Reproductive Biology and Endocrinology

RESEARCH

Open Access

Cordycepin improves hyperactivation and acrosome reaction through adenosine receptors during human sperm capacitation in vitro

Lijun Shan[1,2], Linmei Gao[1,2], Yuhao Chai[1,2], Kun Li[1,3], Jianmin Yu[1], Fei Liang[2], Jiangfeng Qin[2], Ya Ni[1,3] and Peibei Sun[1,3*]

Abstract

Background Sperm capacitation is a prerequisite for natural or in vitro fertilization. After capacitation, sperm become hyperactivated and undergo an acrosome reaction, which helps them penetrate the oocyte. Cordycepin, a bioactive compound first isolated from *Cordyceps militaris*, is an adenosine analog with numerous physiological activities. However, its effects on sperm capacitation remain unclear. This study aims to elucidate the effects and mechanisms of cordycepin on human sperm capacitation.

Methods During in vitro capacitation culture, healthy human sperm were treated with cordycepin (20, 100, 500 μM). Sperm motility and hyperactivation were detected using a computer-assisted sperm analyzer. Sperm acrosome reaction was measured using fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin. Sperm protein kinase A (PKA) activity was analyzed using an ELISA kit. The levels of sperm protein tyrosine phosphorylation were detected by western blotting. Sperm DNA damage was detected by a sperm chromatin dispersion assay. Reactive oxygen species (ROS) were measured using the fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate. The expression and localization of adenosine receptors were analyzed by western blotting and immunofluorescence. The specific inhibitors of adenosine receptors were used to confirm their effects on cordycepin-induced sperm capacitation. Finally, molecular docking was performed to analyze the interaction between cordycepin and adenosine receptors.

Results Cordycepin improved hyperactivated sperm motility, acrosome reaction, PKA activity, and protein tyrosine phosphorylation during capacitation while having no obvious effects on sperm ROS or DNA damage. Four adenosine receptor subtypes were expressed in human sperm, but their localizations differed. Inhibition of adenosine receptors significantly decreased cordycepin-induced sperm hyperactivation and the acrosome reaction. Molecular docking showed that cordycepin can bind to the four subtypes of adenosine receptors.

Conclusion Cordycepin may promote human sperm capacitation through adenosine receptor-mediated signaling pathways. These findings may be useful for assisted reproductive technology and animal breeding.

Keywords Acrosome reaction, Adenosine receptor, Capacitation, Cordycepin, Human sperm, Hyperactivation

*Correspondence:
Peibei Sun
peibeisun@hmc.edu.cn
[1]School of Pharmacy, Hangzhou Medical College, Hangzhou, Zhejiang, China
[2]School of Basic Medical Sciences and Forensic Medicine, Hangzhou Medical College, Hangzhou, Zhejiang, China
[3]Zhejiang Provincial Laboratory of Experimental Animal's & Nonclinical Laboratory Studies, Hangzhou Medical College, Hangzhou, Zhejiang, China

Background

Sperm capacitation is a prerequisite for natural fertilization. Human sperm are not capable of fertilization immediately after ejaculation. They must first reside in the female reproductive tract where they undergo physiological and biochemical changes for fertilization, which is known as sperm capacitation [1]. When using assisted reproductive technologies, such as in vitro fertilization (IVF), sperm also need to complete capacitation in the culture medium, which mimics the constituents of human tubal fluid (HTF). During capacitation, sperm cholesterol efflux is accompanied by changes in membrane permeability and increases in intracellular Ca^{2+} and HCO_3^- ion concentrations. Ca^{2+} and HCO_3^- activate soluble adenylyl cyclase and subsequently open the downstream cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathways. PKA activates other tyrosine kinases and indirectly increases tyrosine phosphorylation of sperm proteins, which is crucial for sperm capacitation [2]. Subsequently, capacitated sperm become hyperactivated and an acrosome reaction (AR) occurs. AR is an acrosome exocytosis mechanism that promotes the ability of sperm to hydrolyze the zona pellucida and complete sperm-oocyte fusion [3]. Hyperactivation is a sperm motility pattern characterized by increased curvilinear motion that helps sperm penetrate the zona pellucida and cumulus cells surrounding the oocyte [4]. Therefore, enhancing sperm capacitation may improve fertilization and fertility. However, the mechanisms during sperm capacitation are still elusive.

Adenosine receptors (AdoRs) play important roles in sperm capacitation. The AdoRs contain four subtypes: A1, A2a, A2b, and A3. They all belong to the G protein-coupled receptor (GPCR) family [5]. Among them, A2a and A2b AdoRs are coupled to G_s, increase cAMP levels, and are considered stimulatory. In contrast, A1 and A3 AdoRs couple to G_i/G_0, decrease cAMP levels and are considered inhibitory [6]. It has been reported that A1 AdoR was localized in the acrosome and equatorial region of the sperm head, and midpiece of sperm tail [7]. A1 AdoR-knockout mice are subfertile, and their sperm capacitation is disrupted [8]. A1 AdoR inhibition also decreases HCO_3^- and cAMP-mediated AR [9], whereas A1 AdoR agonists promote capacitation [10]. Recently, Chen et al. also found that A2a AdoR was expressed in human sperm. Its antagonist decreased sperm motility, intracellular Ca^{2+} concentration, and penetration ability [11]. Adenosine and its analogs can stimulate sperm motility, capacitation, and fertilization through the A2 AdoR [6]. Therefore, we are curious about the effects of adenosine analogs on sperm capacitation.

Cordyceps militaris is a fungal parasite similar to *Cordyceps sinensis*. They belong to the same fungal order and are used as folk tonics in traditional Chinese medicine [12]. They are also believed to improve sexual functions. Cordycepin (3′-deoxyadenosine) was first isolated from *Cordyceps militaris* in the 1950s [13]. It is an adenosine analog that lacks a hydroxyl group at the 3′ site of the ribose moiety [14]. Cordycepin and adenosine can sometimes be indistinguishable by some proteins in the body. Therefore, cordycepin may have special functions, such as interacting with AdoRs or polyadenylates and affecting downstream signaling pathways [15, 16]. Huang et al. reported that cordycepin improves cognitive function through the A2a AdoR in a mouse model of Parkinson's disease [15]. Leu et al. also found that cordycepin activates steroidogenesis in Leydig cells via AdoRs [17]. Cordycepin also has numerous physiological and pharmacological properties, including antitumor, antiviral, anti-inflammatory, antioxidant, and antimicrobial effects [18]. Cordycepin can also play an essential role in male reproduction. For example, it increases steroidogenesis in mouse Leydig cells [19], ameliorates testicular function [20], and improves sperm motility [21] and quality [22]. However, the role and mechanism of cordycepin in human sperm capacitation remain unknown. The structure of cordycepin is similar to that of adenosine; therefore, we hypothesize that cordycepin regulates human sperm capacitation through AdoRs.

In this study, we explored the effects of cordycepin on human sperm capacitation. The results showed that cordycepin significantly increased sperm AR and hyperactivated motility during capacitation. However, cordycepin had no significant effects on reactive oxygen species (ROS) and DNA damage in human sperm. Cordycepin also tended to increase PKA activity and protein tyrosine phosphorylation, indicating a potential connection to the cAMP/PKA signaling pathway. We also explored the expression and localization of all AdoRs in human sperm. Specific inhibitors were used to distinguish between the types of AdoRs involved in cordycepin-stimulated capacitation. Molecular docking predicted that cordycepin could bind to four AdoR subtypes. These results suggest that cordycepin promotes human sperm capacitation through AdoRs. These findings may be useful for IVF and animal breeding in the future.

Methods

Reagents

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and cordycepin were obtained from Solarbio Life Sciences (Beijing, China). Propidium iodide (PI), fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA), and HTF medium were bought from Sigma-Aldrich (St. Louis, MO, USA). SDS lysis buffer, the bicinchoninic acid (BCA) kit, and Fluo-3 AM were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). The enhanced chemiluminescence

(ECL) kit and prestained protein marker were bought from Thermo Fisher Scientific (Waltham, MA, USA). The protease and phosphatase inhibitor cocktails were provided by Roche (Mannheim, Germany). The PKA activity assay kit, antibodies to A2a AdoR (ab3461), A2b AdoR (ab229671), A3 AdoR (ab197350), and phosphotyrosine (ab179530), as well as the Alexa Fluor 488-conjugated goat anti-rabbit (ab150077), Alexa Fluor 555-conjugated goat anti-mouse (ab150118), horseradish peroxidase (HRP)-conjugated goat anti-rabbit (ab6721), and HRP-conjugated goat anti-mouse (ab6789) secondary antibodies were obtained from Abcam (Cambridge, UK). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (BK7021) was purchased from Baoke Biotechnology (Hangzhou, China). The anti-A1 AdoR antibody (sc-66193) was provided by Santa Cruz Biotechnology (Dallas, TX, USA). A sperm chromatin dispersion (SCD) kit was provided by BRED Life Science Technology (Shenzhen, China). The inhibitors to the A1 (DPCPX; HY-100937), A2b (MRS1754; HY-14121), and A3 (MRS1191; HY-124543) AdoRs were purchased from MedChemExpress (Shanghai, China). The A2a AdoR inhibitor (DMPX; S37466) was purchased from Yuanye Biotechnology (Shanghai, China).

Sperm capacitation culture

The study was performed in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Hangzhou Medical College (LL2022-16). We recruited 20 male semen donors (aged 20–38) who had passed physical examinations to exclude disease of the reproductive system or other serious conditions [23, 24]. All participants provided written informed consent. Their semen parameters are shown in Table S1. All donors met the criteria of the World Health Organization laboratory manual, with sperm survival rate ≥ 58%, sperm motility ≥ 40%, sperm concentration ≥ 15×10^6 sperm/mL, and normal sperm morphology ≥ 4%. After abstinence for 3–7 d, fresh samples from at least three donors were randomly obtained for each experiment. The semen samples were collected and liquefied in a 5% CO_2 incubator at 37℃ for 30 min. Then, the samples were mixed. Dead spermatozoa and cell debris were removed by 40% and 80% discontinuous Percoll gradient centrifugation. The sperm precipitate was washed with phosphate-buffered saline, and then some sperm samples were collected as the control group at 0 h. Subsequently, the residual samples were adjusted to 20×10^6 sperm cells/mL using HTF and were divided into different groups: cordycepin (20, 100, and 500 μM), vehicle control (dimethyl sulfoxide [DMSO] 0.1% v/v), A1 AdoR inhibitor (DPCPX 0.001, 0.01, and 0.1 μM), A2a AdoR inhibitor (DMPX 0.001, 0.01, and 0.1 μM), A2b AdoR inhibitor (MRS1754 0.001, 0.01, and 0.1 μM), A3 AdoR inhibitor (MRS1191 0.001, 0.01, and 0.1 μM), and PKA inhibitor (H-89 50 μM). Finally, samples were cultured in a 5% CO_2 incubator at 37℃ for 3 h.

Acrosome reaction evaluation

As only capacitated sperm can undergo AR, the sperm AR ratio was evaluated using FITC-PSA staining. Before capacitation culture, spontaneous AR was analyzed to serve as a control. After incubation with cordycepin for 3 h, the AR of the other groups was induced with progesterone (15 μM) for 15 min. Additionally, we tested whether cordycepin could directly induce the AR. After a 3-hour capacitation incubation (without cordycepin), sperm AR was induced using DMSO (vehicle control), different concentrations of cordycepin, progesterone, or a combination of cordycepin and progesterone for 15 min, respectively. Next, PI (0.03 μM) was used to stain dead sperm cells. After washing, sperm were fixed with 95% ethanol and then smeared on slides. Subsequently, sperm were stained using FITC-PSA (25 mg/L) at 4℃ overnight. After washing, the slides were observed by fluorescence microscopy. For each group, at least 600 sperm cells were counted. AR sperm showed only weak fluorescence in the equatorial zone or almost no fluorescence at the sperm head. The acrosome integrity (AI) sperm did not undergo AR, and bright fluorescence was observed at the sperm head. Bright red fluorescence can be observed at the head of dead sperm. The AR ratio and nonviable cell percentage (NVC%) were calculated.

Sperm motility parameters evaluation

Capacitated sperm exhibit hyperactivated motility; therefore, a computer-assisted sperm analyzer (CASA) was used to evaluate sperm motility parameters, including progressive motility, sperm motility, amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), straight-line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), straightness (STR, VSL/VAP × 100), linearity (LIN, VSL/VCL × 100), and hyperactivated sperm. Hyperactivated sperm have the following characteristics: VCL ≥ 150 μm/s, LIN ≤ 50%, and ALH ≥ 7.0 μm [25].

PKA activity assessment

Human sperm PKA activity was detected using a PKA activity ELISA kit. Sperm were lysed using NP40 lysis buffer according to the manufacturer's instructions. Next, the lysate protein concentration was analyzed using a BCA kit. The sperm lysate was then added to a microplate coated with PKA-specific substrates. The PKA in sperm lysate can phosphorylate the substrates. Next, a specific primary antibody was added to detect phosphorylated substrates. HRP-conjugated secondary antibodies were used to interact with the primary antibodies. The

color reaction was performed using 3,3′,5,5′-tetramethylbenzidine (TMB). A microplate reader was used to measure the color intensity.

Western blotting

Protein tyrosine phosphorylation levels and adenosine receptor expression were detected by western blotting. After capacitation, sperm samples were collected and ultrasonically lysed in SDS lysis buffer supplemented with phosphatase and protease inhibitor cocktails. Protein concentrations were detected using a BCA kit. Subsequently, 10% SDS-PAGE was used to separate different group proteins. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 3% bovine serum albumin (BSA) for 1 h, the membranes were then incubated with antibodies to phosphotyrosine, A1 AdoR, A2a AdoR, A2b AdoR, or A3 AdoR at 4℃ overnight. After washing with tris-buffered saline, HRP-conjugated secondary antibody was used to incubate the membranes for 2 h at 25℃. An ECL kit was used for the color reaction. The membranes were observed using a gel imaging system. Thereafter, antibodies were removed. The membranes were blocked using BSA again, and incubated with an antibody to GAPDH. The gray intensity was analyzed using ImageJ software.

Sperm chromatin dispersion assay

Sperm DNA fragmentation was analyzed using an SCD assay kit according to the manufacturer's instructions. After mixing with fused agar, the sperm samples were pipetted onto a slide and covered. The samples were solidified at 4℃. After removing the coverslips, the slides were immersed in solution A (acidic solution) and solution B for 7 min and 25 min, respectively. After dehydrating in an ethanol series (70-90-100%) and air drying, the sperm samples were stained using Wright's dye solution. At least 500 sperm cells from each group were observed under a microscope. Sperm with halo widths greater than the diameter of the core were considered to show DNA integrity (DI), whereas others were regarded as having DNA fragmentation (DF).

ROS level detection

Sperm ROS levels were detected using the fluorescent probe DCFH-DA. First, sperm were labeled using 10 μM DCFH-DA for 30 min in the dark. After washing, the free DCFH-DA was removed. Sperm were then added to a 96-well plate (10^6 cells/mL). The fluorescence signals were recorded using a microplate reader at 488 nm. Initial recordings were acquired before the addition of DMSO or different concentrations of cordycepin. Data were recorded every 3 min for 30 min. After capacitation for 3 h, data were acquired again. The initial values were normalized.

Indirect immunofluorescence assay

Sperm samples before or after capacitation for 3 h were fixed in 4% paraformaldehyde for 1 h. Subsequently, they were smeared on slides and air-dried. After permeabilizing with 0.1% Triton X-100, sperm samples were blocked using 10% goat serum. Thereafter, sperm were incubated with antibodies to the A1, A2a, A2b, or A3 AdoRs overnight at 4 ℃. Negative control samples were incubated with normal mouse or rabbit IgG. After washing to remove uncombined primary antibodies, Alexa Fluor 488-conjugated anti-rabbit secondary antibody or Alexa Fluor 555-conjugated anti-mouse secondary antibody was incubated for 1 h at 25℃. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, the sperm were observed using fluorescence microscopy.

Molecular docking

A1, A2a, and A2b AdoRs protein structure files (PDB ID: 5N2S, 5NLX, and 8HDO, respectively) were obtained from the PDB database. The A3 AdoR protein structure file (AF-P0DMS8-F1) was downloaded from UniProt. As there is no published crystal structure for A3 AdoR, the structure was predicted using AlphaFold. All protein structures were preprocessed using PyMol to remove water, ligands, and other proteins. The SDF file of the cordycepin 3D structure was obtained from the PubChem database and converted to mol2 using Open Babel. Molecular docking of the cordycepin and AdoRs was performed using AutoDock 4. The protein-ligand conformation with the lowest binding energy was obtained. PyMol was used to visualize the final images.

Statistical analysis

Statistical analyses were carried out using the Social Science Statistics Software Package (SPSS). Statistical significances among groups were analyzed using one-way analysis of variance (ANOVA). Dunnett's T3 test was used if the homogeneity test for variance was $P<0.05$; otherwise, the least significant difference test was used. Data were presented as mean±standard deviation (SD). Statistical significance was set at $P<0.05$.

Results

Cordycepin promotes the human sperm acrosome reaction

The effects of cordycepin on human sperm AR were examined using FITC-PSA staining [26]. Sperm with the AI pattern showed uniform and bright green fluorescence in the head region, as FITC-PSA can specifically combine with glycoproteins in the acrosome area. After AR, the glycoprotein was lost through exocytosis. Thus, sperm with the AR pattern had almost no fluorescence in the head region or only some fluorescence in the equatorial region. As shown in Fig. 1A and B, the spontaneous AR ratio at 0 h was low, approximately 16.48±3.95%.

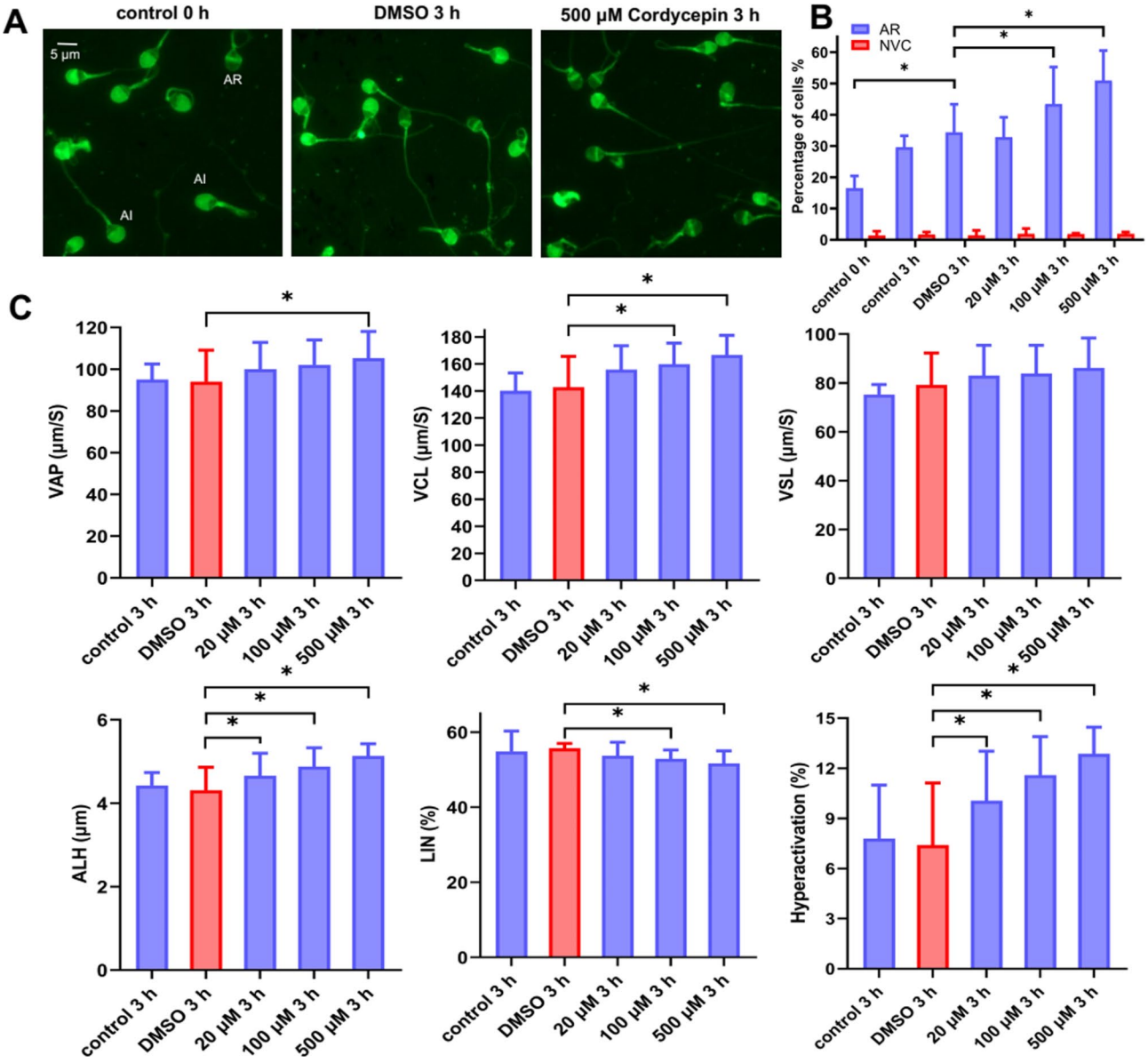

Fig. 1 Cordycepin increased acrosome reaction and hyperactivation during human sperm capacitation. (**A**) Human sperm was stained using fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA). Sperm with uniform and bright green fluorescence in the head were considered to have acrosome integrity (AI). Sperm that only had weak fluorescence in the equatorial region, or even no fluorescence in the head were regarded as exhibiting an acrosome reaction (AR). (**B**) The number of AR sperm divided by the total number of sperm was calculated to yield the AR ratio. Dead sperm were stained using propidium iodide (PI) to calculate nonviable cell percentage (NVC%). Data are presented as means ± standard deviation (SD; $n = 3$). *$P < 0.05$ (compared with the dimethyl sulfoxide [DMSO] group). (**C**) The effects of different concentrations of cordycepin on sperm motility parameters. Data are presented as means ± SD ($n = 5$). *$P < 0.05$ (compared with the DMSO group)

After capacitation for 3 h, the AR ratios of the control group (without DMSO or cordycepin) and the vehicle control group (DMSO) were approximately 29.68 ± 3.62% and 34.36 ± 9.00%, respectively, with no significant differences between them. Since cordycepin was dissolved in DMSO, the vehicle control group was used for comparison with the cordycepin-treated groups. The vehicle control group (DMSO) showed a higher AR ratio than spontaneous AR ($P = 0.000$). High concentrations of cordycepin significantly promoted AR compared to the vehicle control group. When sperm were incubated with 100 or 500 μM cordycepin for 3 h, the AR ratio increased to 43.43 ± 11.85% ($P = 0.046$) and 50.97 ± 9.59% ($P = 0.000$), respectively. PI staining showed that cordycepin had no significant effect on NVC% (Fig. 1B), suggesting that the cordycepin concentration used in this study had no obvious effect on sperm viability. We also tested whether cordycepin can directly induce AR. The results

showed that cordycepin alone could not directly induce AR. However, when AR was induced with cordycepin and progesterone simultaneously, the AR ratio increased significantly (Figure S1), suggesting that cordycepin can promote human sperm capacitation and AR.

Cordycepin promotes hyperactivation of human sperm

After capacitation, the sperm became hyperactivated. Hyperactivated sperm exhibit higher curve rates and swing amplitudes, helping the sperm penetrate the oocyte. As shown in Fig. 1C and Table S2, after capacitation, the hyperactivated sperm of the control group (without DMSO or cordycepin) and the vehicle control group (DMSO) were approximately 7.80±3.19% and 7.40±3.72% ($P>0.05$), respectively. Compared to the vehicle control, 20, 100, and 500 μM cordycepin increased hyperactivated sperm to 10.07±2.96% ($P=0.020$), 11.60±2.29% ($P=0.006$), and 12.87±1.60% ($P=0.000$), respectively, suggesting that cordycepin promoted sperm capacitation. We also found that cordycepin dose-dependently increased VAP, VCL, and ALH, but decreased LIN, which increased the swing amplitude of sperm and decreased linearity, consistent with the definition of hyperactivation.

Cordycepin increases PKA activity and protein tyrosine phosphorylation of human sperm

The cAMP/PKA signaling pathway was important in sperm capacitation [27]. Therefore, we explored whether cordycepin influenced PKA activity and downstream protein tyrosine phosphorylation levels. PKA activity was analyzed using an ELISA kit. We found that when the concentration of cordycepin increased, PKA activity showed a dose-dependent upward trend (Fig. 2A). Tyrosine phosphorylation in the sperm was then analyzed by western blotting. Generally, the total protein tyrosine phosphorylation level increased after sperm capacitation, as shown by the western blotting results (Fig. 2B). Compared to the vehicle control, sperm treated with high concentrations of cordycepin during capacitation tended to have increased tyrosine-phosphorylated protein levels (Fig. 2B). In Fig. 2C, the ratio of tyrosine phosphorylation to the internal reference (GAPDH) was analyzed. Bands at 45, 65, 90, and 110 KDa revealed that cordycepin enhanced protein tyrosine phosphorylation compared to the vehicle control (Fig. 2C). The results suggest that cordycepin was involved in the PKA and protein tyrosine phosphorylation signaling pathway during human sperm capacitation.

Cordycepin has no effects on sperm DNA fragmentation

To explore whether cordycepin may damage sperm, an SCD test was used. After acid denaturation and nucleoprotein removal, the undamaged sperm chromatin can diffuse and form a halo, which is considered to indicate DI. Sperm with DNA damage instead have very small halos and thus are labeled DF [28]. Microscopic results (Fig. 3A) and statistical graphs (Fig. 3B) showed that there were no significant differences between the control and the cordycepin-treated groups, suggesting that the concentrations of cordycepin used did not cause DNA damage to human sperm.

Cordycepin has no significant effects on sperm ROS levels

ROS is a double-edged sword for sperm capacitation. During capacitation, sperm ROS levels increase slightly and activate downstream tyrosine kinases. However, an excessive increase in ROS levels can cause DNA damage and induce sperm apoptosis [29]. Therefore, DCFH-DA was used to determine whether cordycepin affects

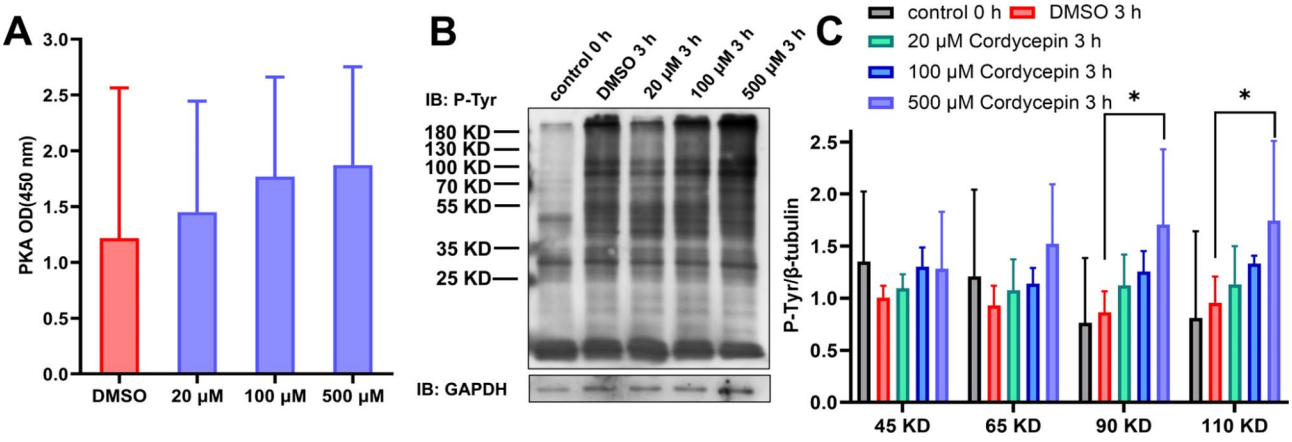

Fig. 2 Cordycepin increased protein kinase A (PKA) activity and protein tyrosine phosphorylation during human sperm capacitation. (**A**) An ELISA-based PKA activity assay kit measured human sperm PKA activity after capacitation for 3 h. The optical density at 450 nm (OD_{450}) was recorded. Data are presented as means ± standard deviation (SD; $n=3$). *$P<0.05$ (compared with the dimethyl sulfoxide [DMSO] group). (**B**) Western blotting was used to measure the protein tyrosine phosphorylation of human sperm. (**C**) The ratio of protein tyrosine phosphorylation levels to that of GAPDH. Data are presented as means ± standard deviation (SD; $n=3$). *$P<0.05$ (compared with the DMSO group)

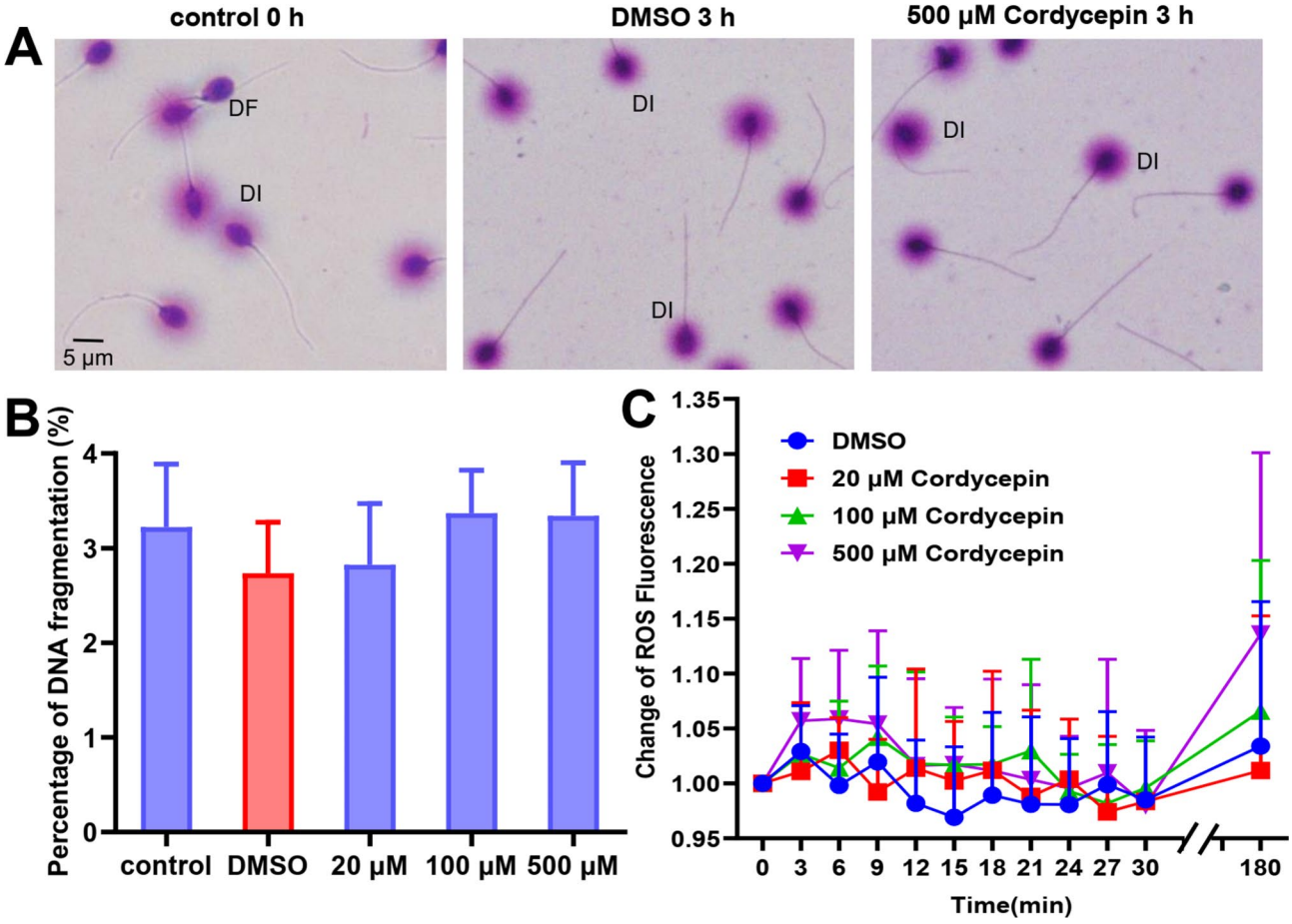

Fig. 3 Cordycepin had no significant effects on sperm DNA fragmentation and ROS levels during sperm capacitation. (**A**) A sperm chromatin dispersion (SCD) assay kit was used to test sperm DNA fragmentation. Sperm with halo widths larger than the diameter of the core were considered to show DNA integrity (DI). Sperm with halo widths less or equal to the core was considered to show DNA fragmentation (DF). (**B**) The percentage of DNA fragmentation was calculated by dividing the number of DF sperm by the total number of sperm. Data are presented as means ± standard deviation (SD; $n = 3$). *$P < 0.05$ (compared with the dimethyl sulfoxide [DMSO] group). (**C**) Sperm ROS levels were detected using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The first data were recorded before cordycepin was added and used for normalization. After adding cordycepin, the data were dynamically observed for 30 min at 3 min intervals during capacitating incubation. After capacitation for 3 h, the fluorescence signal was acquired once again. Data are presented as means ± standard deviation (SD; $n = 3$). *$P < 0.05$ (compared with the DMSO group)

sperm ROS levels during capacitation (Fig. 3C). The results showed that when capacitated for 30 min, the fluorescence intensity of the cordycepin groups tended to increase, revealing that ROS levels were slightly increased in human sperm. After capacitation for 3 h, high concentrations of cordycepin (100 and 500 μM) also tended to increase sperm ROS levels. However, there were no significant differences between the cordycepin and control groups, suggesting that the cordycepin-induced increase in ROS may promote sperm capacitation without causing damage.

Expression and localization of adenosine receptors in human sperm

To explore the mechanisms of action of cordycepin in human sperm capacitation, the expression and localization of four AdoR subtypes were examined. Western blotting showed that all four AdoR subtypes (A1, A2a, A2b, and A3) were expressed in human sperm (Fig. 4A). There were no significant differences in expression levels after capacitation. High concentrations of cordycepin had no obvious effect on their expression (Fig. 4B). The localization of the AdoRs was analyzed using indirect immunofluorescence (Fig. 5). We found that A1 AdoR was mainly localized in the tail middle piece and equatorial region of human sperm, with a small amount distributed in the principal piece of the tail. A2a AdoR staining revealed bright fluorescence in the tail region; however, almost no fluorescence was observed in the human sperm head. A2b AdoR was mainly localized in the sperm equatorial zone and had little activity in the tail middle piece. The A3 AdoR is mainly expressed in the whole tail and equatorial regions of human sperm. The

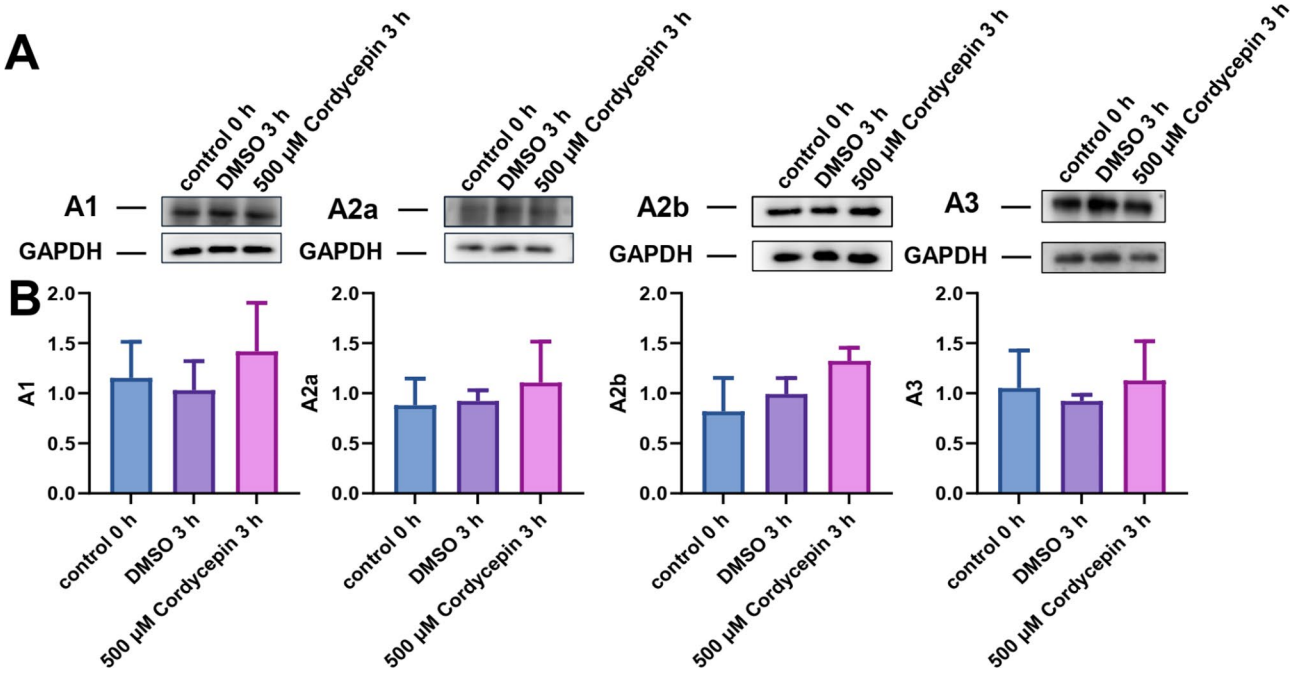

Fig. 4 Expression of four subtypes of adenosine receptors (AdoR) in human sperm. (**A**) Western blotting was used to detect the expression of four AdoR subtypes (A1, A2a, A2b, and A3) in human sperm. The expression before and after capacitation and cordycepin treatment were analyzed. (**B**) The ratio of adenosine receptor levels to GAPDH. Data are presented as means ± standard deviation (SD; $n = 3$). *$P < 0.05$ (compared with the DMSO group)

3 h capacitation culture had no significant effect on their localization and expression.

Adenosine receptor inhibitors decrease acrosome reaction and hyperactivation

To explore whether AdoRs mediate cordycepin-induced capacitation, sperm were treated with cordycepin and adenosine receptor inhibitors simultaneously, and sperm AR and hyperactivation were evaluated. As shown in Fig. 6A and B, spontaneous AR before capacitation and the AR ratio of the vehicle control after capacitation were about 16.48±3.95% and 34.36±9.00%, respectively. Cordycepin (500 μM) increased the AR ratio to approximately 50.97±9.59%, with significant differences observed between the vehicle control group and the 500 μM cordycepin group. However, all the AdoR inhibitors decreased the AR ratio. When sperm were simultaneously treated with 500 μM cordycepin and 0.1 μM A1 AdoR inhibitor DPCPX, the AR ratio decreased significantly to about 33.45±13.22% (P=0.001, compared to the 500 μM cordycepin group), which was similar to the vehicle control. Compared with the 500 μM cordycepin group, all concentrations (0.001, 0.01, and 0.1 μM) of inhibitors to the A2a (DMPX), A2b (MRS1754), or A3 (MRS1191) AdoRs decreased the AR ratio significantly. Figure 6C shows the hyperactivation results. Detailed sperm motility data are presented in Tables S3-S6. Cordycepin greatly increased the number of hyperactivated sperm, whereas all four adenosine receptor inhibitors significantly decreased hyperactivation. The results suggest that all AdoR subtypes are involved in cordycepin-mediated capacitation. Since PKA may be involved in cordycepin-induced capacitation, sperm were treated simultaneously with the PKA inhibitor H-89 (50 μM) and cordycepin. The results showed that inhibition of PKA decreased the cordycepin-mediated increase in AR and hyperactivation (Fig. 6B and C, Table S7), suggesting that cordycepin promotes capacitation through the PKA signaling pathway.

Cordycepin has docking binding activity with adenosine receptors

We hypothesized that cordycepin mimics adenosine, and promotes capacitation by binding to AdoRs. Therefore, molecular docking was used to predict whether cordycepin can bind to the four AdoR subtypes. As shown in Fig. 7, cordycepin can form hydrogen bonds with amino acid residues of A1, A2a, A2b, and A3 AdoRs, and the binding energy was −2.31, -2.25, -2.14, and −1.58 kcal/mol respectively. Docking energy less than −1.2 kcal/mol was considered the standard of significant binding capacity [30]. The results showed that cordycepin could effectively bind to all AdoR subtypes. We also found that cordycepin bound to all four AdoR subtypes on the extracellular side, suggesting that cordycepin may further activate downstream GPCR signaling pathways and ultimately affect capacitation.

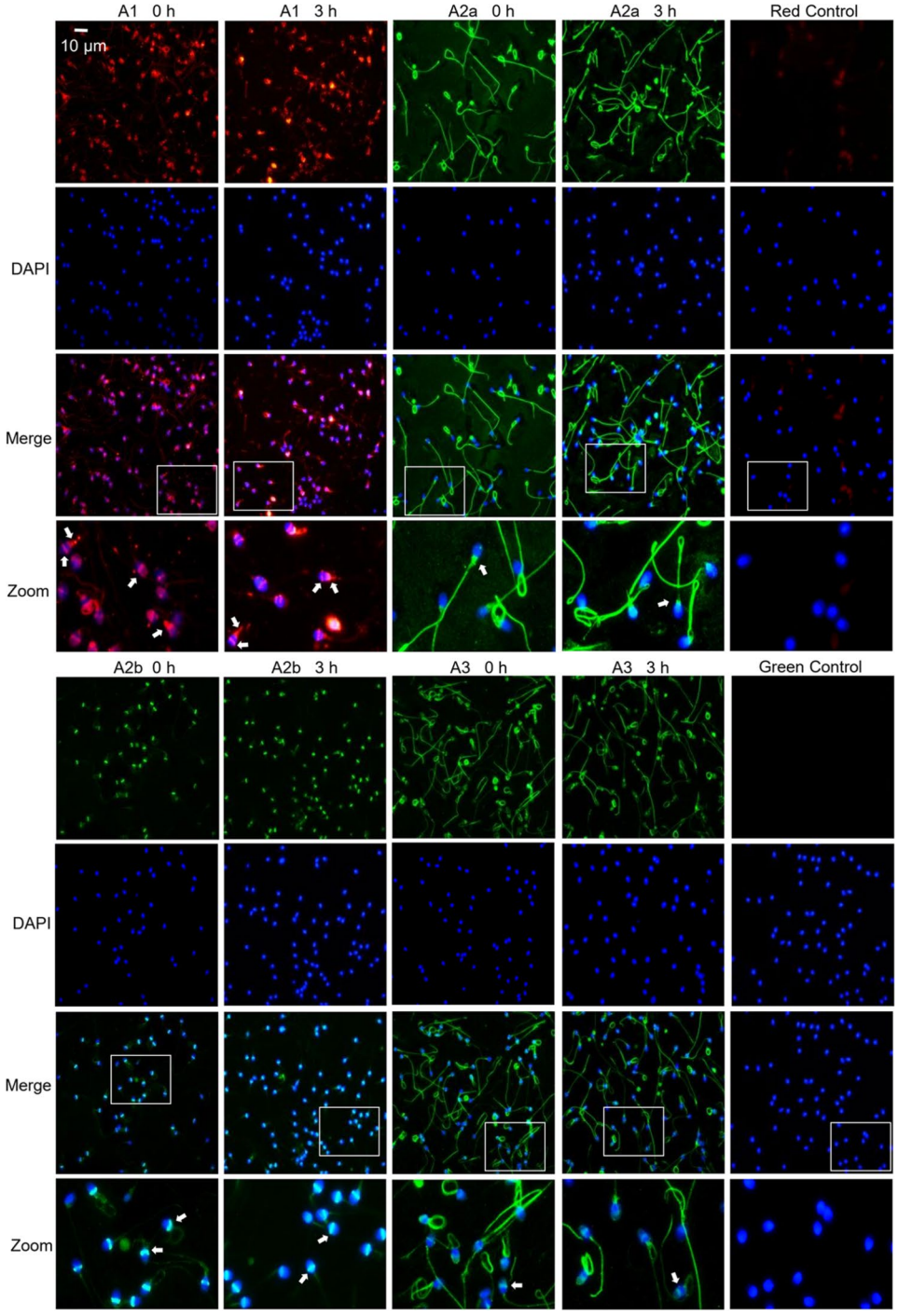

Fig. 5 Localization of four adenosine receptor (AdoR) subtypes in human sperm. Indirect immunofluorescence was used to detect the localization of the four subtypes of AdoRs in human sperm. The localization before and after capacitation was analyzed. A1 (red). A2a, A2b, and A3 (green). Sperm nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (blue). Negative controls were incubated with normal mouse or rabbit IgG as the primary antibody. Fluorescence in the equatorial region and the middle piece of the tail are labeled by arrows. The figure is representative of three independent experiments

Discussion

Cordyceps militaris is a nourishing food in traditional Chinese medicine. It belongs to the Clavicipitaceae family and is considered beneficial to male reproduction [31]. Cordycepin is a bioactive compound isolated from *Cordyceps militaris* [14]. It has antitumor, antiviral, anti-inflammatory, antioxidant, and antimicrobial effects [32–34]. Cordycepin also plays a role in male reproduction

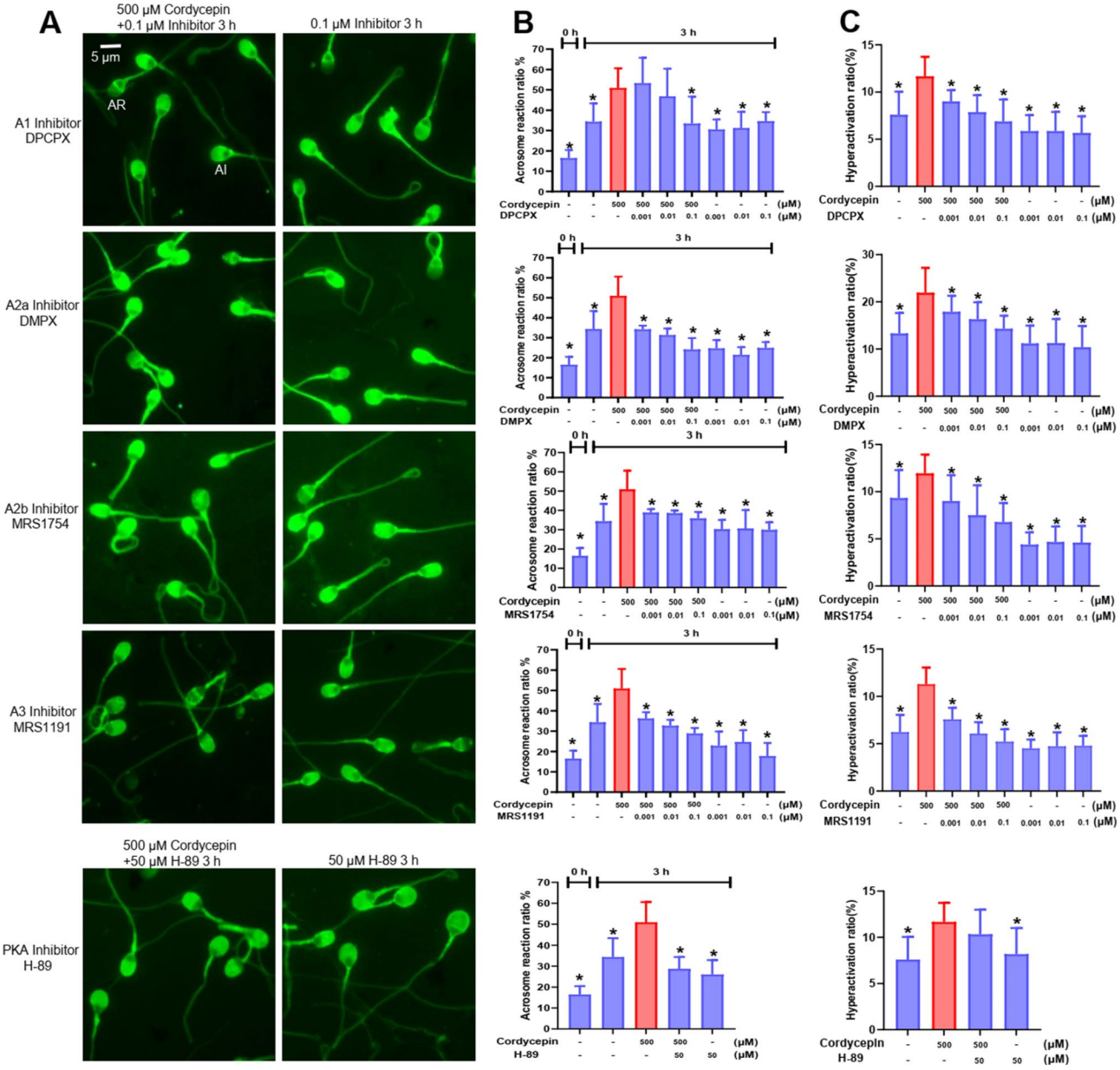

Fig. 6 Adenosine receptor (AdoR) inhibitors decreased cordycepin-promoted acrosomal reaction (AR) and hyperactivation in human sperm. (**A**) Human sperm was stained using fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA), which can distinguish the AR and acrosome integrity (AI) easily. Sperm were treated with cordycepin and AdoR inhibitors separately or simultaneously. Inhibitors to the A1 (DPCPX), A2a (DMPX), A2b (MRS1754), and A3 (MRS1191) AdoRs were used. The PKA inhibitor H-89 was used as the positive control. (**B**) Statistical analysis of AR ratio. Data are presented as means ± standard deviation (SD; $n = 3$). *$P < 0.05$ (compared with 500 μM cordycepin group). (**C**) The effects of AdoR inhibitors on hyperactivation. The PKA inhibitor H-89 was used as the positive control. Data are presented as means ± SD ($n = 5$). *$P < 0.05$ (compared with 500 μM cordycepin group)

by ameliorating testicular damage and improving sperm quality [19, 21, 35, 36]. However, its role and mechanism of action in human sperm capacitation remain unknown.

Sperm capacitation is a prerequisite for fertilization. Capacitation begins when sperm enters the female reproductive tract, which causes physiological and biochemical changes in human sperm [37]. First, sperm membrane cholesterol efflux increases sperm membrane permeability. Intracellular Ca^{2+} and HCO_3^- concentrations increase, and then activate soluble adenylyl cyclase and open the cAMP/PKA signaling pathway. PKA activates downstream tyrosine kinases [38]. Sperm then undergo AR and hyperactivation, thereby facilitating the final fertilization. Therefore, the classical cAMP/PKA pathways are involved in sperm capacitation. Further studies have shown that adenosine and its receptors

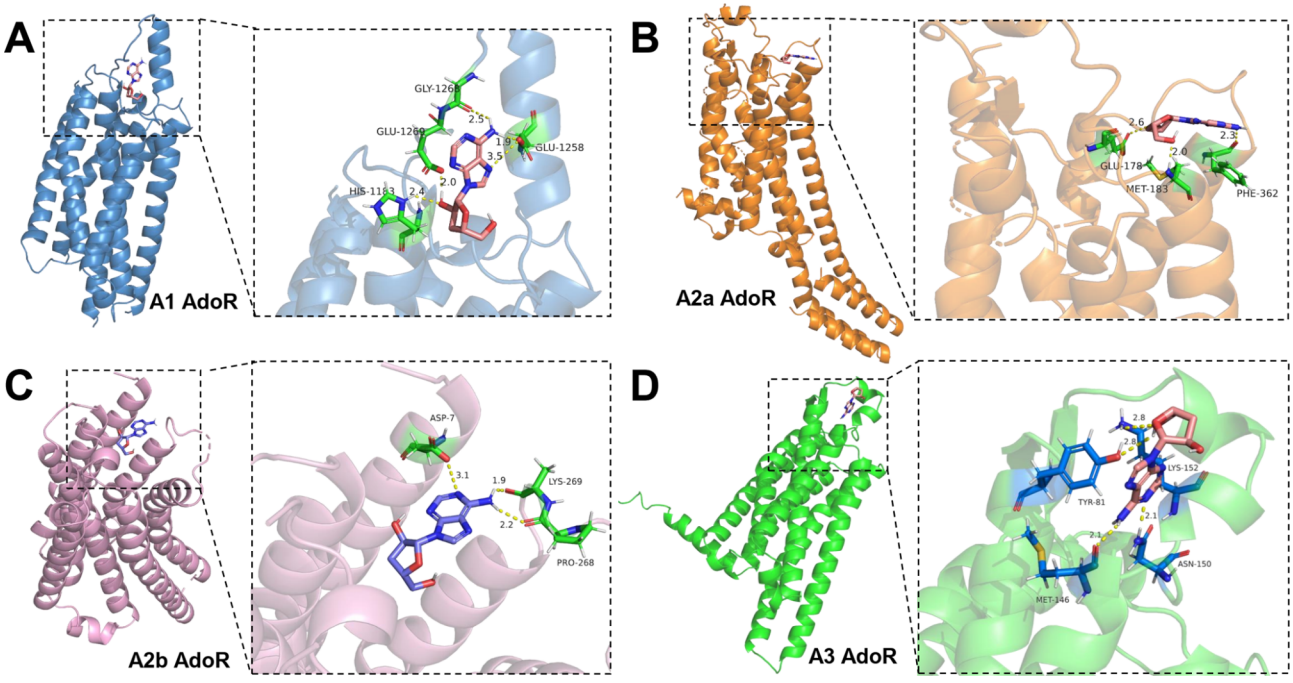

Fig. 7 Docking results of cordycepin with adenosine receptors. (**A**) Molecular docking of cordycepin with A1 AdoR (PDB ID: 5N2S). (**B**) Molecular docking of cordycepin with A2a AdoR (PDB ID: 5NLX). (**C**) Molecular docking of cordycepin with A2b AdoR (PDB ID: 8HDO). (**D**) Molecular docking of cordycepin with A3 AdoR (AF-P0DMS8-F1). The amino acid residues interacting with cordycepin and the hydrogen bonds are shown

may also play a role in sperm capacitation [6, 7]. Adenosine is found at high micromolar concentrations in the female reproductive tract, which is involved in sperm capacitation [6]. AdoRs all belong to the GPCR family, and adenosine can bind to their extracellular domains and activate downstream GPCR signaling pathways. Adenosine promotes capacitation in mouse sperm by interacting with these AdoRs [39]. Adenosine can also affect intracellular Ca^{2+}, soluble adenylyl cyclase, cAMP levels, and PKA activity in sperm [40], suggesting that adenosine regulates sperm capacitation through the cAMP/PKA signaling pathway. Inhibition of A2a AdoR decreased Ca^{2+} levels, motility, and the penetration ability of human sperm [11]. Cordycepin, also known as 3′-deoxyadenosine (Fig. 8B), has a similar structure to adenosine (Fig. 8A). Compared with adenosine, cordycepin only lacks a hydroxyl group at the 3′ position. However, it remains unclear whether cordycepin affects sperm capacitation by mimicking adenosine and binding to adenosine receptors.

Here, we have demonstrated that cordycepin promotes human sperm capacitation. Our research showed that cordycepin increased the sperm AR ratio and hyperactivation without increasing their ROS levels or DNA fragmentation, suggesting that cordycepin may help the process of sperm-oocyte fusion. We also found that cordycepin tended to increase PKA activity, and a high concentration of cordycepin significantly increased protein tyrosine phosphorylation, suggesting that cordycepin regulates sperm capacitation through the cAMP/PKA and downstream tyrosine kinase signaling pathways. We hypothesized that cordycepin binds to AdoRs and exerts its effects.

It has been reported that AdoRs are involved in sperm capacitation. Fenichel et al. found that A2 AdoR agonist 5′-N-ethylcarboxamidoadenosine increased cAMP, modified protein tyrosine phosphorylation, and accelerated the capacitation of human sperm [41]. Human sperm A1 AdoR is localized to the acrosome region, equatorial segment, and middle piece [7]. A1 AdoR agonists also stimulate human sperm capacitation [9]. However, it has remained unclear whether cordycepin binds to AdoRs to promote capacitation. Thus, we then examined the expression of all four AdoRs in human sperm and found that they all existed. Capacitation culture and cordycepin treatment did not alter their expression levels. The A1 AdoR is mainly localized in the equatorial zone and middle piece regions, which is consistent with previous studies [7]. Recently, Chen et al. also found that A2a AdoR is localized mainly in the tail of human sperm, which is consistent with our findings [11]. To the best of our knowledge, this is the first study to investigate the localization of A2b and A3 AdoRs in human sperm. When sperm were co-treated with cordycepin and an AdoR inhibitor, cordycepin-induced increases in AR and hyperactivation were significantly inhibited. These results revealed that A1, A2a, A2b, and A3 AdoRs are all involved in the promotion of human sperm capacitation by cordycepin.

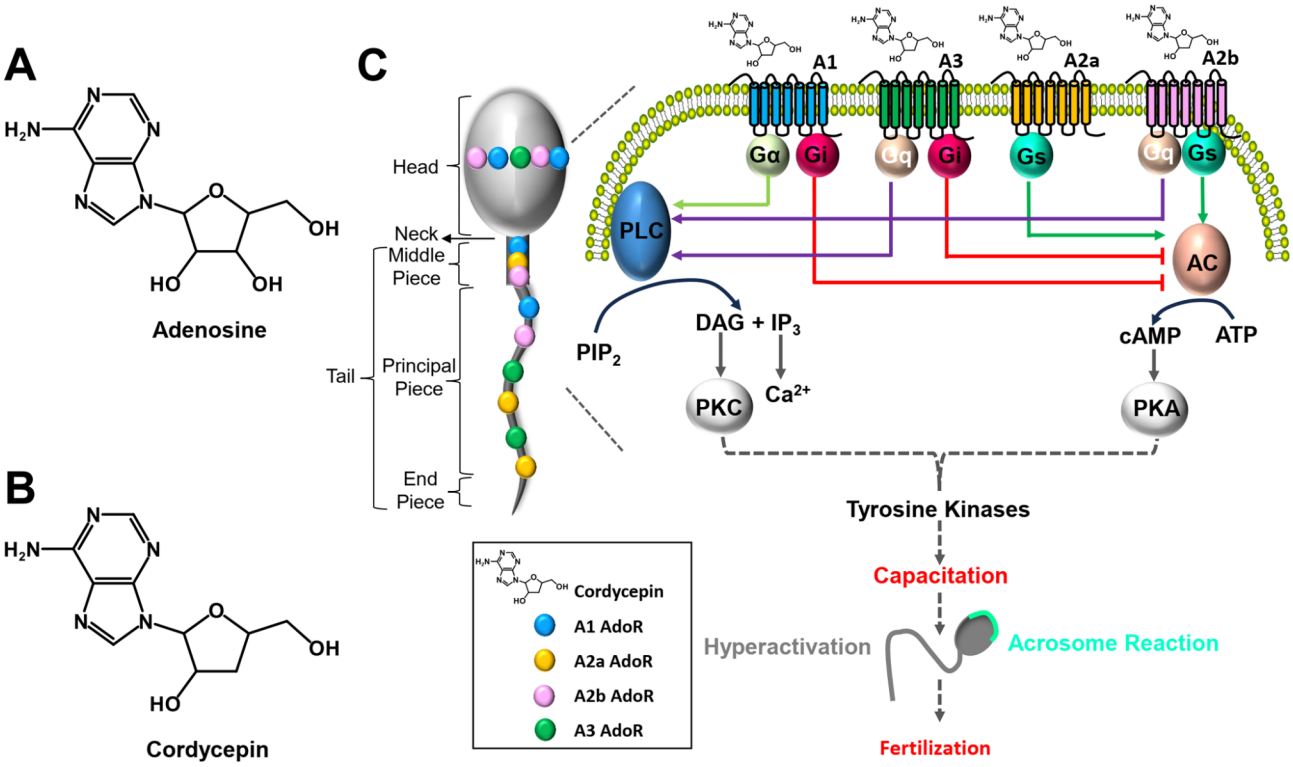

Fig. 8 Schematic representation of the mechanisms of cordycepin regulating human sperm capacitation. (**A**) Chemical structure of adenosine. (**B**) Chemical structure of cordycepin. (**C**) Four subtypes of adenosine receptors (AdoRs) are expressed in human sperm. Cordycepin can bind to AdoRs and open downstream G protein-coupled receptor (GPCR) signaling pathways. A2a and A2b AdoRs couple with stimulatory G_s and activate the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling pathway. A1 and A3 AdoRs coupled with inhibitory G_i, decreasing the level of cAMP and inhibiting PKA activity. A1 or A3 AdoRs also bind to G_q and G_α and activate the protein kinase C (PKC) signaling pathways. Finally, PKA and PKC indirectly cause sperm protein tyrosine phosphorylation and promote human sperm capacitation

Cordycepin can reportedly improve testicular function through AdoRs [17]; however, this is the first study to show that cordycepin promotes human sperm capacitation through AdoRs.

All AdoRs are GPCR family proteins that contain seven transmembrane helixes. Molecular docking showed that cordycepin could interact with the four AdoR subtypes on the extracellular side (Fig. 7), which may further open downstream signaling pathways through the G protein. Based on their effects on cAMP, A1, A2a, A2b, and A3 AdoRs can be divided into two groups: First are the A2a and A2b AdoRs, which couple with stimulatory G_s and activate the cAMP/PKA signaling pathway [42]. When cordycepin binds to the A2a or A2b AdoRs, it may increase downstream cAMP levels, enhance PKA activity, and improve human sperm capacitation. Second are the A1 and A3 AdoRs. In contrast to A2a and A2b AdoRs, these couple with inhibitory G_i, decreasing cAMP levels and inhibiting PKA activity [5, 43]. When cordycepin binds to the A1 or A3 AdoRs, it appears to inhibit the cAMP/PKA pathways and sperm capacitation. However, our experiments showed that inhibiting A1 and A3 AdoRs decreased cordycepin-mediated capacitation. In other words, the binding of cordycepin to A1 or A3 AdoR promotes human sperm capacitation. These may be explained that besides G_i, A1 AdoR and A3 AdoR are also bound to other G proteins, such as G_q and G_α [42]. Subsequently, they activate phospholipase C (PLC) and convert phosphatidylinositol diphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 then increases intracellular Ca^{2+} concentration, which can activate the classical cAMP/PKA signaling pathway and promote sperm capacitation. DAG and Ca^{2+} activate downstream protein kinase C (PKC) [5, 10]. Fujinoki et al. found that PKC inhibitors significantly suppressed hyperactivation, suggesting that PKC also promotes sperm capacitation [44]. Previously, we also found that PKC can regulate the phosphorylation of Hsp90 and affect tyrosine kinases, which are essential for sperm capacitation [45]. Therefore, our study suggested that cordycepin binds to the four AdoR subtypes and ultimately promotes sperm capacitation (Fig. 8C). Further research is warranted to elucidate how cordycepin influences the signaling pathways downstream of the adenosine receptor and their G proteins during capacitation.

Nowadays, the infertility ratio is increasing globally. In China, about 15% of childbearing-age couples suffer from infertility [46]. About 50% of these are caused by

male factors. In clinical practice, assisted reproductive technologies are sometimes used to treat patients with oligoasthenozoospermia and teratozoospermia. During IVF treatment, sperm also need to be capacitated in the culture medium before they can fertilize an oocyte. Our study found that cordycepin may aid the process of capacitation, which could help improve IVF success rates. Furthermore, these findings may also be helpful in the field of animal breeding. Therefore, the safety of cordycepin must be considered. However, studies on cordycepin toxicity in sperm are rare. A systematic review indicated that cordycepin had IC_{50} values ranging from 15 μM to 2 mM for various cell lines with incubation times of 24–48 h [34]. In our study, human sperm treated with up to 500 μM cordycepin for 3 h showed no significant differences in ROS levels, DNA fragmentation, and NVC% compared to the vehicle control. The results suggest that cordycepin may be beneficial in assisted reproduction in vitro. However, there are limitations in this study, including the sample size of sperm donors and the functional variability of sperm from different individuals. Furthermore, the elimination half-life of adenosine and cordycepin in humans or animals is relatively short [47]. Therefore, the effects and mechanisms of oral cordycepin on male reproduction require further investigation.

Conclusions

In summary, this study showed that cordycepin promotes human sperm AR and hyperactivation during capacitation, potentially aiding in final fertilization. Mechanistically, we found that cordycepin may bind to the A1, A2a, A2b, and A3 AdoRs, activating downstream signaling pathways and promoting human sperm capacitation. Collectively, these results suggest that cordycepin could be a candidate for enhancing fertilization in assisted reproduction and animal breeding.

Abbreviations

AI	Acrosome integrity
AdoR	Adenosine receptor
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
AR	Acrosome reaction
BCA	Bicinchoninic acid
BCF	Beat-cross frequency
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CASA	Computer-assisted sperm analyzer
DAG	Diacylglycerol
DAPI	4′,6-diamidino-2-phenylindole
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DF	DNA fragmentation
DI	DNA integrity
DMPX	3,7-Dimethyl-1-(2-propynyl)xanthine
DMSO	Dimethyl sulfoxide
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FITC-PSA	Fluorescein isothiocyanate-conjugated Pisum sativum agglutinin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein coupled receptors
HRP	Horseradish peroxidase
HTF	Human tubal fluid
IP_3	Inositol triphosphate
IVF	In vitro fertilization
LIN	Linearity
MRS1191	3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate
MRS1754	8-[4-[((4-Cyanophenyl) carbamoylmethyl) oxy] phenyl]-1,3-di(n-propyl) xanthine
NECA	5′-N-ethyl carboxamidoadenosine
NVC%	Non-viable cell percentage
PBS	Phosphate-buffered saline
PI	Propidium iodide
PIP_2	Phosphatidylinositol diphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PVDF	Poly vinylidene fluoride
ROS	Reactive oxygen species
SCD	Sperm chromatin dispersion
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPSS	Social Science Statistics Software Package
STR	Straightness
TBS	Tris-buffered saline
TMB	3, 3′, 5, 5′-tetramethylbenzidine
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-024-01318-3.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

We thank all donors who participated in this study.

Author contributions

P.S. and L.S. designed the study; L.S., L.G., Y.C., J.Y., F.L. and J.Q. performed the experiments; L.S., L.G., Y.C., K.L. and Y.N. analyzed the data; P.S. and L.S. drafted the paper; all authors reviewed the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (81801525), Natural Science Foundation of Zhejiang Province (LY23H040007), Medical Science and Technology Project of Zhejiang Province (2023KY648), Zhejiang Traditional Chinese Medicine Administration (2023ZR007), Zhejiang Province Health High-level Talent Training (WJW2023004).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was performed in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Hangzhou Medical College (LL2022-16). All semen donors provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 4 June 2024 / Accepted: 6 November 2024
Published online: 12 November 2024

References

1. Puga Molina LC, Luque GM, Balestrini PA, Marin-Briggiler CI, Romarowski A, Buffone MG. Molecular basis of human sperm capacitation. Front Cell Dev Biol. 2018;6:72.
2. Aitken RJ, Nixon B. Sperm capacitation: a distant landscape glimpsed but unexplored. Mol Hum Reprod. 2013;19(12):785–93.
3. Breitbart H, Grinshtein E. Mechanisms that protect mammalian sperm from the spontaneous acrosome reaction. Int J Mol Sci. 2023;24(23).
4. Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update. 2008;14(6):647–57.
5. Borea PA, Gessi S, Merighi S, Vincenzi F, Varani K. Pharmacology of Adenosine receptors: the state of the art. Physiol Rev. 2018;98(3):1591–625.
6. Bellezza I, Minelli A. Adenosine in sperm physiology. Mol Aspects Med. 2017;55:102–9.
7. Allegrucci C, Liguori L, Mezzasoma I, Minelli A. A1 adenosine receptor in human spermatozoa: its role in the fertilization process. Mol Genet Metab. 2000;71(1–2):381–6.
8. Minelli A, Liguori L, Bellazza I, Mannucci R, Johansson B, Fredholm BB. Involvement of A1 adenosine receptors in the acquisition of fertilizing capacity. J Androl. 2004;25(2):286–92.
9. Osycka-Salut C, Diez F, Burdet J, Gervasi MG, Franchi A, Bianciotti LG, et al. Cyclic AMP efflux, via MRPs and A1 adenosine receptors, is critical for bovine sperm capacitation. Mol Hum Reprod. 2014;20(1):89–99.
10. Minelli A, Bellezza I, Collodel G, Fredholm BB. Promiscuous coupling and involvement of protein kinase C and extracellular signal-regulated kinase 1/2 in the adenosine A1 receptor signalling in mammalian spermatozoa. Biochem Pharmacol. 2008;75(4):931–41.
11. Chen H, Xing G, Xu W, Chen Y, Xia L, Huang H, et al. The adenosine A2A receptor in human sperm: its role in sperm motility and association with in vitro fertilization outcomes. Front Endocrinol (Lausanne). 2024;15:1410370.
12. Jedrejko KJ, Lazur J, Muszynska B. Cordyceps militaris: an overview of its Chemical constituents in relation to Biological Activity. Foods. 2021;10(11).
13. Cunningham KG, Manson W, Spring FS, Hutchinson SA. Cordycepin, a metabolic product isolated from cultures of cordyceps militaris (Linn.) Link. Nature. 1950;166(4231):949.
14. Khan MA, Tania M. Cordycepin and kinase inhibition in cancer. Drug Discov Today. 2023;28(3):103481.
15. Huang SY, Su ZY, Han YY, Liu L, Shang YJ, Mai ZF, et al. Cordycepin improved the cognitive function through regulating adenosine A(2A) receptors in MPTP induced Parkinson's disease mice model. Phytomedicine. 2023;110:154649.
16. Cao ZP, Dai D, Wei PJ, Han YY, Guan YQ, Li HH, et al. Effects of cordycepin on spontaneous alternation behavior and adenosine receptors expression in hippocampus. Physiol Behav. 2018;184:135–42.
17. Leu SF, Poon SL, Pao HY, Huang BM. The in vivo and in vitro stimulatory effects of cordycepin on mouse leydig cell steroidogenesis. Biosci Biotechnol Biochem. 2011;75(4):723–31.
18. Ashraf SA, Elkhalifa AEO, Siddiqui AJ, Patel M, Awadelkareem AM, Snoussi M et al. Cordycepin for Health and Wellbeing: a potent bioactive metabolite of an entomopathogenic cordyceps Medicinal Fungus and its nutraceutical and therapeutic potential. Molecules. 2020;25(12).
19. Chen YC, Chen YH, Pan BS, Chang MM, Huang BM. Functional study of Cordyceps sinensis and cordycepin in male reproduction: a review. J Food Drug Anal. 2017;25(1):197–205.
20. Kopalli SR, Cha KM, Cho JY, Kim SK, Koppula S. Cordycepin from Medicinal Fungi cordyceps Militaris mitigates Inflammaging-Associated testicular damage via regulating NF-kappaB/MAPKs signaling in naturally aged rats. Mycobiology. 2022;50(1):89–98.
21. Kopalli SR, Cha KM, Lee SH, Hwang SY, Lee YJ, Koppula S et al. Cordycepin, an active constituent of Nutrient Powerhouse and potential Medicinal Mushroom cordyceps Militaris Linn., ameliorates Age-related testicular dysfunction in rats. Nutrients. 2019;11(4).
22. Chang Y, Jeng KC, Huang KF, Lee YC, Hou CW, Chen KH, et al. Effect of Cordyceps militaris supplementation on sperm production, sperm motility and hormones in Sprague-Dawley rats. Am J Chin Med. 2008;36(5):849–59.
23. Huerta-Retamal N, Saez-Espinosa P, Robles-Gomez L, Aviles M, Romero A, Aizpurua J, et al. Human sperm chaperone HSPA2 distribution during in vitro capacitation. J Reprod Immunol. 2021;143:103246.
24. Gomez-Torres MJ, Huerta-Retamal N, Robles-Gomez L, Saez-Espinosa P, Aizpurua J, Aviles M et al. Arylsulfatase a remodeling during human sperm in Vitro Capacitation using Field Emission scanning Electron Microscopy (FE-SEM). Cells. 2021;10(2).
25. Shan L, Chai Y, Gao T, Li K, Yu J, Liang F, et al. Perfluorooctane sulfonate and perfluorooctanoic acid inhibit progesterone-responsive capacitation through cAMP/PKA signaling pathway and induce DNA damage in human sperm. Environ Toxicol Pharmacol. 2023;100:104165.
26. Sun P, Wang Y, Gao T, Li K, Zheng D, Liu A, et al. Hsp90 modulates human sperm capacitation via the Erk1/2 and p38 MAPK signaling pathways. Reprod Biol Endocrinol. 2021;19(1):39.
27. Ickowicz D, Finkelstein M, Breitbart H. Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. Asian J Androl. 2012;14(6):816–21.
28. Kuroda S, Karna KK, Kaiyal RS, Cannarella R, Lundy SD, Vij SC, et al. Novel sperm chromatin dispersion test with artificial intelligence-aided halo evaluation: a comparison study with existing modalities. Andrology. 2023;11(8):1581–92.
29. Aitken RJ. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol Reprod Dev. 2017;84(10):1039–52.
30. Ma X, Zhao Y, Yang T, Gong N, Chen X, Liu G, et al. Integration of network pharmacology and molecular docking to explore the molecular mechanism of Cordycepin in the treatment of Alzheimer's disease. Front Aging Neurosci. 2022;14:1058780.
31. Phull AR, Ahmed M, Park HJ. Cordyceps Militaris as a Bio Functional Food source: pharmacological Potential, anti-inflammatory actions and related molecular mechanisms. Microorganisms. 2022;10(2).
32. Tan L, Song X, Ren Y, Wang M, Guo C, Guo D et al. Anti-inflammatory effects of cordycepin: a review. Phytother Res. 2021; 35(3):1284-97.
33. Verma AK. Cordycepin: a bioactive metabolite of cordyceps militaris and polyadenylation inhibitor with therapeutic potential against COVID-19. J Biomol Struct Dyn. 2022;40(8):3745–52.
34. Radhi M, Ashraf S, Lawrence S, Tranholm AA, Wellham PAD, Hafeez A, et al. A systematic review of the Biological effects of Cordycepin. Molecules. 2021;26:19.
35. Huang T, Zhou Y, Lu X, Tang C, Ren C, Bao X, et al. Cordycepin, a major bioactive component of Cordyceps Militaris, ameliorates diabetes-induced testicular damage through the Sirt1/Foxo3a pathway. Andrologia. 2022;54(1):e14294.
36. Okur MH, Arslan S, Aydogdu B, Zeytun H, Basuguy E, Arslan MS, et al. Protective effect of Cordycepin on Experimental Testicular Ischemia/Reperfusion Injury in rats. J Invest Surg. 2018;31(1):1–8.
37. Lavanya M, Selvaraju S, Krishnappa B, Krishnaswamy N, Nagarajan G, Kumar H. Microenvironment of the male and female reproductive tracts regulate sperm fertility: impact of viscosity, pH, and osmolality. Andrology. 2022;10(1):92–104.
38. Battistone MA, Da Ros VG, Salicioni AM, Navarrete FA, Krapf D, Visconti PE, et al. Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. Mol Hum Reprod. 2013;19(9):570–80.
39. Fraser LR, Adeoya-Osiguwa S. Modulation of adenylyl cyclase by FPP and adenosine involves stimulatory and inhibitory adenosine receptors and g proteins. Mol Reprod Dev. 1999;53(4):459–71.
40. Schuh SM, Carlson AE, McKnight GS, Conti M, Hille B, Babcock DF. Signaling pathways for modulation of mouse sperm motility by adenosine and catecholamine agonists. Biol Reprod. 2006;74(3):492–500.
41. Fenichel P, Gharib A, Emiliozzi C, Donzeau M, Menezo Y. Stimulation of human sperm during capacitation in vitro by an adenosine agonist with specificity for A2 receptors. Biol Reprod. 1996;54(6):1405–11.
42. Moro S, Gao ZG, Jacobson KA, Spalluto G. Progress in the pursuit of therapeutic adenosine receptor antagonists. Med Res Rev. 2006;26(2):131–59.
43. Mazziotta C, Rotondo JC, Lanzillotti C, Campione G, Martini F, Tognon M. Cancer biology and molecular genetics of A(3) adenosine receptor. Oncogene. 2022;41(3):301–8.
44. Fujinoki M. Progesterone-enhanced sperm hyperactivation through IP(3)-PKC and PKA signals. Reprod Med Biol. 2013;12(1):27–33.
45. Li K, Sun P, Wang Y, Gao T, Zheng D, Liu A, et al. Hsp90 interacts with Cdc37, is phosphorylated by PKA/PKC, and regulates src phosphorylation in human sperm capacitation. Andrology. 2021;9(1):185–95.

46. Qiao J, Wang Y, Li X, Jiang F, Zhang Y, Ma J, et al. A Lancet Commission on 70 years of women's reproductive, maternal, newborn, child, and adolescent health in China. Lancet. 2021;397(10293):2497–536.
47. Tsai YJ, Lin LC, Tsai TH. Pharmacokinetics of adenosine and cordycepin, a bioactive constituent of Cordyceps sinensis in rat. J Agric Food Chem. 2010;58(8):4638–43.

Publisher's note