Asian Journal of Andrology (2024) 26, 490–499
www.asiaandro.com; https://journals.lww.com/ajandrology

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ORIGINAL ARTICLE

Male Infertility

Scriptaid is a prospective agent for improving human asthenozoospermic sample quality and fertilization rate *in vitro*

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Male infertility is a global issue caused by poor sperm quality, particularly motility. Enhancement of the sperm quality may improve the fertilization rate in assisted reproductive technology (ART) treatment. Scriptaid, with a novel human sperm motility-stimulating activity, has been investigated as a prospective agent for improving sperm quality and fertilization rate in ART. We evaluated the effects of Scriptaid on asthenozoospermic (AZS) semen, including its impact on motility stimulation and protective effects on cryopreservation and duration of motility, by computer-aided sperm analysis (CASA). Sperm quality improvement by Scriptaid was characterized by increased hyaluronan-binding activity, tyrosine phosphorylation, adenosine triphosphate (ATP) concentration, mitochondrial membrane potential, and an ameliorated AZS fertilization rate in clinical intracytoplasmic sperm injection (ICSI) experiments. Furthermore, our identification of active Scriptaid analogs and different metabolites induced by Scriptaid in spermatozoa lays a solid foundation for the future biomechanical exploration of sperm function. In summary, Scriptaid is a potential candidate for the treatment of male infertility *in vitro* as it improves sperm quality, prolongs sperm viability, and increases the fertilization rate.
Asian Journal of Andrology (2024) **26**, 490–499; doi: 10.4103/aja202416; published online: 11 June 2024

Keywords: asthenozoospermia; HDAC6; ICSI; Scriptaid; sperm motility

INTRODUCTION

Infertility is defined by the inability to attain pregnancy after 12 consecutive months of engaging in unprotected sexual intercourse. *In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are assisted reproductive technologies (ART) used to treat male infertility. The success of these techniques is highly dependent on sperm quality, particularly motility.[1,2] Poor sperm motility is a major factor in male infertility and low fertilization and pregnancy rates. Therefore, sperm motility enhancement could improve the success rate of ART.

Sperm motility is a complex process involving various biological processes, including posttranslational modifications.[3–5] Lysine acetylation, a general posttranslational modification, occurs on the ε-NH_2 of protein lysine residues and is catalyzed by histone acetyltransferases and deacetylases (HDACs). Acetyl-lysine proteomic studies have revealed acetylation in the human sperm tail and its association with sperm motility.[6,7] In asthenozoospermia, lysine acetylation levels in spermatozoa cells are significantly lower than those in normozoospermia.[8] We previously reported different acetyltransferase and deacetylase expression levels in asthenozoospermic (AZS) and normozoospermic (NZS) semen samples,[7] suggesting that acetylation may play an important role in maintaining normal sperm function, particularly motility.

Our exploratory compound screening indicates that Scriptaid, an HDAC6 inhibitor, may act as a potent agent for enhancing sperm motility in patients with AZS. However, its effects on sperm motility-related activity and potential clinical use require further evaluation. HDAC6, a histone deacetylase, decreases acetylation in histones[9–11] and plays a critical role in regulating microtubule stability and cellular motility.[12] It is reportedly distributed in the tail of spermatozoa cells in Holtzman rats.[13] HDAC6 deacetylated alpha-tubulin, and after treatment with HDAC6 inhibitors, HDAC6 levels in spermatozoa cell tails decreased.[13] Compared with wild-type controls, HDAC6-deficient male mice produce offspring through natural mating without any significant differences in litter size or fertility, suggesting that loss or reduction in HDAC6 function does not affect normal male reproductive function.[14] Treatment with Scriptaid has the potential to promote embryonic development,[15] and significantly increased numbers of blastocysts and total cells in embryos,[16] suggesting that Scriptaid has a positive effect on both sperm function and embryonic development in patients with AZS. However, there are no reports confirming the relationship between Scriptaid and sperm motility.

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Received: 28 September 2023; Accepted: 04 March 2024

To address this, we investigated the potential of Scriptaid as an agent for the clinical promotion of sperm motility and ICSI success in male AZS patients.

PARTICIPANTS AND METHODS

Semen preparation

This study was approved by the Institutional Ethics Committee of Zhongshan Hospital Affiliated with Fudan University (Shanghai, China; Approval No. B2020-097R). Written informed consent was obtained from all participants. Semen samples were collected from June 2020 to June 2021 in the Reproductive Medicine Center of Zhongshan Hospital (Shanghai, China). All participants were asked to abstain from ejaculation for 2–3 days before sample collection. In accordance with the standard criteria of the World Health Organization (WHO) manual (5th edition),[17] only semen samples that tested negative for white blood cells were included in this study. AZS and NZS semen samples were defined as progressive sperm motility of <32% and ≥32%, respectively. Liquefied semen samples were centrifuged using a 5804R centrifuge (Eppendorf, Hamburg, Germany) at 300*g* for 15 min. The pellet obtained was washed twice with Biggers–Whitten–Whittingham (BWW) medium supplemented with 5% (*w/v*) medium human serum albumin (HSA; Healthgen Biotechnology, Wuhan, China). Subsequently, the pellet was resuspended in BWW containing 5% (*w/v*) HSA to obtain a spermatozoa concentration of 10×10^6–20×10^6 ml^{-1}.

Testicular sperm aspiration (TESA) was performed under local anesthesia. A 21-gauge needle attached to a 10-ml syringe was used to puncture the skin of the scrotum and enter the testis. Multiple punctures were made in the testicular parenchyma to obtain testicular tissue. The aspirate was collected in a sterile container.

Sperm motility and cryopreservation

To test the effect of Scriptaid on sperm motility promotion, sperm motility parameters were assessed using computer-aided sperm analysis (CASA; IVOSII; Hamilton Thorne, Beverly, MA, USA) or by counting motile spermatozoa under a microscope (Leica DMi1; Leica, Wetzlar, Germany). To ascertain the effect of Scriptaid on sperm motility, we performed a comparative analysis with various sperm culture media, namely, the basic sperm culture medium BWW, clinical fertilization medium G-IVF (Vitrolife, Gothenburg, Sweden), and a commercial sperm motility enhancer (Fasrun; GaoHong Biotechnology, Shanghai, China). Sperm culture media samples were divided into four groups: Scriptaid (TargetMol, Boston, MA, USA), BWW, G-IVF, and commercial sperm motility enhancers (Com).

Spermatozoa were cryopreserved as follows. A modified cryoprotectant Tyrode's glucose glycerol (TGG) was used according to the WHO manual (5th edition).[17] The total semen sample was divided into three groups: one group was mixed with cryoprotectants and supplemented with 10 μmol l^{-1} Scriptaid, another group was treated with commercial cryoprotectant 1 (comcry 1), and the third group was treated with commercial cryoprotectant 2 (comcry 2). For each sample, a 100-μl sperm suspension was loaded into 0.25-ml plastic freezing straws (IMV Technologies, Paris, France) from a 1-ml syringe along with 15 mm of air. Both ends of the straw were heat sealed and the sealed straw was transferred to a freezing canister and cooled in a liquid nitrogen vapor layer 5–10 cm above liquid nitrogen for 30 min. The straws were then plunged directly into liquid nitrogen and stored for at least 1 month before thawing.

For sperm motility assessment, straws were removed from the liquid nitrogen and thawed in a water bath at 37°C. Subsequently, they were washed three times in phosphate-buffered saline (PBS), followed by centrifugation at 600*g* for 5 min (5804R; Eppendorf). Finally, the sperm concentration was adjusted to 10×10^6–20×10^6 ml^{-1} in BWW to assess sperm motility parameters.

Hyaluronan-binding assay

To assess the ability of spermatozoa to bind to hyaluronic acid (HA), commercially available HBA kits (Sperm–hyaluronan-binding assay; Cooper Surgical, Trumbull, CT, USA) were used as per the manufacturer's instructions (on 100–200 spermatozoa). Briefly, a 7-μl drop of sperm suspension with AZS (incubated as controls or exposed to 10 μmol l^{-1} Scriptaid) or spermatozoa from NZS semen was carefully placed on the HBA® slide, and a cover slip was immediately placed on it. Slides were then incubated at room temperature (25°C) for 10 min to allow sperm to bind to the surface of the HA-treated glass slides. Unbound and bound motile spermatozoa were counted in a phase-contrast microscope (Leica) at 200× total magnification with the same number of grid squares.

Adenosine triphosphate (ATP) concentration

The processed sperm suspension was divided into six aliquots (1.2×10^6 per group) and incubated with Scriptaid at concentrations of 0, 1.25 μmol l^{-1}, 2.5 μmol l^{-1}, 5 μmol l^{-1}, 10 μmol l^{-1}, or 20 μmol l^{-1} for 30 min. The sperm suspension was centrifuged at 1000*g* (Eppendorf) for 3 min and then treated with the Enhanced ATP Assay Kit, as per the manufacturer's instructions (Beyotime, Shanghai, China) in duplicate. To minimize ATP consumption, all samples were tested within 20–30 min at room temperature.

Detection of mitochondrial membrane potential (MMP)

Sperm suspensions from AZS patients were treated with Scriptaid at a concentration of 10 μmol l^{-1} for 30 min. Subsequently, the suspension was centrifuged at 800*g* (Eppendorf) for 5 min, followed by staining with JC-1 (Beyotime) at a concentration of 2.5 μg l^{-1} for 20 min at room temperature. After two washes in PBS, samples were analyzed using the LSR Fortessa cytometer (Becton Biosciences, Franklin Lakes, NY, USA).

Clinical ICSI

We recruited 82 couples in which the male partner was diagnosed with AZS for the clinical ICSI study. Among the participants, 25 AZS patients with motile spermatozoa were assigned to the untreated group; 57 AZS patients for whom motile spermatozoa could not be found in the semen were assigned to the Scriptaid-treated group. Oocytes were collected from the participants by follicular aspiration and subjected to *in vitro* maturation (IVM) to ensure that they reached appropriate stage for subsequent ICSI. The sperm of the Scriptaid-treated group was treated with Scriptaid at concentrations of 0.2 μmol l^{-1}, 1 μmol l^{-1}, and 2.5 μmol l^{-1} and then subjected to ICSI along with spermatozoa from the control group. During ICSI, specialized micromanipulation techniques were used to inject a single spermatozoon into each mature oocyte. To ensure consistency, the same technician (BY) performed each injection. Oocyte fertilization rates were evaluated for both the Scriptaid-treated and untreated groups.

Mouse in vitro fertilization

Oocyte collection was performed as follows: superovulation of 6–8-week-old C57 female mice was achieved by injecting 5 IU of pregnant mare serum gonadotropin (PMSG), followed by 5 IU of human chorionic gonadotropin (hCG) after 48 h. Female mice were sacrificed by cervical dislocation 13–15 h after hCG injection. Cumulus–oocyte complexes (COCs) were isolated from the oviducts with a sterile pipette viewed in a stereomicroscope. Isolated COCs were incubated in potassium simplex optimized medium (KSOM) supplemented with 4 mg ml^{-1} bovine serum albumin (BSA)

and 25 mmol l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 37°C in 5% (*v*/*v*) CO_2 until ready for IVF.

Sperm preparation was performed as follows. C57 male mice (8–12-week-old) were sacrificed and the cauda epididymidis was dissected and transferred into a 1.5 ml capacity microcentrifuge tube containing human tubal fluid (HTF). Spermatozoa were allowed to swim out of the cauda epididymidis in HTF at 37°C for 15–20 min. Sperm suspensions (5×10^6 ml^{-1}) were collected and divided into four equal parts; each part was cultured for 30 min in media containing different concentrations of Scriptaid (0, 0.2 μmol l^{-1}, 1 μmol l^{-1}, and 2.5 μmol l^{-1}).

The prepared oocyte suspension (50 μl) was mixed with an equal volume of the prepared sperm suspension in a 200 μl drop of HTF in the presence of mineral oil. The HTF drop was incubated at 37°C in 5% (*v*/*v*) CO_2 for 4–6 h. After IVF, presumptive zygotes were transferred to fresh drops of KSOM supplemented with 4 mg ml^{-1} BSA, 25 mmol l^{-1} HEPES, and 2 μmol l^{-1} Scriptaid. The embryos were cultured at 37°C in 5% (*v*/*v*) CO_2 for 3 days, with daily monitoring for cleavage and blastocyst formation. The number of blastocysts was recorded.

Western blot and immunofluorescence staining

For western blot analysis, the protein concentration of sperm lysates was determined by the bicinchoninic acid (BCA) assay. Equal amounts of protein were loaded onto a polyacrylamide gel and separated by electrophoresis. The proteins were then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) and blocked with 5% (*w*/*v*) nonfat milk in Tris-buffered saline (TBS) buffer. The membrane was incubated with primary antibodies against HDAC6 (1:1000; HUABIO, Hangzhou, China) or β-tubulin (1:5000; HUABIO), followed by incubation with a secondary antibody conjugated with horseradish peroxidase (HRP; Beyotime). Protein bands were visualized by an enhanced chemiluminescence detection system (Thermo Fisher, Waltham, MA, USA).

For immunofluorescence staining, sperm smears were fixed with paraformaldehyde and permeabilized with Triton X-100 (Sango Biotech, Shanghai, China), blocked using 5% (*w*/*v*) BSA in PBS, incubated with a primary antibody against HDAC6 (1:1000), α-tubulin (1:1000; HUABIO) or Kac α-tubulin (1:1000; HUABIO) in a humid box for 30 min, and then incubated with a fluorescently labeled secondary antibody in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime). Images were captured in a confocal microscope (Nikon, Tokyo, Japan) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA).

Metabolomics analysis

Sperm samples were delivered to BioProfile Technology Co., Ltd. (Shanghai, China) for metabolite isolation and widely targeted metabolomic analysis. Each group consisted of 10 samples as biological replicates. Each sample was obtained from a single ejaculate. Briefly, metabolites were extracted from cell residue with 1 ml precooled methanol/acetonitrile/water solution (2:2:1, *v*/*v*/*v*), sonicated in an ice bath for 60 min, stored for 1 h at −20°C, and centrifuged at 14 000*g* (Eppendorf) for 20 min at 4°C. Sodium dodecyl sulfate dithiothreitol Tris-HCl (SDT) lysis buffer (300 μl) was used to resuspend precipitated proteins, which were quantified using BCA assay. From the protein contents, metabolites from each sample were vacuum dried. For mass spectrometry analysis, 100 μl of acetonitrile–water solution (1:1, *v*/*v*) was added for resuspension, followed by centrifugation at 14 000*g* for 15 min at 4°C. The supernatant was collected for injection and analysis.

Chromatographic conditions are as follows. The sample was injected automatically at 4°C and separated using an Agilent 1290 Infinity LC HILIC column (Agilent, Santa Clara, CA, USA) with mobile phases A (25 mmol l^{-1} ammonium hydroxide in water) and B (acetonitrile). The gradient elution procedure was as follows: 95% of B for 0.5 min, linearly changing from 95% to 65% over 6.5 min, linearly changing from 65% to 40% for 1 min, maintaining 40% for 1 min, linearly changing from 40% back to 95% over 0.1 min, and finally, maintaining 95% for 2.9 min.

Mass spectrometry conditions are as follows. The Triple TOF-6600 mass spectrometer (AB Sciex, Franklin, MA, USA) was used with electrospray ionization in both positive and negative ion modes. The positive voltage, negative voltage, and temperature were 5000 V, 4500 V, and 500°C, respectively; the cluster removal voltage, collision voltage, collision energy dispersion, and collision energy were 60 V, 30 V, 15 V, and 30 V, respectively.

Partial least squares regression was used to model the relationship between metabolite expression and sample class to predict the sample class. Orthogonal partial least squares discriminant analysis (OPLS–DA) with variable importance for the projection of > 1 and $P < 0.05$ were used as significant difference cut-offs in metabolite screening. Functions of metabolites and metabolic pathways were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

We searched for proteins that interacted with the Scriptaid target protein HDAC6 and the sperm motility-related protein TUBULIN, with high reliability (combined score >0.7), in the STRING database.[18,19] We then used MetScape in Cytoscape to construct associations between metabolites and proteins at the metabolic pathway level.[20,21]

Statistical analyses

Data were presented as the mean ± standard deviation (s.d.). The results were statistically analyzed using analysis of variance (ANOVA). The Holm–Sidak test was used for multiple comparisons, and the Dunnett's test was used for comparisons with the control group. Prism was used to analyze and visualize data. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of Scriptaid on sperm motility and cryopreservation

After treatment with 10 μmol l^{-1} Scriptaid for 30 min, AZS samples showed improved total and progressive motility (PR) compared with those in the control group ($P < 0.001$), as per the CASA results (**Figure 1a**; each group consisted of three biological replicates). In the images of sperm motion trajectories, Scriptaid-treated spermatozoa displayed longer blue tracks, indicating faster sperm movement than the controls. **Supplementary Video 1** and **2** present sperm motility before and after Scriptaid treatment, respectively. Scriptaid showed concentration-dependent promotion of sperm motility ranging from 1 μmol l^{-1} to 10 μmol l^{-1} ($P < 0.001$; **Figure 1b**). However, when the concentration of Scriptaid was increased to 20 μmol l^{-1}, there was no significant difference in sperm motility compared with that for the 10 μmol l^{-1} concentration. Therefore, 10 μmol l^{-1} of Scriptaid was used to test the duration of its effect (**Figure 1b**). Spermatozoa treated with Scriptaid for 1 h and 5 h exhibit higher motility than that in the control group ($P < 0.05$). Other motility parameters are presented in **Supplementary Figure 1a**. The average path (VAP) and beat cross frequency (BCF) were higher in the Scriptaid-treated group than those in the control group. For testicular spermatozoa obtained by percutaneous TESA, treatment with 10 μmol l^{-1} Scriptaid also significantly increased the number of motile cells (**Figure 1c**), facilitating easy and efficient capture of active spermatozoa for ICSI. Videos of sperm motility before and after Scriptaid treatment are shown in **Supplementary Video 3** and **4**, respectively.

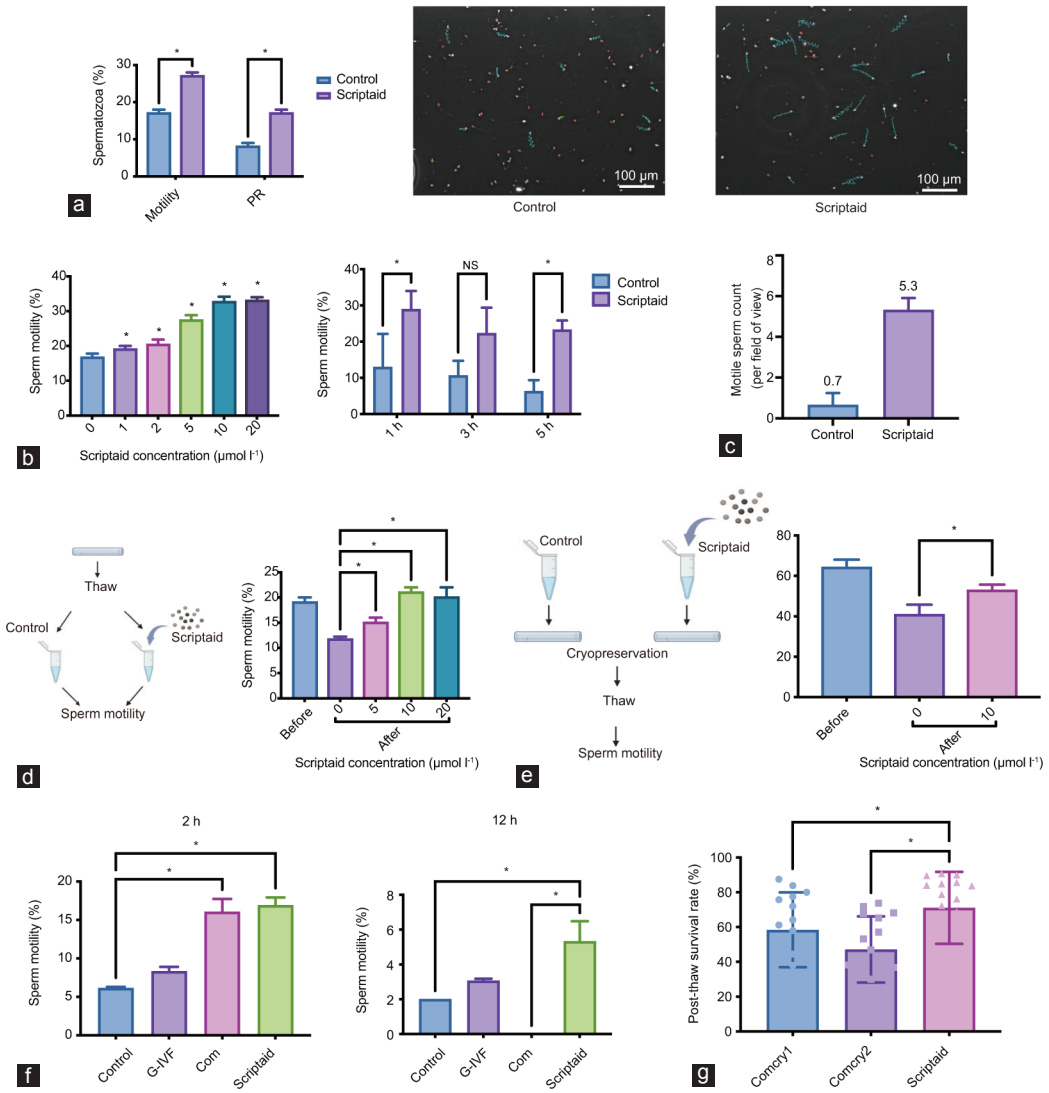


Figure 1: Effects of Scriptaid on sperm motility and cryopreservation. (**a**) AZS sperm motility and progressive motility of spermatozoa treated with 10 μmol l^{-1} Scriptaid. Data analysis was performed using a paired *t*-test. (**b**) AZS sperm motility of spermatozoa treated with various concentrations (0, 1 μmol l^{-1}, 2 μmol l^{-1}, 5 μmol l^{-1}, 10 μmol l^{-1}, and 20 μmol l^{-1}) of Scriptaid for different durations (1 h, 3 h, and 5 h). One-way ANOVA was used to determine the effect of different Scriptaid concentrations on sperm motility. Two-way ANOVA was employed to analyze the effect of Scriptaid treatment duration on sperm motility. (**c**) Effects of 10 μmol l^{-1} Scriptaid on the counts of motile testicular sperm following percutaneous biopsy. (**d**) Effects of Scriptaid on postthaw sperm motility of cryopreserved sperm. (**e**) Effects of Scriptaid on sperm cryopreservation. (**f**) Effects of 10 μmol l^{-1} Scriptaid on AZS sperm motility compared to that of commercial sperm culture media and sperm viability solutions *in vitro*. (**g**) Comparison of the effects of laboratory-made freezing medium supplemented with 10 μmol l^{-1} Scriptaid and commercial sperm cryopreservation solutions. More than 2000 sperm were calculated in each sample except **c**. Data were analyzed using one-way ANOVA. Data were expressed as mean ± s.d. $^{*}P < 0.05$, $n \geq 3$ semen samples in each group. PR: progressive motility; AZS: asthenozoospermic; ANOVA: analysis of variance; s.d.: standard deviation; Com: commercial sperm motility enhancers; NS: not significant.

Scriptaid also promoted the motility of cryopreserved spermatozoa after thawing (**Figure 1d**). For each group, consisting of three biological replicates, incubation of thawed spermatozoa with 5 μmol l^{-1}, 10 μmol l^{-1}, and 20 μmol l^{-1} Scriptaid significantly improved sperm motility (all $P < 0.05$), reaching a level comparable to that of prefrozen spermatozoa. When 10 μmol l^{-1} Scriptaid was added to the freezing medium for sperm cryopreservation (**Figure 1e**), increased sperm motility was observed after thawing for 10 min ($P < 0.05$), suggesting that Scriptaid had a positive effect on preserving sperm activity during freezing.

The effects of Scriptaid were compared with those of the control (BWW basic culture medium), G-IVF (sperm culture medium for ICSI), and Com (commercialized sperm motility solution) groups. Each group consisted of three samples as biological replicates. CASA was performed after 2 h and 12 h (**Figure 1f**). Sperm motility was significantly higher in the Com and Scriptaid groups than that in the control group ($P < 0.0001$). However, after 12 h of incubation, all spermatozoa in the Com group became static, while those in the Scriptaid-containing groups remained motile, suggesting that the protective effect of Scriptaid on sperm motility is durable. There was a decrease in motility when comparing 2 h with 12 h (17.0% *vs* 5.3%). We compared the protective effects of Scriptaid with those of a commercial freezing medium on the postthaw survival rate of spermatozoa, which was calculated by dividing the motility of postthaw spermatozoa by that of precryopreservation spermatozoa. Each group consisted of 16 biological replicates. After a 30-min treatment, we observed a significant

increase in sperm postthaw survival rate in the Scriptaid group. It is important to note that Scriptaid was added to the cryoprotectant (TGG) during the experiment according to WHO manual (5th edition),[17] as shown in **Figure 1g** ($P < 0.05$).

Effects of Scriptaid on sperm HA binding, ATP level, and MMP

We observed a significant increase in the percentage of AZS spermatozoa with high HA-binding ability following treatment with Scriptaid (47.8%) compared with that in the control group (21.2%), as shown in **Figure 2a** ($P < 0.05$); each group consisted of three biological replicates. These results indicate that Scriptaid enhances the HA-binding ability of spermatozoa, which is important for successful fertilization and embryonic development.[22,23]

Treatment with Scriptaid significantly increased ATP concentration in AZS spermatozoa (**Figure 2b**); each group consisted of three biological replicates. Increasing concentrations of Scriptaid (1.25 μmol l^{-1}, 2.5 μmol l^{-1}, 5 μmol l^{-1}, 10 μmol l^{-1}, and 20 μmol l^{-1}) led to increasing ATP levels; 20 μmol l^{-1} Scriptaid stimulated the highest increase in ATP concentration ($P < 0.05$). Scriptaid may have a positive effect on sperm energy metabolism, which may be beneficial for improving sperm function and fertility.

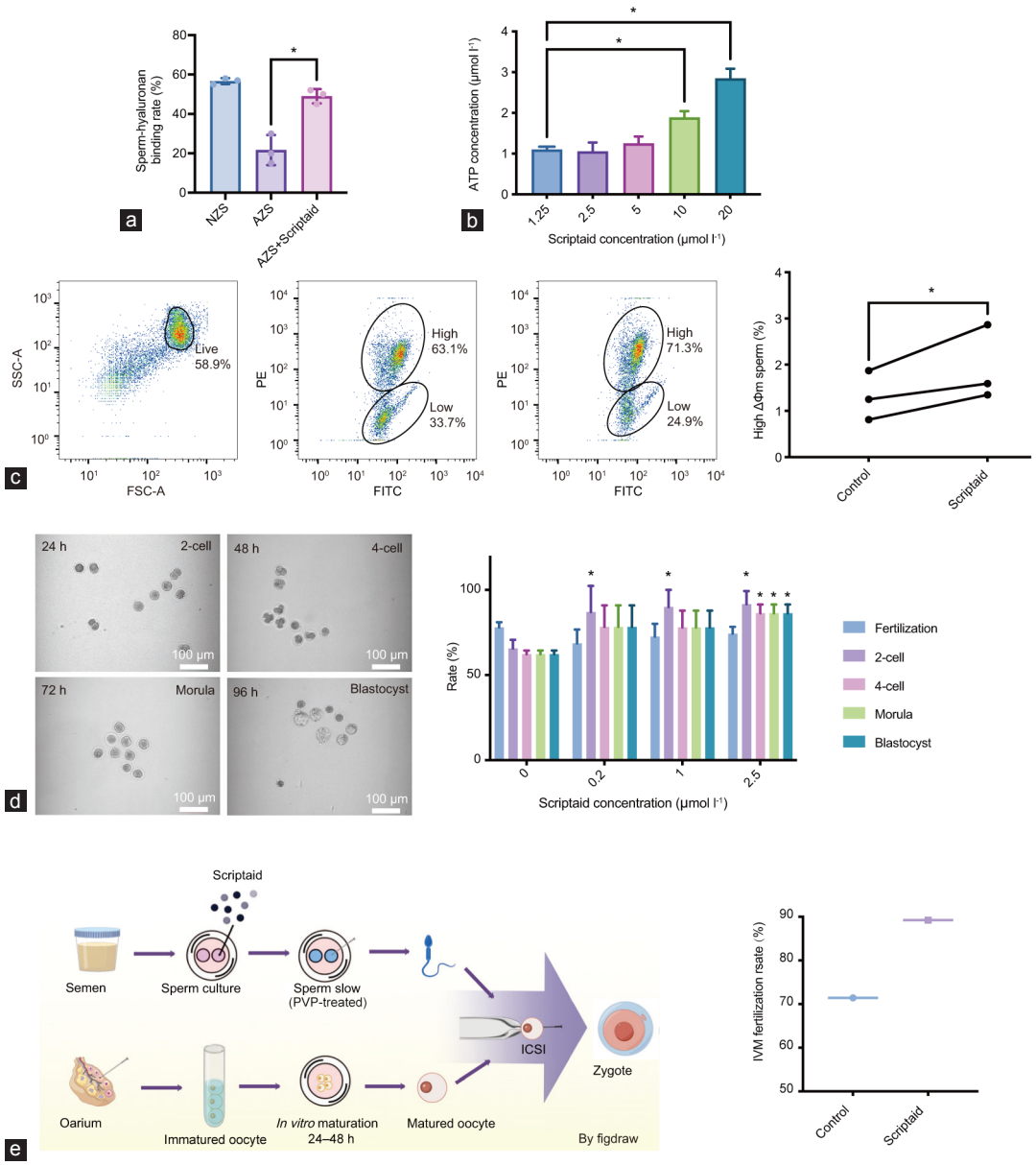


Figure 2: Impact of Scriptaid on sperm function and embryo development. (**a**) Impact of 10 μmol l^{-1} Scriptaid on hyaluronic acid-binding rate of AZS human sperm. Two hundred spermatozoa were calculated for each sample. (**b**) ATP concentration in AZS sperm after a 30-min treatment with different concentrations of Scriptaid. (**c**) FACS analysis of MMP in AZS sperm treated with 10 μmol l^{-1} Scriptaid for 30 min; 100 oocytes were counted for each sample. (**d**) Mouse IVF embryo development after exposure of sperm to different concentrations of Scriptaid. Two-way ANOVA. (**e**) Schematic diagram of ICSI procedure using *in vitro* maturation (IVM) oocytes with Scriptaid treatments and fertilization rate after treating AZS sperm with 10 μmol l^{-1} Scriptaid. One-way ANOVA. Data were expressed as mean ± s.d. $^*P < 0.05$, $n \geq 3$ samples in each group except $n \geq 25$ samples in **e**. ATP: adenosine triphosphate; FACS: fluorescence-activated cell sorting; MMP: mitochondrial membrane potential; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection; FITC: fluorescein isothiocyanate; PE: phycoerythrin; SSC-A: side scatter-area; FSC-A: forward scatter-area; s.d: standard deviation; ANOVA: analysis of variance.

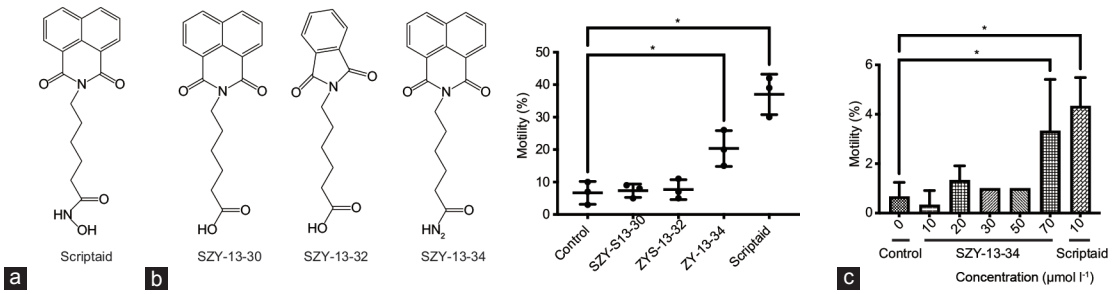


Figure 3: Chemical structure of Scriptaid and analogs, and their effects on sperm motility. (**a**) Chemical structure of Scriptaid. (**b**) Chemical structure of Scriptaid analogs (SZY-13-30, SZY-13-32, and SZY-13-34), and their effects on sperm motility rate at a concentration of 10 μmol l^{-1} after 30 min. More than 2000 sperm were calculated in each sample. (**c**) Effects of different concentrations of SZY-13-34 on sperm motility compared to that of Scriptaid. More than 2000 sperm were calculated in each sample. One-way ANOVA. Data were expressed as mean ± s.d. $^*P < 0.05$, $n \geq 3$ semen samples in each group. ANOVA: analysis of variance; s.d.: standard deviation.

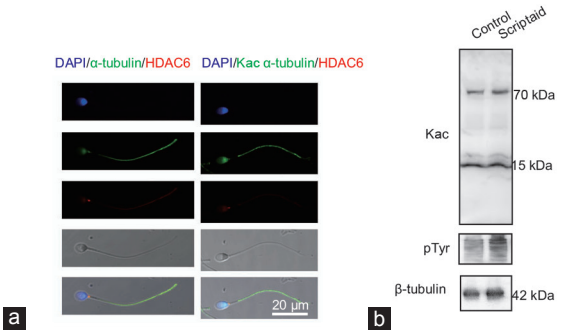


Figure 4: Sperm protein lysine acetylation, tyrosine phosphorylation, and localization of HDAC6 in human spermatozoa. (**a**) Immunofluorescence staining of HDAC6, alpha-tubulin (α-tubulin), and Kac α-tubulin in human sperm. (**b**) Western blot profiles of protein Kac and tyrosine phosphorylation (pTyr) in human sperm following treatment with 10 μmol l^{-1} Scriptaid. HDAC: histone deacetylase; Kac: lysine acetylation; DAPI: 4',6-diamidino-2-phenylindole.

We observed a significant increase in MMP, an essential parameter reflecting sperm quality, in spermatozoa treated with Scriptaid compared with those in the control group ($P < 0.05$; **Figure 2c**). Upon the addition of Scriptaid to the basic cryopreservation solution (TGG), a higher MMP was observed in the thawed spermatozoa compared to the control group with TGG. However, no significant differences were found in MMP between the TGG cryopreservation solution and the commercial cryopreservation reagents comcry 1 and comcry 2 ($P = 0.18$ and $P = 0.22$, respectively; **Supplementary Figure 1b**).

Scriptaid treatment effect on mouse IVF fertilization and clinical ICSI fertilization rates

We treated the mouse spermatozoa with Scriptaid at concentrations of 0.2 μmol l^{-1}, 1 μmol l^{-1}, and 2.5 μmol l^{-1} before IVF. Each group contained approximately 100 oocytes. The Scriptaid-treated group exhibited higher embryo developmental potency than the control group. Moreover, the 2-cell rates of Scriptaid-treated groups were significantly higher than that of the control group ($P < 0.01$). The rates of 2-cell, 4-cell, morula, and blastocyst stages in the group treated with 2.5 μmol l^{-1} Scriptaid were significantly higher than those in the control group (all $P < 0.01$). Embryos derived from IVF using Scriptaid-treated spermatozoa had the ability to develop into the blastocyst stage (**Figure 2d**). Finally, Scriptaid treatment significantly increased the fertilization rate of IVM oocytes after ICSI to a rate of 89.2%, compared with 71.4% in the untreated group (**Figure 2e**).

Structure–function relationship of Scriptaid and its analogs

To explore the major functional groups of Scriptaid (**Figure 3a**) that promote sperm motility, Scriptaid analogs SZY-13-30, SZY-13-32, and SZY-13-34 were synthesized and tested at a concentration of 10 μmol l^{-1} (**Figure 3b**). Each group consisted of three biological replicates. SZY-13-34 was the only analog that exhibited significant sperm motility promotion compared with the control group ($P < 0.01$). However, it did not reach the level of motility enhancement observed upon Scriptaid treatment. At a relatively high concentration (70 μmol l^{-1}), the SZY-13-34 analog exhibited high stimulatory activity on sperm motility. However, even at this concentration, it did not reach the level of activity observed upon Scriptaid treatment at 10 μmol l^{-1} (**Figure 3c**).

Localization of HDAC6 on spermatozoa and enhancement of tyrosine phosphorylation by Scriptaid

The postulated target protein of Scriptaid, HDAC6, was mainly identified in the centriole area of human spermatozoa (**Figure 4a**), as shown using immunofluorescent staining with the HDAC6 antibody. Each group consisted of three biological replicates. As spermatozoa with poor motility have lower acetylation and higher HDAC6 expression,[8] Scriptaid, an inhibitor of HDAC6 (deacetylase), may increase sperm motility by increasing protein acetylation levels. Western blot showed that the total protein acetylation level of spermatozoa did not change after Scriptaid treatment, whereas the tyrosine phosphorylation level, which is positively correlated with sperm motility,[24] showed a notable increase (**Figure 4b**). Each group consisted of three biological replicates.

Differential metabolites of Scriptaid-treated sperm

OPLS–DA was used to compare metabolomic data between control and Scriptaid-treated spermatozoa for pairwise comparisons, with the goal of maximizing separation within groups (**Figure 5a**). OPLS–DA model quality was evaluated using permutation tests. OPLS–DA score plots showed clear separation between different treatment groups. R^2 (close to 1) and Q^2 (> 0.5) values indicated that the models were stable and appropriate for fitness and prediction.[25] These results confirm that sperm metabolites following Scriptaid treatment contained distinct ingredients compared with those in the control.

Volcano plots revealed abundant differential metabolites between the Scriptaid-treated spermatozoa and control groups (**Figure 5b**). There were 64 metabolites with increased levels and 24 metabolites with decreased levels in the Scriptaid group (**Figure 5c** and **Table 1**).

The differential metabolites between the two groups could be categorized into three major classes in KEGG (**Figure 5d**):

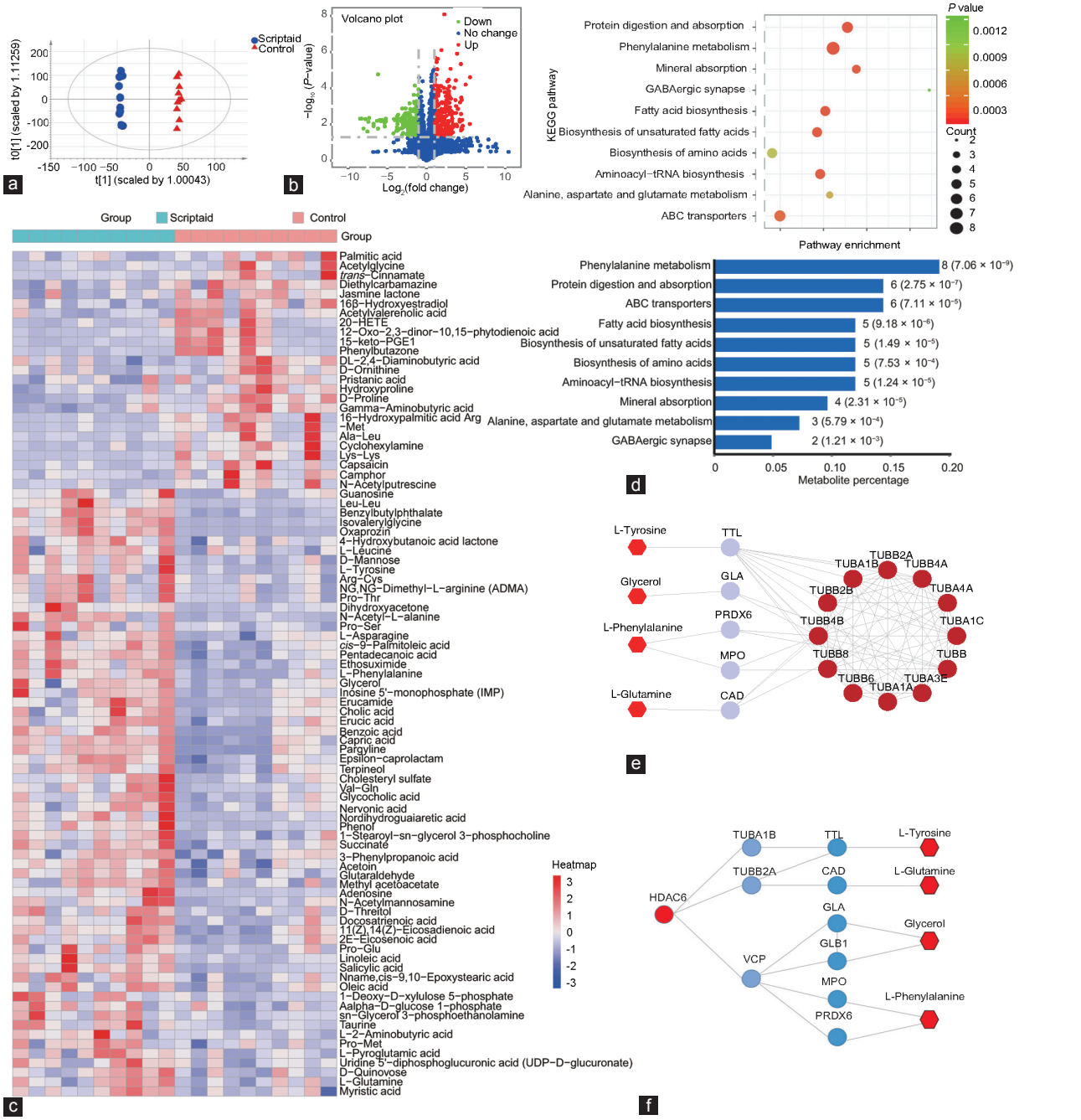


Figure 5: Metabolomic analysis of human sperm after treatment with Scriptaid. (**a**) OPLS-DA analysis of profiling data. R^2 (0–1) measures data explanation; higher values indicate better interpretation. Q^2 assesses predictive performance; above 0.5 implies good prediction. t[1]: score of the first predictive component; t0[1]: score of the first orthogonal component. (**b**) The volcano plot of AZS control group control versus AZS Scriptaid-treated group. (**c**) Heatmap visualization of DEG expression levels. Colors ranging from red (high expression level) to blue (low expression level) represent the FPKM values. (**d**) KEGG enrichment pathways of differential metabolites. (**e**) Connecting protein between sperm motility-related protein (circle) and metabolites (hexagon). (**f**) Connecting proteins (circle) between HDAC6 (red circle) and significantly different metabolites (hexagon) observed in AZS sperm after treatment with 10 μmol l^{-1} Scriptaid for 30 min. n = 10. OPLS-DA: orthogonal projections to latent structures discriminant analysis; DEG: differentially expressed gene; FPKM: fragments per kilobase of exon model per million mapped fragments; KEGG: Kyoto Encyclopedia of Genes and Genomes; AZS: asthenozoospermic; HDAC6: histone acetyltransferases and deacetylases.

phenylalanine metabolism, protein digestion and absorption, and ATP-binding cassette transporter (ABC) transporters.

Among the identified differential metabolites, L-tyrosine, glycerol, L-phenylalanine, and L-glutamine are associated with the sperm motility-related protein tubulin via the connecting proteins tubulin tyrosine ligase (TTL), alpha-galactosidase A (GLA), peroxiredoxin-6 (PRDX6), myeloperoxidase (MPO), and carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), as shown in **Figure 5e**. The target protein HDAC6 of Scriptaid was associated with the connecting proteins tubulin alpha-1B chain (TUBA1B), tubulin beta-2A chain (TUBB2A), and valosin-containing protein (VCP), as shown in **Figure 5f**.

Table 1: Metabolites associated with sperm-related functions

Metabolite	*Function related with sperm*	*Reference*
L-phenylalanine	Stimulate low levels of ROS production; improve the success rate of IVF	51
Oleic acid	Enhance ATP production; improve boar sperm motility	52
Succinate	Increase the MMP of sperm	53
L-leucine	Increase sperm motility	54
Palmitic acid	Increase sperm motility; elevate levels of MMP and ATP	52
Gamma-aminobutyric acid	Improve sperm motility and capacitation	55
Phenylalanine	Stimulate sustained low levels of ROS production; sperm energy supply, and acquisition processes; improve IVF rate in ART	56

ROS: reactive oxygen species; IVF: *in vitro* fertilization; ATP: adenosine triphosphate; MMP: mitochondrial membrane potential; ART: assisted reproductive technology

DISCUSSION

We demonstrated that Scriptaid, a histone deacetylase inhibitor, exhibits novel and long-lasting effects on sperm motility stimulation and protection against AZS and may be beneficial for clinical sperm cryopreservation, IVF, or ICSI outcomes.

As cryopreservation often results in decreased sperm motility and fertilization rates,[26] Scriptaid, in either freezing or postthaw media, may have an important clinical application to improve sperm motility and improve ART fertilization rates. For example, a basic freezing medium supplemented with Scriptaid can achieve the protective effects of commercial media, which are well-optimized. Scriptaid also has potential for male fertility preservation, especially for spermatozoa retrieved using the microtesticular sperm extraction technique, as the ICSI outcome is not affected by freezing[27] and is even superior for testicular spermatozoa compared with ejaculated counterparts.[28]

Direct application of Scriptaid *in vitro* showed rapid and durable stimulatory and protective effects on sperm motility. Herein, the effects of Scriptaid lasted for, but were not confined to, 5 h, making ART application comparatively efficient for the rapid selection of viable spermatozoa for ICSI, while also extending the duration of sperm viability, thereby providing greater flexibility in ART application. The motility of Scriptaid-treated spermatozoa decreased by approximately 60% after 12 h compared with 2 h, but the control group samples exhibited a similar trend. This suggests that the decline in motility might be a general phenomenon attributable to changes in the cellular physiological state owing to the extended incubation time (*e.g.*, a decline in energy metabolism or alterations in other biological processes). Despite the decline in motility of Scriptaid-treated samples during incubation, it remained significantly higher compared with that of samples in commercial media after 12 h, suggesting superior performance over extended periods. Owing to oocytes sensitivity to fluctuations in pH and temperature, prolonged manipulation outside the incubator can cause irreversible damage to their spindle apparatus,[29] which significantly impacts subsequent embryonic development and clinical outcomes. Therefore, strict control over sperm selection time is crucial for oocyte manipulation. Shortening the duration of sperm selection for normal morphology is important for favorable outcomes. Efficient techniques improve fertilization rates for ICSI.[30] Scriptaid treatment may shorten the selection process for motile spermatozoa, which would improve fertilization rates and promote embryonic development. In ICSI for male factor infertility, microscopic observation is necessary to select spermatozoa with the best fertilization and embryo development potential.[31] However, this process is time-consuming and nonmotile cells tend to increase over time, which ultimately hinders optimal spermatozoa selection. We conclude that Scriptaid can effectively maintain sperm motility for up to 5 h, offering clinicians a substantial timeframe for sperm selection.

In the study of Kong *et al.*,[16] Scriptaid has been proved a safe agent for embryos at working concentrations of 0.125–1 μmol l^{-1}. VAP is an indicator of sperm motility, fertilization capacity, sperm quality, and health status.[32,33] A longer average path is typically associated with better swimming ability and higher velocity.[32] Spermatozoa that exhibit a straight, stable, and regular average path are more likely to achieve successful fertilization.[32] BCF is the frequency at which sperm tails beat or oscillate during forward movement. Sperm with higher BCF exhibit more active and coordinated tail movement, so BCF serves as an important indicator of sperm quality and reproductive potential.[34,35] Mouse embryonic development can be enhanced by Scriptaid treatment.[16] Scriptaid treatment can promote embryonic development, enhance embryonic safety, and improve developmental potential in various species (*e.g.*, pigs,[36] mini-pigs,[37] buffalo,[38] and bovines[39]). To avoid species bias and direct embryonic effects of trace Scriptaid that could be introduced by sperm incubation, we used Scriptaid-pretreated human spermatozoa in a washout process. In the clinical experiments, we employed IVM oocytes, which are suitable for studying fertilization and embryonic development as they have the potential to develop into blastocysts, and allow the collection and maturation of oocytes in a short timeframe, reducing the treatment cycle duration. The IVM oocyte fertilization rate in the Scriptaid-pretreated group improved after ICSI, suggesting that Scriptaid may be a potential therapeutic option for targeting spermatozoa to improve the success rates of ICSI. Although the data suggest that Scriptaid may have potential as an agent for promoting sperm motility or improving ART outcomes (*e.g.*, ICSI), comprehensive pharmacological and toxicological studies are needed.

Although Scriptaid activities in embryo development are well recognized, its activities in spermatozoa, with respect to cellular and molecular mechanisms, are novel. We explored primary changes induced by Scriptaid treatment of human spermatozoa from the perspectives of energy supply, fertilization potential, posttranslational modifications, and metabolic changes. Higher levels of ATP and MMP induced by Scriptaid are possible explanations from the energy perspective response, as spermatozoa with higher ATP are more likely to have better motility and fertilization potential,[40,41] and spermatozoa with higher MMP are more likely to have better motility, fertilization potential,[42] and embryo development.[43] As HA-binding ability is an important predictor of fertilization potential, spermatozoa with high HA-binding ability, in response to Scriptaid treatment, are associated with intact membranes, improved motility, and other changes that promote fertilization. Thus, Scriptaid serves as a stimulating agent for the selection of high-quality spermatozoa. While our experiments primarily focused on AZS samples, our preliminary observations suggest that Scriptaid also exerts a certain promotive effect on the motility of normal sperm samples. Additionally, studies indicate a close positive correlation between spermatozoa quality and sperm

motility, implying that Scriptaid treatment may favor the selection of high-quality spermatozoa with improved motility characteristics. The postulated Scriptaid target, HDAC6, is located in the neck region (connecting piece) of human spermatozoa, but in the tail of rat sperm.[13] The increase in tyrosine phosphorylation levels induced by Scriptaid provides further evidence of the crucial role of HDAC6 in regulating sperm motility.[44] However, the intact acetylation profile of sperm proteins did not support the deacetylation pathway. The exact target proteins and molecular mechanisms of Scriptaid action in human sperm warrant further investigation. Phosphorylated tyrosine residues play a critical role by regulating proteins related to sperm motility in sperm.[45,46] For instance, tyrosine phosphorylation can alter the structure and function of proteins, thereby affecting the activity of the cellular cytoskeleton and motility proteins, subsequently influencing sperm flagellar movement and motility; the underlying mechanisms and pathways may provide potential therapeutic targets for male infertility. Significant changes in spermatozoa metabolites after Scriptaid treatment were associated with tubulin and HDAC6, which is consistent with previous findings that tubulin plays a vital role in maintaining the structural integrity of sperm flagella and is associated with sperm motility.[47] The differential metabolites identified in our study suggest potential roles in the regulation of sperm motility, such as L-phenylalanine, oleic acid, succinate, L-leucine, gamma-aminobutyric acid, and phenylalanine, and that Scriptaid positively affects sperm function and fertilization rate by improving overall spermatozoa quality through complex mechanisms.

HDAC6, as a potential target of Scriptaid, is indirectly supported by its relationship with sperm motility and related functional proteins (*e.g.*, tubulin alpha 1b [TUBA1B], tubulin beta 2A class IIa [TUBB2A], and VCP). Some studies show that protein acetylation affects sperm function, especially lysine acetylation, and could modulate mouse sperm capacitation.[48,49] This suggests that Scriptaid may promote sperm motility by affecting acetylation levels of diverse proteins through HDAC6 inhibition. Scriptaid may alter the acetylation levels of numerous proteins by inhibiting HDAC6, which could serve as a crucial starting point for influencing other mechanisms. By suppressing specific protein acetylation related to motion, ATP concentration and MMP increase, which could enhance the spermatozoa energy supply, consequently boosting motility. The increase in ATP might also further drive phosphorylation of tyrosine residues. Tyrosine phosphorylation plays a pivotal role in sperm motility, and this process could potentially enhance motility by improving energy conversion. Simultaneously, changes in metabolic byproducts, which could be a result of heightened sperm motility, might also further contribute to enhancing sperm movement.

Scriptaid could potentially enhance sperm motility and fertilization through these interconnected and synergistic mechanisms. While each mechanism could independently impact sperm functionality, their combined effects might yield more significant outcomes. Currently, the distribution of Scriptaid following cellular treatment can be determined using fluorescence-based techniques,[50] enabling the determination of target protein expression and acetylation levels. Using active Scriptaid analogs as baits, it is possible to identify the true targets of Scriptaid in human spermatozoa. We synthesized a series of Scriptaid analogs and revealed that SZY-13-34 exhibited significant stimulatory activity at a concentration of 20 μmol l^{-1}, although it did not reach the level observed for Scriptaid. SZY-13-34 demonstrated the highest stimulatory activity at a concentration of 70 μmol l^{-1}, indicating the potential of using this active Scriptaid analog for target protein identification. Our findings also provide insights into the relationship between Scriptaid structure and activity and its analogs. This may be useful for designing potent compounds, identifying novel target proteins, and providing a foundation for further optimization of the molecular design of Scriptaid analogs, emphasizing the need for a precise balance of functional groups to achieve desired bioactivity.

In summary, our study provides new insights into the potential use of Scriptaid for the stimulation and protection of sperm motility, particularly in sperm cryopreservation, IVF, and ICSI, by regulating sperm energy metabolism, protein modification, sperm motility, sperm metabolites, and proteins associated with fertilization function. These findings have important implications for improving sperm function and fertility in the field of reproductive medicine and for ARTs in clinical settings. Because of limited number of cases, our study only explored the impact of Scriptaid treatment on fertilization rate, and more observation would be made in future in terms of reproduction.

AUTHOR CONTRIBUTIONS

HGY designed and supervised the study. SYL provided clinical guidance and conducted patient recruitment. HD supervised the study and improved the manuscript. YTY performed sperm parameter analysis and immunoassays, and drafted the manuscript. BY collected semen samples, conducted ICSI and fertilization rate statistics, and helped draft the manuscript. LNG collected clinical information and helped analyze the data. ML helped conduct the clinical ethics application and obtained approval. YHL helped perform the experiments. ZYS synthesized the chemicals. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

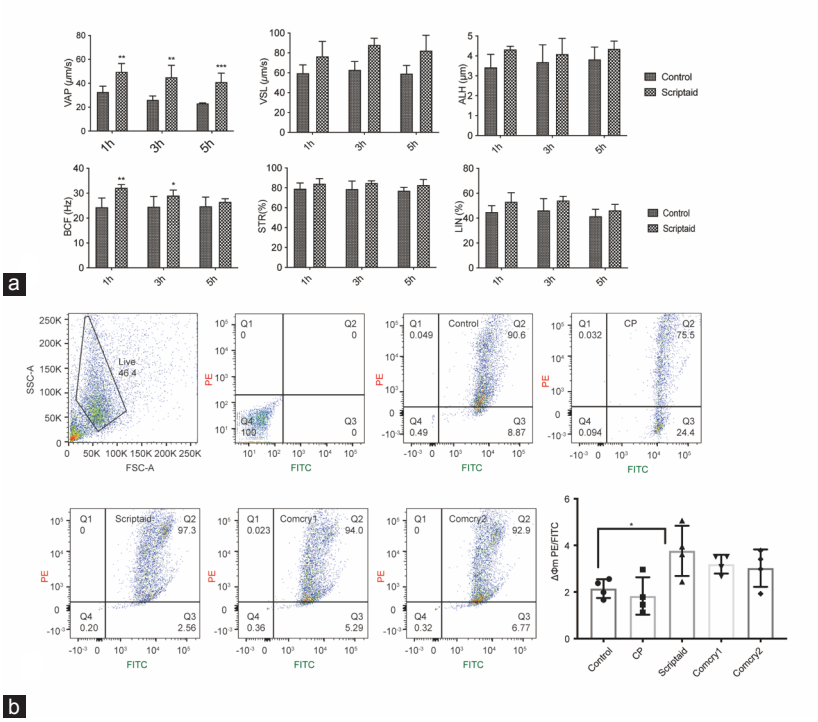
This work was supported by the Science and Technology Commission of Shanghai Municipality (grant No. 20ZR1448100). **Figure 1d** and **1e** were created with BioRender.com (https://www.biorender.com/).

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

REFERENCES

1 Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, *et al*. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 2001; 345: 1388–93.
2 Villani MT, Morini D, Spaggiari G, Falbo AI, Melli B, *et al*. Are sperm parameters able to predict the success of assisted reproductive technology? A retrospective analysis of over 22,000 assisted reproductive technology cycles. *Andrology* 2022; 10: 310–21.
3 Serrano R, Garcia-Marin LJ, Bragado MJ. Sperm phosphoproteome: unraveling male infertility. *Biology* 2022; 11: 659.
4 Cheng YM, Hu XN, Peng Z, Pan TT, Wang F, *et al*. Lysine glutarylation in human sperm is associated with progressive motility. *Hum Reprod* 2019; 34: 1186–94.
5 Cheng YM, Peng Z, Chen HY, Pan TT, Hu XN, *et al*. Posttranslational lysine 2-hydroxyisobutyrylation of human sperm tail proteins affects motility. *Hum Reprod* 2020; 35: 494–503.
6 Sun G, Jiang M, Zhou T, Guo Y, Cui Y, *et al*. Insights into the lysine acetylproteome of human sperm. *J Proteomics* 2014; 109: 199–211.
7 Yu H, Diao H, Wang C, Lin Y, Yu F, *et al*. Acetylproteomic analysis reveals functional implications of lysine acetylation in human spermatozoa (sperm). *Mol Cell Proteom* 2015; 14: 1009–23.
8 Bhagwat S, Dalvi V, Chandrasekhar D, Matthew T, Acharya K, *et al*. Acetylated α-tubulin is reduced in individuals with poor sperm motility. *Fertil Steril* 2014; 101: 95–104.e3.
9 Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, *et al*. HDAC6 is a microtubule-associated deacetylase. *Nature* 2002; 417: 455–8.
10 Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, *et al*. *In vivo* destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J* 2002; 21: 6820–31.
11 North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. The human Sir2 ortholog,

SIRT2, is an NAD+-dependent tubulin deacetylase. *Mol Cell* 2003; 11: 437–44.
12 Palazzo A, Ackerman B, Gundersen GG. Cell biology: tubulin acetylation and cell motility. *Nature* 2003; 421: 230.
13 Parab S, Shetty O, Gaonkar R, Balasinor N, Khole V, *et al*. HDAC6 deacetylates alpha tubulin in sperm and modulates sperm motility in holtzman rat. *Cell Tissue Res* 2015; 359: 665–78.
14 Yang Y, Ran J, Liu M, Li D, Li Y, *et al*. CYLD mediates ciliogenesis in multiple organs by deubiquitinating Cep70 and inactivating HDAC6. *Cell Res* 2014; 24: 1342–53.
15 Zhang H, Xiao Y, Wang X, Riaz H, Li W, *et al*. Effects of histone deacetylase inhibitors on the early development of bovine androgenetic embryos. *Cell Reprogram* 2014; 16: 54–64.
16 Kong P, Yin M, Chen D, Li S, Li Y, *et al*. Effects of the histone deacetylase inhibitor "Scriptaid" on the developmental competence of mouse embryos generated through round spermatid injection. *Hum Reprod* 2017; 32: 76–87.
17 World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010.
18 Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, *et al*. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* 2017; 45: D362–8.
19 Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, *et al*. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res* 2015; 43: D447–52.
20 Karnovsky A, Weymouth T, Hull T, Tarcea VG, Scardoni G, *et al*. Metscape 2 bioinformatics tool for the analysis and visualization of metabolomics and gene expression data. *Bioinformatics* 2012; 28: 373–80.
21 Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; 13: 2498–504.
22 Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after *in vitro* fertilization. *Fertil Steril* 2004; 82: 378–83.
23 Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, *et al*. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril* 2003; 79 Suppl 3: 1616–24.
24 Sati L, Cayli S, Delpiano E, Sakkas D, Huszar G. The pattern of tyrosine phosphorylation in human sperm in response to binding to zona pellucida or hyaluronic acid. *Reprod Sci* 2014; 21: 573–81.
25 Xia X, Chen C, Yang L, Wang Y, Duan A, *et al*. Analysis of metabolites in young and mature *Docynia delavayi* (Franch.) Schneid leaves using UPLC-ESI-MS/MS. *PeerJ* 2022; 10: e12844.
26 Huang C, Tang YL, Hu JL, Zhou WJ, Huang ZH, *et al*. Update on techniques for cryopreservation of human spermatozoa. *Asian J Androl* 2022; 24: 563–9.
27 Yu Z, Wei Z, Yang J, Wang T, Jiang H, *et al*. Comparison of intracytoplasmic sperm injection outcome with fresh versus frozen-thawed testicular sperm in men with nonobstructive azoospermia: a systematic review and meta-analysis. *J Assist Reprod Genet* 2018; 35: 1247–57.
28 Kang YN, Hsiao YW, Chen CY, Wu CC. Testicular sperm is superior to ejaculated sperm for ICSI in cryptozoospermia: an update systematic review and meta-analysis. *Sci Rep* 2018; 8: 7874.
29 Khoudja R, Li T, Ding C, Xu Y, Liu Y, *et al*. Effect of co-incubation of oocytes with a decreasing number of spermatozoa on embryo quality. *Reprod Biomed Online* 2013; 26: 353–9.
30 Sakkas D, Ramalingam M, Garrido N, Barratt CL. Sperm selection in natural conception: what can we learn from mother nature to improve assisted reproduction outcomes? *Hum Reprod Update* 2015; 21: 711–26.
31 Moretti E, Signorini C, Noto D, Corsaro R, Collodel G. The relevance of sperm morphology in male infertility. *Front Reprod Health* 2022; 4: 945351.
32 Vasan SS. Semen analysis and sperm function tests: how much to test? *Indian J Urol* 2011; 27: 41–8.
33 Sloter E, Schmid TE, Marchetti F, Eskenazi B, Nath J, *et al*. Quantitative effects of male age on sperm motion. *Hum Reprod* 2006; 21: 2868–75.
34 Nakao S, Takeo T, Watanabe H, Kondoh G, Nakagata N. Successful selection of mouse sperm with high viability and fertility using microfluidics chip cell sorter. *Sci Rep* 2020; 10: 8862.
35 Larsen L, Scheike T, Jensen TK, Bonde JP, Ernst E, *et al*. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. The Danish first pregnancy planner study team. *Hum Reprod* 2000; 15: 1562–7.
36 Li W, Zheng H, Yang Y, Xu H, Guo Z. A diverse English keyword search reveals the value of scriptaid treatment for porcine embryo development following somatic cell nuclear transfer. *Reprod Fertil Dev* 2022; 34: 798–803.
37 Zhang L, Huang Y, Wu Y, Si J, Huang Y, *et al*. Scriptaid upregulates expression of development-related genes, inhibits apoptosis, and improves the development of somatic cell nuclear transfer mini-pig embryos. *Cell Reprogram* 2017; 19: 19–26.
38 Panda SK, George A, Saha A, Sharma R, Singh AK, *et al*. Effect of scriptaid, a histone deacetylase inhibitor, on the developmental competence of handmade cloned buffalo (*Bubalus bubalis*) embryos. *Theriogenology* 2012; 77: 195–200.
39 Wang LJ, Zhang H, Wang YS, Xu WB, Xiong XR, *et al*. Scriptaid improves *in vitro* development and nuclear reprogramming of somatic cell nuclear transfer bovine embryos. *Cell Reprogram* 2011; 13: 431–9.
40 Sanchez-Rodriguez A, Sansegundo E, Tourmente M, Roldan ER. Effect of high viscosity on energy metabolism and kinematics of spermatozoa from three mouse species incubated under capacitating conditions. *Int J Mol Sci* 2022; 23: 15247.
41 Moustakli E, Zikopoulos A, Sakaloglou P, Bouba I, Sofikitis N, *et al*. Functional association between telomeres, oxidation and mitochondria. *Front Reprod Health* 2023; 5: 1107215.
42 Paoli D, Gallo M, Rizzo F, Baldi E, Francavilla S, *et al*. Mitochondrial membrane potential profile and its correlation with increasing sperm motility. *Fertil Steril* 2011; 95: 2315–9.
43 Malić Vončina S, Golob B, Ihan A, Kopitar AN, Kolbezen M, *et al*. Sperm DNA fragmentation and mitochondrial membrane potential combined are better for predicting natural conception than standard sperm parameters. *Fertil Steril* 2016; 105: 637–44.e1.
44 Chawan V, Yevate S, Gajbhiye R, Kulkarni V, Parte P. Acetylation/deacetylation and microtubule associated proteins influence flagellar axonemal stability and sperm motility. *Biosci Rep* 2020; 40: BSR20202442.
45 Leclerc P, de Lamirande E, Gagnon C. Cyclic adenosine 3',5'monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod* 1996; 55: 684–92.
46 Jiménez-Trejo F, Tapia-Rodríguez M, Cerbón M, Kuhn DM, Manjarrez-Gutiérrez G, *et al*. Evidence of 5-HT components in human sperm: implications for protein tyrosine phosphorylation and the physiology of motility. *Reproduction* 2012; 144: 677–85.
47 Zhao W, Li Z, Ping P, Wang G, Yuan X, *et al*. Outer dense fibers stabilize the axoneme to maintain sperm motility. *J Cell Mol Med* 2018; 22: 1755–68.
48 Bowker Z, Goldstein S, Breitbart H. Protein acetylation protects sperm from spontaneous acrosome reaction. *Theriogenology* 2022; 191: 231–8.
49 Ritagliati C, Luque GM, Stival C, Baro Graf C, Buffone MG, *et al*. Lysine acetylation modulates mouse sperm capacitation. *Sci Rep* 2018; 8: 13334.
50 Fleming CL, Ashton TD, Nowell C, Devlin M, Natoli A, *et al*. A fluorescent histone deacetylase (HDAC) inhibitor for cellular imaging. *Chem Commun* 2015; 51: 7827–30.
51 Houston B, Curry B, Aitken RJ. Human spermatozoa possess an IL4I1 L-amino acid oxidase with a potential role in sperm function. *Reproduction* 2015; 149: 587–96.
52 Zhu Z, Li R, Feng C, Liu R, Zheng Y, *et al*. Exogenous oleic acid and palmitic acid improve boar sperm motility via enhancing mitochondrial B-oxidation for ATP generation. *Animals (Basel)* 2020; 10: 591.
53 Paventi G, Lessard C, Bailey JL, Passarella S. In boar sperm capacitation L-lactate and succinate, but not pyruvate and citrate, contribute to the mitochondrial membrane potential increase as monitored via safranine O fluorescence. *Biochem Biophys Res Commun* 2015; 462: 257–62.
54 Zhang J, Zhang X, Liu Y, Su Z, Dawar FU, *et al*. Leucine mediates autophagosome-lysosome fusion and improves sperm motility by activating the PI3K/Akt pathway. *Oncotarget* 2017; 8: 111807–18.
55 Kurata S, Hiradate Y, Umezu K, Hara K, Tanemura K. Capacitation of mouse sperm is modulated by gamma-aminobutyric acid (GABA) concentration. *J Reprod Dev* 2019; 65: 327–34.
56 Serafini S, O'Flaherty C. Redox regulation to modulate phosphorylation events in human spermatozoa. *Antioxid Redox Signal* 2022; 37: 437–50.



Supplementary Figure 1: Effects of Scriptaid on other sperm motility parameters apart from motility and progressive motility, as well as differential effects of various cryopreservation solutions on sperm mitochondrial membrane potential (MMP). (**a**) Time effects of 10 μmol l^{-1} Scriptaid on sperm average path velocity, straight line velocity, amplitude of lateral head displacement, beat cross frequency, straightness, and linearity. More than 2000 sperm were calculated in each sample. Two-way analysis of variance (ANOVA). (**b**) Fluorescence-activated cell sorting analysis of the MMP of human sperm cryopreserved in different cryopreservation solutions. One-way ANOVA. Bar: mean ± standard deviation. $^{*}P < 0.05$, $n \geq 3$ semen sample in each group. FITC: Fluorescein Isothiocyanate; PE Phycoerythrin; SSC-A: Side Scatter-Area; FSC-A: Forward Scatter-Area.