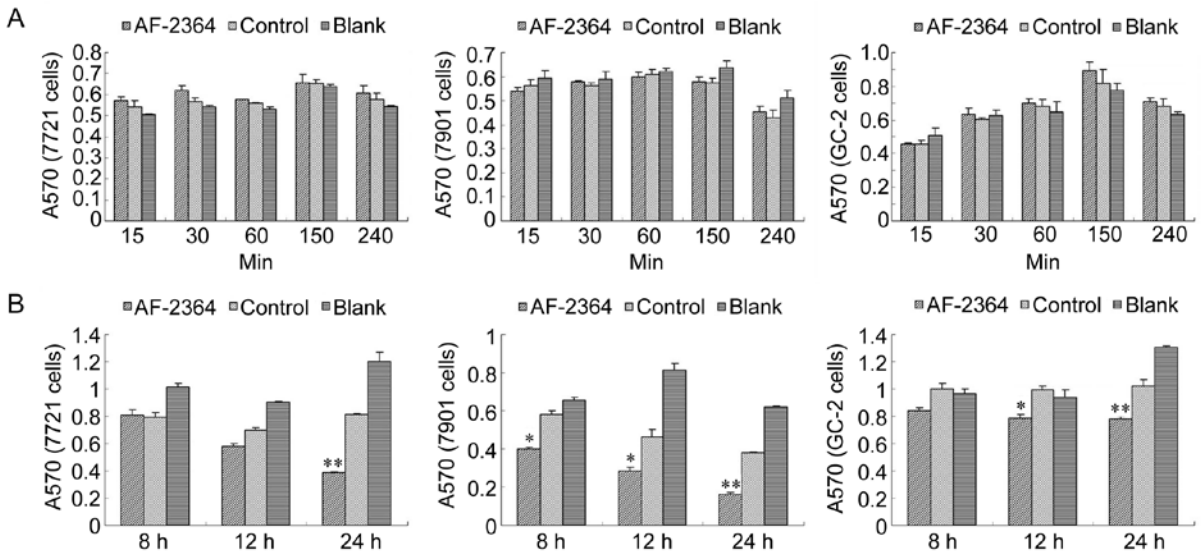

Figure 10. Viability for human SMMC-7721, SGC-7901 and mouse GC-2 cells with the incubation of AF-2364 for 15, 30, 60, 150, 240 min (A) or 8, 12 and 24 h (B). The live cell population was represented by the absorbance at 570 nm (A570). $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the control.

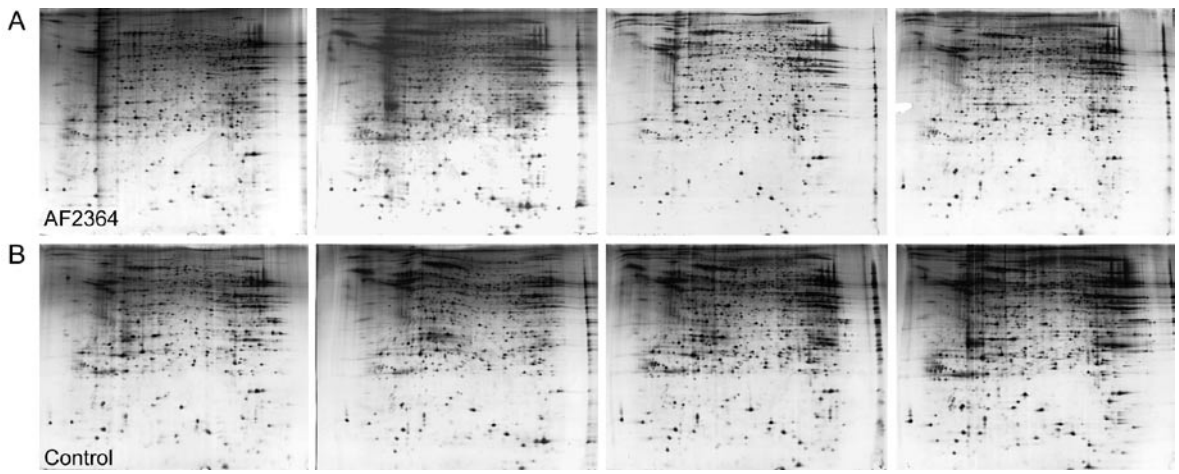

Figure 11. Eight two-dimensional electrophoresis (2-DE) sperm gels for four individuals treated with AF-2364 or ethanol in our first time 2-DE experiment. (A): Gels for four different human sperm samples in AF-2364 group one by one. (B): Gels for the same four individuals in control group consequently.

deliver the condensed nucleus to the ovum, while the tail or flagellum includes the mid-piece, tightly packed with mitochondria, and the principal piece; both are required for sperm motility [24]. During spermatogenesis, the sperm mitochondria undergo substantial programmed modifications that mirror metabolic differentiation [25, 26]. In addition, some OXPHOS subunits are exclusively expressed or show higher activities in spermatozoa [27], and mitochondrial membrane potential, which is dependent on OXPHOS activity, increases by up to sixfold throughout spermatogenesis [28, 29]. These dramatic and specialized mitochondrial changes indicate their significant role in sperm function and make sperm mitochondria a promising target for contra-

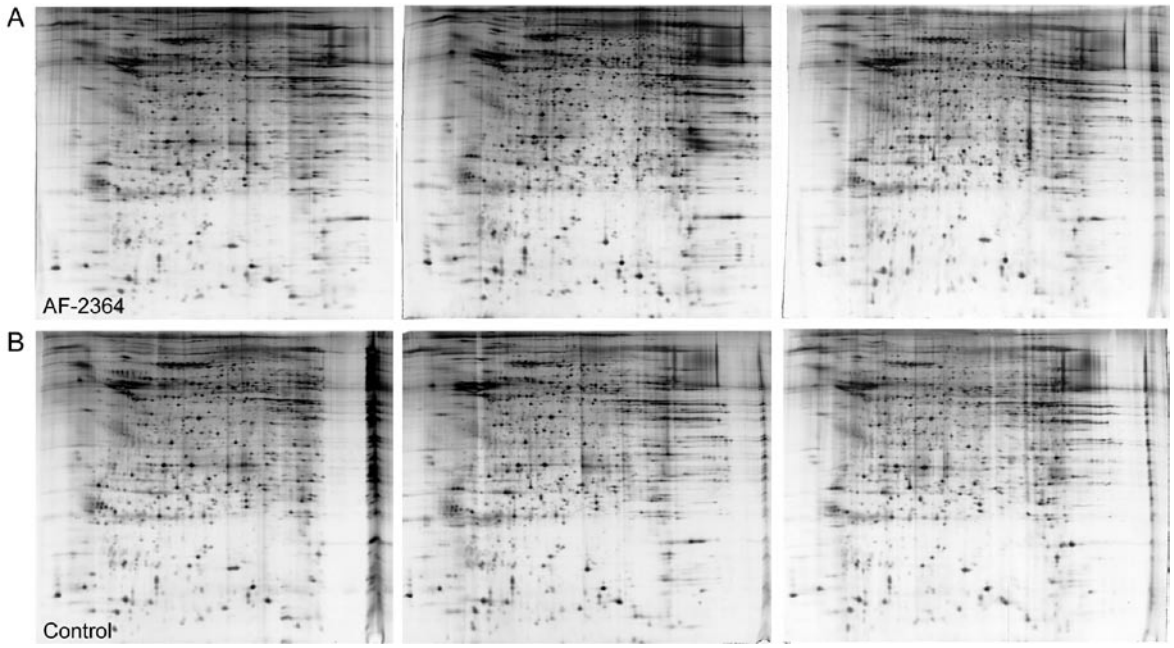

Figure 12. Six two-dimensional electrophoresis (2-DE) gels for three combined sperm samples (four individual sperm together as one combined sample) treated with AF-2364 or ethanol in our second time 2-DE experiment. (A): Gels for three combined sperm samples in AF-2364 group. (B): Gels for the same three combined sperm samples in control group consequently.

ception. Based on previous research, we predicted that AF-2364, an analogue of LND, would target human sperm mitochondria and impair sperm function. Our results showed that AF-2364 has a concentration- and time-dependent spermicidal effect (Figure 1). Notably, high concentrations of ethanol (250 mmol L^{-1}) reportedly induce membrane damage in the form of loss of the acrosome and equatorial segment in human sperm [30]. The ethanol used in these experiments (concentrations C and D are 410 and 550 μmol L^{-1}, or 1.9% and 2.5%, respectively) was at a much lower concentration than in the reported data, and we did not detect the aforementioned structural changes by transmission electronic microscopy (data not shown). However, we did observe that ethanol had some effect on sperm viability and sperm motility; so we present the Δ effect data (AF-2364 minus ethanol) as Supplementary Figure 1. The data revealed that AF-2364 itself does have a dose- and time-dependent spermicidal effect, although the effect might be enhanced by the ethanol solvent.

4.2 AF-2364 targets sperm mitochondria

Further experiments focused on the direct effects of AF-2364 on sperm mitochondria. Typically, mitochondrial inner and outer membranes contain an MPT pore composed of the following proteins: voltage-dependent anion channel, adenine nucleotide translocator (ANT) and cyclophilin-D (CyD) [31, 32]. Further, ΔΨm is a parameter required for many key mitochondrial functions, including ion transport, protein import, biogenesis and energy conservation [32–35]. The relationship between sperm mitochondrial ΔΨm and ATP production in the context of sperm motility has been frequently reported [6, 9, 36]. In the present study, exposure to AF-2364 contributed to the loss of sperm ΔΨm in a time-dependent manner (Figures 2–5). We also combined mitochondrial ΔΨm stain JC-1 with the viability stain PI to determine whether JC-1-negative sperm were alive or dead and revealed that some of the JC-1-negative sperm were indeed still alive (Supplementary Figure 2). As a consequence of the downregulation of sperm ΔΨm, we observed a significant downregulation of ATP (Figure 7), suggesting that AF-2364 might interfere with energy production by inhibiting sperm mitochondrial potential, similar to the action of LND in tumour cells [37, 38].

As glycolysis and OXPHOS both contribute to the energy resources required for sperm motility [2], we

also measured glucose levels in sperm samples after exposure to AF-2364 (Supplementary Figure 2). However, there were no significant differences between the two groups, indicating that mitochondria are the main targets of AF-2364 in this cell type.

To explore whether AF-2364 inhibits sperm motility through a direct effect on sperm mitochondria via the PT pore, similar to LND in tumour cells, we co-incubated sperm with AF-2364 and CsA and observed rescued motility. CsA is a widely used PT pore inhibitor that reportedly prevents CyD binding to the ANT and PT activation [31, 39, 40]. The fact that the mitochondria of mice lacking CyD were resistant to CsA-sensitive PT *in vitro* validated the use of CsA to characterize the involvement of PT [41, 42]. In our research, we found that the addition of CsA partially rescued the AF-2364-induced inhibition of sperm motility *in vitro* in a dose-dependent manner (Figure 6). These observations provide evidence that AF-2364 provokes a disruption in the mitochondrial trans-membrane potential through a direct effect on the MPT pore. Taken together, the above results indicate that AF-2364 might target the sperm MPT pore and induce the loss of sperm $\Delta\Psi m$, which in turn impairs mitochondrial function and decreases respiratory ATP generation.

As LND can interfere with cellular energy production and lead to apoptosis [43–45], and because cell survival status may dramatically influence ATP detection, we performed the eosin test to confirm that the ATP changes observed after incubation with AF-2364 were not part of the sequence of sperm death (Figures 7–9). It is interesting to note that although sperm ATP production decreased significantly in the AF-2364 group relative to the control group after a 30-min treatment, both groups showed an increase at the 60-min time point, followed by another drop at 150 min (Figure 7). Recent research suggests that the opening of the PT pore is the direct cause of the MPT. As the MPT extends to more and more mitochondria, autophagy, apoptosis and necrosis progressively develop in proportion to the number of injured mitochondria and the extent of ATP depletion, a phenomenon of necrapoptosis [46]. When the MPT is limited to a few mitochondria, mitophagy occurs and cells use autophagy to remove damaged structures and maintain the minimum ATP provisions required for survival [47] (although whether autophagy promotes or defends against cell death remains controversial [48]). As more mitochondria undergo MPT, apoptosis is stimulated, and finally, as the majority of mitochondria experience MPT, OXPHOS fails and ATP levels plummet. Based on our sperm ATP detection and eosin-staining results, we presume that lower concentrations of AF-2364 (concentration A, B, and C) might induce mitophagy, while the higher concentration of AF-2364 (concentration D) might induce the apoptotic process, contributing to the changes in sperm ATP production, and finally cause sperm death. Further experiments with the MTT assay revealed that all effects of AF-2364 on human sperm cells at concentrations < 96 μmol L^{-1} for less than 240 min were sperm-specific and not the cytotoxicity reactions of the compound. However, the increased cell death rates observed with extended incubation times supported our presumption that higher concentrations of AF-2364 exposure will eventually induce cell apoptosis (Figure 10). As the sperm cell is dependent on mitochondria, we believe that AF-2364's spermicidal effect would be specific to spermatozoa and less toxic to other somatic cells in the vagina, especially with a short exposure.

4.3 Other mechanisms by which AF-2364 might inhibit sperm motility

Besides restrictions on energy supplies for the sperm tail, sperm motility can be regulated by two other important factors, signal pathways and the cytoskeletal network [49, 50]. In previous studies, AF-2364 was reported to induce reversible germ cell loss from the seminiferous epithelium by disrupting cell AJs between Sertoli and spermatogenic cells. This effect was mediated by an initial activation of the integrin/cadherin/testin multi-protein complex at the site of ectoplasmic specialization (a testis-specific cell–cell actin-based AJ), followed by downstream activation of the ROCK/LIM kinase/cofilin signalling pathways via phosphorylation, which then adjusted actin cytoskeleton organization/reorganization pertinent to AJ dynamics [14, 51–53]. We assessed whether this pathway was affected by AF-2364 treatment in human sperm, as the actin-based cytoskeleton was reported to be involved in sperm motility [54–57]. However, no significant differences in cofilin or phosphorylated cofilin expression levels were detected in human sperm after incubation with AF-2364 for 1, 2, 3 or 4 h. The F-actin status of the treated sperm was also coherent (data not shown). Likewise, the 2-DE analysis did not find any significantly differentially expressed proteins between the AF-2364-treated and control groups (Figure 11) from the individual or combined sperm samples (Figure 12). These results

therefore indicate that AF-2364 did not inhibit sperm motility through regulation of the actin-based cytoskeletal network or by changing protein expression levels or post-translational modifications; sperm mitochondria appear to be the main target of this drug.

In summary, in the present study we found that (1) AF-2364 can inhibit human sperm motility *in vitro* and might be developed into a candidate spermicide. As AF-2364 is beginning to be considered as a male oral contraceptive, its spermicidal effect indicates that a combinatorial delivery approach might also be effective. (2) Further work revealed that AF-2364 interfered with energy production by inhibiting the sperm mitochondrial potential ($\Delta\Psi m$), similar to the effect of LND on tumour cells, and subsequently impaired sperm motility. No significant changes in the cytoskeletal network or in the human sperm proteome were observed after exposure to AF-2364, which suggests that mitochondria are the main targets for motility inhibition *in vitro*. However, more work needs to be done to develop AF-2364 into a real spermicide with clinical applications. Such work would include the evaluation of *in vivo* toxicity to the vaginal micro-environment, damage to the vagina or cervical epithelium, detection of proinflammatory cytokines and interleukins, the effects of AF-2364 on HIV [58], and the *in vivo* anti-fertility effect.

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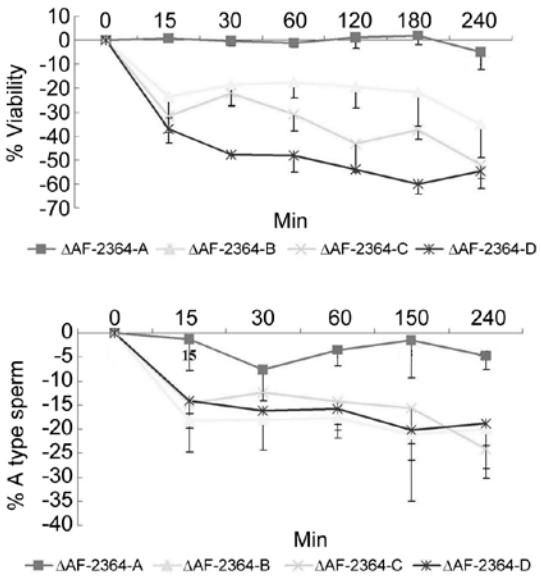
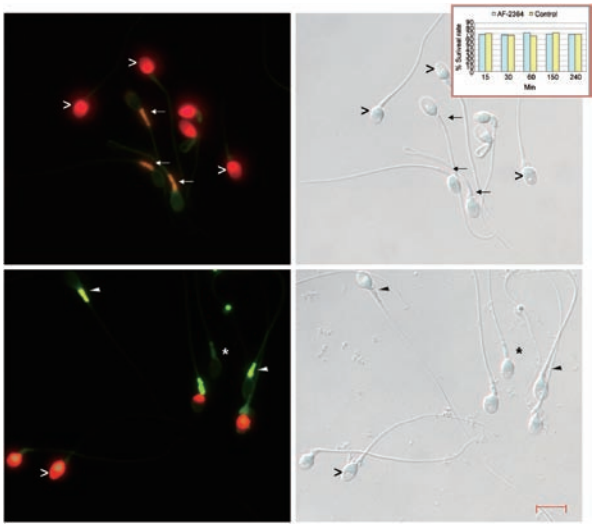
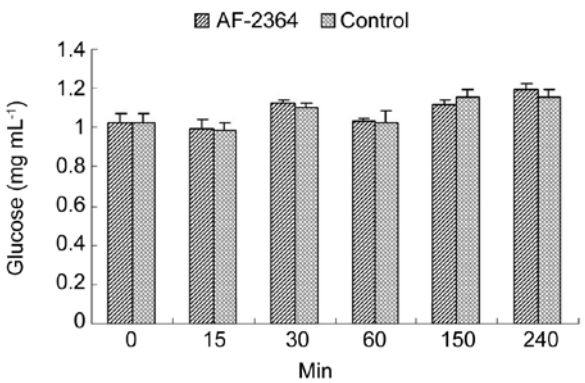


Supplementary Figure 1. Spermicidal effect of AF-2364 minus ethanol for different concentrations (24, 48, 72 or 96 μmol L^{-1}, concentrations A, B, C, D are just as the aboves). To minimize the solvent effect to the statistical analysis in Figure 1, we reanalyzed the data to show the effects of AF-2364 itself on human sperm samples.



Supplementary Figure 2. The combination of JC-1 and propidium iodide (PI) staining of human sperm. The three types of sperm can be seen in the image: Arrows (↑): JC-1 positive and PI negative sperms which means the sperms are alive with a normal mitochondrial membrane potential (Ψm); Arrow heads (▲): JC-1 negative and PI negative sperms which means the sperms are still alive but with a lower Ψm (asterisk [*] also means sperms in this type, but the Ψm even lower); Angles (<): JC-1 negative and PI positive sperms which means the sperms are already dead. The graph in the topright is the survival rates of sperms in concentration C at 15, 30, 60, 150, 240 min calculating of PI positive sperms and the result is similar to Figure 5. Bar = 5 μm.



Supplementary Figure 3. The glucose level in sperm samples after exposure to 72 μmol L^{-1} AF-2364 (concentration C) at 15, 30, 60, 150, 240 min, respectively.