

Seminal plasma exosomes evoke calcium signals via the CatSper channel to regulate human sperm function

Xiaoning Zhang^{†,1,3}, Dandan Song^{†,2}, Hang Kang², Wenwen Zhou¹, Houyang Chen⁴ & Xuhui Zeng^{*,1,2}

[†]These authors contributed equally to this work.

¹Medical School, Institute of Reproductive Medicine, Nantong University, Nantong, China

²Institute of Life Science and School of Life Science, Nanchang University, Nanchang, China

³Genetics and Biochemistry Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA.

⁴Reproductive Medical Center, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi 330006, PR China

Running title: CatSper channel mediates exosome-evoked calcium signal

*Corresponding Author:

Xuhui Zeng, Ph.D.

9 Seyuan R.D., Nantong 226019, China

Tel: +86 19979568728;

E-mail address: zengxuhui@ntu.edu.cn

19 ABSTRACT

20 Seminal plasma exosomes (SPE) have been proposed to regulate intracellular calcium
 21 concentration ($[Ca^{2+}]_i$) and sperm function. However, neither the underlying
 22 mechanisms by which $[Ca^{2+}]_i$ is regulated by SPE nor the physiological and
 23 pathological significance of the SPE-evoked calcium signal are fully understood. Here,
 24 we successfully isolated and characterized SPE by several methods, including
 25 transmission electron microscopy, nanoparticle tracking and nanoflow cytometry
 26 analysis. Application of SPE dose-dependently increased human sperm $[Ca^{2+}]_i$ via
 27 extracellular Ca^{2+} influx. The Ca^{2+} influx was mediated by the sperm-specific CatSper
 28 channel, because the SPE-elevated $[Ca^{2+}]_i$ was suppressed by a CatSper inhibitor, and
 29 SPE potentiated the CatSper current in human sperm. The role of CatSper in the
 30 SPE-induced elevation of $[Ca^{2+}]_i$ was further confirmed by the absence of the
 31 SPE-induced $[Ca^{2+}]_i$ increase and CatSper current in a CatSper-deficient sample.
 32 Furthermore, both protein and no-protein components in SPE were shown to
 33 contribute to the elevated $[Ca^{2+}]_i$, as well as the hyperactivated motility of human
 34 sperm. Interestingly, when sperm were stimulated with exosomes derived from
 35 asthenozoospermic semen, the elevation of $[Ca^{2+}]_i$ was significantly lower than that
 36 by exosomes isolated from normal seminal plasma. The SPE from normal seminal
 37 plasma improved the motility of sperm from asthenozoospermic samples. Taken
 38 together, these findings demonstrated that SPE modulates Ca^{2+} signaling and human
 39 sperm function by activating a CatSper channel. The application of SPE to enhance
 40 sperm motility may provide a new clinical avenue for asthenozoospermic men.

41 **KEYWORDS:** Seminal plasma exosomes; calcium signal; CatSper; sperm function;

42 hyperactivation; asthenozoospermia

43

44 **Introduction**

45 Exosomes are shed from most cell types and exist in a wide variety of body fluids.

46 Increasing evidence indicates that exosomes are important mediators of intercellular

47 communication, rather than non-specific waste from cells [1]. Exosomes can be taken

48 up via specific membrane surface receptors or can release certain signal molecules to

49 regulate recipient cell functions [2, 3]. Mammalian seminal plasma contains

50 membranous exosomes (extracellular vesicles) mainly produced by the epididymis

51 and prostate, traditionally termed epididymosomes or prostasomes, which contain

52 proteins, nucleic acids, and a high content of cholesterol and sphingomyelin [4-7].

53 Previous studies have demonstrated that seminal plasma exosomes (SPE) and their

54 cargos have antibacterial, antioxidant, and immunosuppressive properties, and may be

55 involved in several biological processes indirectly influencing sperm function [8, 9].

56 For example, exosomes reduced ROS overproduction and attenuated oxidative stress

57 in sperm [10], and transferred Ca^{2+} signaling factors to regulate progesterone-induced

58 sperm motility [11]. Directly interacting with sperm, human SPE may play pivotal

59 roles in sperm maturation in the epididymis, as well as sperm function and

60 fertilization [12-15]. Interestingly, SPE isolated from oligoasthenozoospermia donors

61 exhibited different miRNA and protein profiles than SPE from normozoospermic

62 exosomes [16, 17]. Furthermore, normal human exosomes promoted sperm motility

63 and capacitation, while this effect was impaired in exosomes derived from donors

64 with asthenospermia [18]. These studies indicate that SPE act as functional regulators

65 of male fertility, and also suggest that dysfunction of exosomes may contribute to

66 male infertility.

67 Intracellular calcium homeostasis is a key component in the control of sperm
68 motility, and Ca^{2+} influx is important for the maintenance of progressive motility and
69 sperm function [19, 20], especially for the acrosome reaction and hyperactivation [21,
70 22]. All these processes are indispensable for sperm fertility. Consistent with roles in
71 the regulation of sperm function, SPE increased intracellular Ca^{2+} concentrations
72 ($[\text{Ca}^{2+}]_i$), thought to be mediated by membrane fusion with sperm rather than ion
73 exchanges and ATP-dependent ion pumps [23, 24]. Only recently has not convincing
74 data been documented concerning the precise mechanism of exosome actions in the
75 sperm $[\text{Ca}^{2+}]_i$ response.

76 Cation channel of sperm (CatSper), a unique cation channel protein family
77 exclusively expressed in sperm, is composed of at least nine subunits and control Ca^{2+}
78 influx to regulate sperm functions, such as capacitation, chemotaxis, the acrosome
79 reaction and hyperactivation [25]. CatSper channel is essential for male fertility
80 because CatSper-deficient male mice and men with mutations in CatSper genes
81 exhibit infertility due to lack of sperm hyperactivation [26]. CatSper can be activated
82 by many physiological substances, such as progesterone, prostaglandins and certain
83 chemicals [27, 28]. Thus it has been considered as a polymodal chemosensor that
84 regulates sperm function and fertility [28]. However, the role of CatSper in
85 exosome-induced $[\text{Ca}^{2+}]_i$ remains unknown. Also, investigation is required to
86 determine the components of exosomes that exert the regulatory function on human
87 sperm function.

88 The present study investigated the mechanism by which SPE regulate Ca^{2+}
 89 signaling in human sperm, with a focus on the involvement of CatSper. In addition,
 90 the functional and pathological significance of SPE-evoked $[\text{Ca}^{2+}]_i$ responses was also
 91 examined, to provide insight into the potential clinical application of SPE.

92 **Materials and Methods**

93 **Reagents**

94 Human tubal fluid (HTF) medium were purchased from Nanjing Aibei Biotechnology
 95 Co., Ltd (Nanjing, China). Fluo-4 AM and Pluronic F-127 were obtained from
 96 Molecular Probes (Eugen, OR, USA). Triton X-100 and Proteinase K (Pro K) were
 97 obtained from Solarbio (Beijing, China). Mibefradil (Mi), progesterone (P4), 1, 2-bis
 98 (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid (BAPTA), EGTA and A23187
 99 were purchased from Sigma-Aldrich (Shanghai, China). CD9 and CD63 antibodies
 100 were obtained from proteintech (Wuhan, China). Prostaglandin E1 (PGE1) and E2
 101 (PGE2) were obtained from Aladdin (Shanghai, China) and TCI Development Co.,
 102 Ltd (Shanghai, China), respectively.

103 **Sperm sample preparation**

104 The collection of semen samples and experiments in this study were approved by the
 105 Institutional Ethics Committee on human subjects of the Nanchang Reproductive
 106 Hospital and Jiangxi Maternal and Child Health Hospital. Informed consent
 107 documents were signed by the donors. Semen viscosity and volume, sperm motility,
 108 and sperm concentration were assessed according to the WHO laboratory manual for

109 the examination and processing of human semen (WHO 2010,
110 <https://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/>).
111 Samples were subjected to high-saline solution (HS, 135 mM NaCl, 5 mM KCl, 1
112 mM MgSO₄, 2 mM CaCl₂, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid and 1
113 mM Na-pyruvate, adjusted to pH 7.4 with NaOH) for washing or a swim-up
114 purification method for the experiments, as described in previous studies with some
115 modifications [29, 30].

116 **Exosome extraction**

117 After semen was centrifuged at 7,850 g for 30 min at 4°C, the supernatant was
118 collected and stored at –80°C until use. Before isolation, seminal plasma was filtered
119 successively with 0.45 and 0.22 µm MF-Millipore™ membranes (Millipore, MA,
120 USA). The filtered seminal plasma was centrifuged at 10,000 g for 30 min at 4°C to
121 remove cell debris and other impurities. Remaining supernate was centrifuged at
122 68,000 g for 90 min at 4°C. Then, the precipitate was suspended in 35 mL of 0.32 M
123 sucrose and centrifuged at 100,000 g for 90 min at 4°C. Next, the precipitate was
124 washed with PBS at 100,000 g for 90 min at 4°C. Finally, the exosomal pellets were
125 resuspended in 3.5 mL HS and stored at –80°C for further study.

126 **Nanoflow analysis**

127 The diameter range and concentration of exosomes were analyzed with a Flow
128 NanoAnalyzer N30 (NanoFCM Inc, Xiamen, China). Marker protein expression in
129 exosomes was analyzed by nanofluidic methods. The exosomes were labeled by

130 CD63 antibody (1:100) and corresponding secondary antibody (1:5000, Invitrogen,
131 CA, USA). During the experiment, primary or secondary antibodies alone were added
132 to samples as controls. In addition, exosomes with secondary antibody alone, and
133 CD63 antibody with PBS were used as background controls. After a final dilution
134 (1:5000), each sample was analyzed by a Flow NanoAnalyzer N30 (NanoFCM Inc,
135 Xiamen, China).

136 **Transmission electron microscopy (TEM)**

137 The exosomes were observed with a TEM (JEM-1200EX, Japan Electronics Co.,
138 Ltd.). First, 5–10 μ L exosomes were sedimented on copper mesh for 3 min and liquid
139 absorbed with filter paper. After the sample was rinsed with PBS, it was dyed with
140 phosphotungstic acid. Finally, it was dried at room temperature for 2 min and images
141 were captured on the TEM (electron microscopy operating voltage 80–120 kV).

142 **Computer-aided sperm analysis system (CASA)**

143 To evaluate the effect of exosomes on sperm motility, seminal plasma and
144 exosome-free seminal plasma were prepared. Seminal plasma was prepared as follows:
145 cell debris and sperm were removed from intact seminal plasma by centrifugation
146 and > 220 nm vesicles were filtered with a $0.22 \mu\text{m}$ MF-Millipore[™] membrane
147 (Millipore, MA, USA). The filtered seminal plasma was centrifuged at 68,000 g for
148 90 min and regarded as exosome-free seminal plasma. Sperm was resuspended in
149 seminal plasma and exosome-free seminal plasma, and then placed in a 5% carbon
150 dioxide incubator (SANYO Electric Co., Ltd., Osaka, Japan) at 37°C for different

151 time periods. Sperm parameters were measured by CASA (WLJY-9000, WeiLi. Co.,
152 Ltd., Beijing, China). Total motility, progressive motility (PR), curvilinear velocity
153 (VCL), straight-line (rectilinear) velocity (VSL), average path velocity (VAP),
154 linearity (LIN) and other sperm parameters were recorded.

155 **Evaluation of the ability of sperm to penetrate viscous media**

156 The environment of the female reproductive tract was simulated with 1%
157 methylcellulose, because sperm complete fertilization with an egg after migration
158 through the female reproductive tract. After sperm were selected by a swim-up
159 purification procedure (30 min, 37°C) the sperm in the upper fractions were washed
160 twice in HS buffer and adjusted to a concentration of 2.0×10^7 cells/mL. Exosomes or
161 other drugs were added after capacitation for 2 h (progesterone was added after 2.5 h).
162 Three h later, 20 µL of 1% methylcellulose was drawn into a glass capillary tube, and
163 the capillary placed in the mixed sperm. The number of sperm in 2 and 3 cm (from the
164 base) of the capillary tube were recorded after 1 h.

165 **Assessment of sperm hyperactivation (HA)**

166 Sperm were capacitated in HTF medium for 3 h and treated with exosomes and drug
167 for 1 h at 37°C. After capacitation, 8 µL of sperm samples were used to assess motion
168 characteristics via the CASA system (Hamilton Thorne, Inc., Florida, USA) and at
169 least 200 spermatozoa were counted for each assay. Aliquots of 8 µL non-capacitated
170 sperm were used as a non-hyperactivated control. The ALH, LIN, VCL and other
171 parameters were recorded. Hyperactivation was defined as cells with a curvilinear

172 velocity (VCL) $\geq 150 \mu\text{m/s}$, linearity (LIN) $< 50\%$, and lateral head displacement
173 (ALH) $\geq 7 \mu\text{m}$, as described previously (Alasmari, et al., 2013). The percentage of
174 hyperactivated sperm was calculated as the number of hyperactivated sperm/all motile
175 sperm.

176 **Assessment of intracellular calcium**

177 Intracellular calcium levels in sperm were monitored with Fluo-4 AM dye and F127.
178 In brief, the sperm were washed 3 times with HS and incubated with Fluo-4 AM (2
179 μM) and F127 (0.1%) in HS for 30 min at 37°C in darkness. After washing the cells
180 with HS two times, sperm was exposed to exosomes or drugs and fluorescence
181 intensity was assessed by the EnSpire® Multimode Plate Reader (PerkinElmer,
182 Waltham, MA, USA) with excitation and emission wavelengths of 488 and 525 nm,
183 respectively. To determine a change in the calcium signal, the sperm suspension was
184 added to a 96-well plate, then the base fluorescence intensity (F_0) was measured.
185 After the drug was added, the fluorescence (F) was measured, and the change in
186 sperm $[\text{Ca}^{2+}]_i$ was calculated by $\Delta F/F_0 (\%) = (F - F_0)/F_0 \times 100\%$.

187 **Single cell calcium imaging technology**

188 The samples were purified by a 50% Percoll solution (Percoll:HSA:EBSS:ddH₂O =
189 5:1:1:3). After centrifugation at 500 g for 15 min, the sperm was collected, washed
190 once with HS and resuspended in HS. The sperm was incubated with Fluo-4 AM and
191 F127 in a 5% carbon dioxide incubator at 37°C for 30 min. After excess dye was
192 washed off with HS, the sperm was adjusted to a concentration of 2.0×10^7 cells/mL.

193 Then 200 μ L of sperm suspension was placed evenly into a dish bottom. The sample
194 was adhered the wall and kept away from light for 30 min. After excess fluid was
195 poured off, sperm in the dish were washed with HS. Finally, $[Ca^{2+}]_i$ was recorded in a
196 single-cell calcium imaging system (OLYMPUS, Tokyo, Japan) before and after
197 exosome treatment.

198 Sperm patch-clamp recordings

199 The whole-cell patch-clamp technique was applied to record human sperm CatSper
200 currents as previously described (Zeng et al., 2011). Seals were formed in the sperm
201 cytoplasmic droplet or the neck region using a 15–30 M Ω pipette. The pipette solution
202 for recording CatSper currents contained 135 mM Cs-Mes, 10 mM Hepes, 10 mM
203 EGTA, and 5 mM CsCl, adjusted to pH 7.2 with CsOH. Then, the transition into
204 whole-cell mode involved the application of short (1 ms) voltage pulses (400–650 mV)
205 combined with light suction. The currents were stimulated by 1 s voltage ramps from
206 –100 to +100 mV from a holding potential of 0 mV. For recording the monovalent
207 current of CatSper, divalent-free (DVF) solution (150 mM NaCl, 20 mM HEPES, and
208 5 mM EDTA, pH 7.4) was used to record basal CatSper monovalent currents. Then,
209 different concentrations of exosomes and progesterone were perfused to record
210 CatSper currents. The currents were analyzed with a Clampfit (Axon, Gilze,
211 Netherlands) and figures were plotted with Grapher 8 software (Golden Software, Inc.,
212 Golden, Colorado).

213 Western blotting

214 The extracted exosomes were added to $5 \times$ protein loading buffer and boiled at 95°C
 215 for 5 min to denature proteins for later use. Each protein sample was subjected to
 216 SDS-PAGE and transferred to a PVDF membrane, which was then blocked with 3%
 217 BSA for 1 h. Next, the membrane was incubated with CD63 and CD9 antibody
 218 (1:1000) at 4°C overnight, then washed three times with TBST. The secondary
 219 antibody was added and incubated for 1 h. Then the PVDF membrane was washed
 220 three times with TBST for 5 min, then exposed to ECL chemical reagent and
 221 examined by a gel imaging system (Bio-Rad, CA, USA).

222 ELISA

223 The content of prostaglandins in exosomes was detected with PGE1 and PGE2
 224 enzyme-linked immunosorbent assay kits from Wuhan Shenke Experimental
 225 Technology Co., Ltd. (Wuhan, China) and Nanjing Jiancheng Bioengineering Institute
 226 (Nanjing, China), respectively, according to the operating instructions.

227 Statistical analysis

228 Data are expressed as the mean \pm SEM. Differences between the controls and treated
 229 samples were assessed with the unpaired Student's t test using the statistical software
 230 GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA, USA). $P < 0.05$
 231 was regarded as statistically significant.

232

233

234

235 **Results**

236 **Identification of exosomes from human semen**

237 The SPE were isolated from 12–15 different donors by ultracentrifugation and had an
 238 average diameter of 94.9 ± 21.8 nm (Figure 1(a)). The SPE size ranged from 50 to
 239 200 nm, with most < 100 nm and more than 4.67×10^{13} particles/mL were
 240 examined by the Flow NanoAnalyzer N30 (Figure 1(b)). After treatment with
 241 Triton-X 100, particle numbers decreased by about 18-fold to 2.66×10^{12} particles/mL
 242 and many particles became smaller (Figure 1(c)). The Flow NanoAnalyzer and
 243 western blotting analysis showed the presence of universal exosome markers, CD9
 244 and CD63, in the isolated exosomes (Figure 1(d)–1(f)). TEM further confirmed that
 245 the exosomes contained a single lipid bilayer and appeared as round particles in the
 246 expected size range of exosomes (Figure 1(g)).

247 **Exosomes elevated $[Ca^{2+}]_i$ signals via extracellular Ca^{2+} influx**

248 It was reported that human spermatozoa $[Ca^{2+}]_i$ increased after fusion of sperm with
 249 exosomes [31], however, the source of the $[Ca^{2+}]_i$ burst remained to be clarified. The
 250 present study investigated the effect of isolated exosomes on human sperm $[Ca^{2+}]_i$.
 251 The results indicated that exosomes increased human sperm $[Ca^{2+}]_i$ within 1 min in a
 252 dose-dependent manner (Figure 2(a) and 2(b)). The concentration of applied
 253 exosomes was close to physiological conditions when the ratio of exosome:sperm was
 254 1:1. Next, we further confirmed the exosome-evoked Ca^{2+} response by single-sperm
 255 $[Ca^{2+}]_i$ imaging technology (Figure 2(c)). To determine whether the exosome-induced

256 increase in $[Ca^{2+}]_i$ was due to Ca^{2+} influx or the mobilization of Ca^{2+} stores, we
257 measured the $[Ca^{2+}]_i$ of sperm exposed to a Ca^{2+} -free medium containing Ca^{2+}
258 chelators BAPTA or EGTA. Under these conditions, we found that exosomes had no
259 effect on $[Ca^{2+}]_i$ (Figure 2(d) and 2(e)), indicating that the exosome-induced human
260 sperm $[Ca^{2+}]_i$ resulted from extracellular Ca^{2+} influx.

261 **CatSper-mediated exosome-induced $[Ca^{2+}]_i$ response**

262 In human spermatozoa, CatSper is the predominant Ca^{2+} channel responsible for Ca^{2+}
263 influx [26]. Hence, we examined whether CatSper was involved in the
264 exosome-induced human sperm $[Ca^{2+}]_i$ burst via extracellular Ca^{2+} influx. The
265 CatSper blocker, Mi, significantly impaired the exosome-activated Ca^{2+} response,
266 suggesting that CatSper was involved in the exosome-induced increase in sperm
267 $[Ca^{2+}]_i$ (Figure 3(a) and 3(b)). To further investigate this possibility, the whole-cell
268 patch-clamp technique was applied to examine the activation effect of exosomes on
269 sperm CatSper. The results showed that exosomes potentiated CatSper currents in
270 normal sperm samples (Figure 3(c) and 3(d)), supporting the role of CatSper in the
271 exosome-induced increase of $[Ca^{2+}]_i$. To clarify the overall role CatSper in mediating
272 exosome-induced Ca^{2+} influx, we took advantage of an infertile sperm sample lacking
273 the CatSper2 subunit; thus lacking the CatSper current [32]. The data showed that
274 exosomes did not increase $[Ca^{2+}]_i$ and CatSper current in the CatSper-deficient
275 sample (Figure 3(e) and 3(f)). As a control, a CatSper independent Ca^{2+} signal
276 activator (A23187) was found to increase sperm $[Ca^{2+}]_i$ (Figure 3(f)). Collectively,
277 these results suggested that CatSper controlled exosome-induced $[Ca^{2+}]_i$ signal

278 responses.

279 **Exosome-loading cargos provoked Ca^{2+} signals**

280 To investigate the specific exosomal cargos that controlled calcium signals in human
 281 sperm, we obtained supernatants and precipitates from 0.02% triton 100-X-treated
 282 exosomes using ultracentrifugation. Only the supernatant components induced the
 283 sperm $[\text{Ca}^{2+}]_i$ signal (Figure 4(a) and 4(b)). Furthermore, ultrasonic-disrupted
 284 exosomes had a stronger stimulatory effect inducing the $[\text{Ca}^{2+}]_i$ signal compared with
 285 intact exosomes (Figure 4(c) and 4(d)). To investigate whether the exosome-bearing
 286 proteins mediated the Ca^{2+} effects, the supernatant from exosomes treated with pro K
 287 at 58°C overnight (18 h) reduced the sperm $[\text{Ca}^{2+}]_i$ signal compared with the unheated
 288 supernatant component (Figure 4(e) and 4(f)). Furthermore, high temperature
 289 denaturation of exosome supernatant also reduced the elevation of $[\text{Ca}^{2+}]_i$ (Figure 4(g)
 290 and 4(h)). There were also no Ca^{2+} responses to pro K- treated exosomes and exosome
 291 supernatant in a Ca^{2+} -free medium (Figure S1). These data indicated that both
 292 exosome proteins and other non-protein components promoted the $[\text{Ca}^{2+}]_i$ increase via
 293 extracellular Ca^{2+} influx. Considering prostaglandins detected in exosomes were
 294 reported to increase $[\text{Ca}^{2+}]_i$ signals [32, 33], we investigated the presence of PGE1
 295 and PGE2 in exosomes (Figure S2(a)). We found that both prostaglandins were
 296 present in isolated exosomes, although the detected concentrations were lower than
 297 the concentrations required to induce a $[\text{Ca}^{2+}]_i$ effect in sperm (Figure S2(b)–(e)). We
 298 then examined the role of the intact membrane structure of exosomes for the stability
 299 of their prostaglandin cargos. Our data suggested that exosome membranes

300 maintained cargo stability and long-term physiological activity, because triton
301 X-10-treated exosomes had no detectable PGE1 levels and higher PGE2 levels
302 (Figure S2(a). We propose that exosomes provide the necessary cargo stability to
303 evoke a Ca^{2+} signal.

304 **Exosomes facilitated sperm hyperactivation**

305 In view of CatSper mainly regulates sperm hyperactivation [34], we examined the
306 effect of the CatSper-mediated increase in exosome-induced $[\text{Ca}^{2+}]_i$ upon sperm
307 hyperactivation. As shown in Figure 5(a), exosomes significantly promoted
308 hyperactivation. In addition, the ability of human spermatozoa to penetrate artificial
309 viscous media, another hyperactivation-related parameter, was enhanced by exosomes
310 and impaired by the CatSper inhibitor Mi (Figure 5(b)). Treatment of exosome
311 supernatant with Pro K or heating significantly impaired the hyperactivated sperm
312 count and the ability of sperm to pass through viscous media (Figure 5(c)–5(f)),
313 implying that exosome-induced sperm hyperactivation results from both exosomal
314 protein and non-protein components.

315 **Exosomes improved asthenozoospermic sperm motility**

316 Considering exosomes facilitate sperm motility [18] and function via calcium signals,
317 we further investigated a potential clinical application of exosomes. We found that the
318 elevation of $[\text{Ca}^{2+}]_i$ was significantly lower in exosomes derived from
319 asthenozoospermia than from normal seminal plasma (Figure 6(a) and 6(b)). Next, the
320 effects of exosomes upon sperm motility parameters in seminal plasma were

investigated by CASA. Sperm total motility, progressive motility, VCL, VSL and VAP parameters were markedly higher in normal seminal plasma than in exosome-free seminal plasma isolated after ejaculation (Figure S3). Consistent with these results, exosomes, especially those derived from normozoospermic semen, improved sperm progressive motility and promoted the penetration of asthenozoospermic sperm into viscous medium (Figure 6(c) and 6(d)). Therefore, exosomes partly rescued sperm motility and sperm function impaired by exosome dysfunction.

Discussion

In this study, we for the first time demonstrated that the SPE-evoked $[Ca^{2+}]_i$ resulted from extracellular Ca^{2+} influx, because exosomes had no effect on sperm $[Ca^{2+}]_i$ in Ca^{2+} -free medium. The exosome-induced elevation of $[Ca^{2+}]_i$ was controlled by CatSper activation, as a CatSper inhibitor significantly impaired the exosome-activated Ca^{2+} response, and there were no $[Ca^{2+}]_i$ and CatSper current responses using exosome-stimulated CatSper deficient sperm. In addition, exosomes and their cargos promoted sperm motility, especially hyperactivation.

This study used various methods, such as the Flow NanoAnalyzer, western blotting and TEM, to systematically identify and characterize exosomes isolated from human seminal plasma. Our data showed that we obtained exosomes with vesicle structural characteristics similar to those described in a previous study [35]. The exosome diameters (94.9 ± 21.8 nm) were consistent with past reports [6, 18]. However, the ratio of CD63 positive exosomes was approximately 18.5% in our samples, which may reflect a more complex composition and unique characteristics in

human seminal plasma exosomes than those derived from other body fluids, and the present of some non-classical CD63 negative exosomal extracellular vesicles [35-37]. For examples, seminal fluid from some species, such as the chicken, lacks CD9- and CD44-bearing extracellular vesicles [38].

It was previously reported that exosomes contributed to elevated Ca^{2+} concentrations in sperm via the release of calcium contained in prostasomes during fusion with sperm, and it was proposed that a novel mechanism influenced sperm $[\text{Ca}^{2+}]_i$ independently of ion-exchange systems and ATP-dependent pumps [31]. However, our results show that the exosome-induced sperm $[\text{Ca}^{2+}]_i$ response was still determined by ion-exchange systems (CatSper). Although exosomes increased sperm $[\text{Ca}^{2+}]_i$ in work by Palmerini et al., the elevated $[\text{Ca}^{2+}]_i$ may have derived from the exosomes themselves, as we showed calcium contained in exosomes could also be monitored by the fluorescent probe (Fluo-4 AM) (Figure S4). Palmerini et al. detected $[\text{Ca}^{2+}]_i$ after the exosomes fused to sperm [31], and under these conditions, the detection of a fluorescence signal may directly result from some exosomes retained or integrated onto sperm. Our results demonstrated an ion channel-controlled mechanism involving CatSper-mediated exosome-induced $[\text{Ca}^{2+}]_i$ signal via extracellular Ca^{2+} influx. The fusion or stimulation of sperm with exosomes and the transfer of Ca^{2+} -related cargos to the sperm surface did not cause the $[\text{Ca}^{2+}]_i$ response if CatSper didn't work.

Exosomes reportedly contained proteins, nucleic acid, lipids and some small molecule compounds, such as prostaglandins [32, 39, 40]. Our results showed that

365 triton X-100 and ultrasonic-treated exosomes conferred a stronger stimulatory effect
366 on the $[Ca^{2+}]_i$ signal response compared with intact exosomes. The precipitates
367 isolated from triton X-100-treated exosomes had no effect on sperm $[Ca^{2+}]_i$.
368 Furthermore, we found that exosome protein components contributed to the increased
369 $[Ca^{2+}]_i$, because enhancement of $[Ca^{2+}]_i$ was partially impaired using Pro K- or
370 heat-treated exosome supernatants compared with untreated exosome supernatants.
371 We speculated that exosome protein and non-protein components exerted Ca^{2+} signal
372 activation in human sperm. Our study also found that intact exosomes contained
373 nanomole levels of PGE1 and PGE2, consistent with other reports [32]. Moreover,
374 PGE1 and PGE2 induced the sperm $[Ca^{2+}]_i$ signal in our study (Figure S2(b)–(e)),
375 consistent with previous research [33]. The detected concentrations of PGE1 and
376 PGE2 were lower than the concentrations required for an effect, so we propose that
377 exosomes may carry PGE1, PGE2 and other prostaglandins, as well as other
378 non-protein cargos, that combine to induce sperm $[Ca^{2+}]_i$. Furthermore, we found
379 triton X-100-treated exosomes had no detectable level of PGE1, but had higher levels
380 PGE2 (Figure S2(a)), suggesting that exosomes maintained the stability and
381 physiological activity of cargos, such as PGE1. It has been reported that the use of
382 liposomes to deliver PGEs slowed the release rate, increased the therapeutic effect and
383 diminished adverse reactions [41, 42]. The effects of PGE1 on human sperm function
384 may involve an open CatSper channel, because transient intracellular Ca^{2+} signals
385 induced by PGE1 were fully inhibited by CatSper blockers [43]. Interestingly, the
386 mechanism of PGE1-stimulated CatSper activation may involve a direct effect and

387 differ from a progesterone receptor-dependent pathway [44]. Here, we found that
388 exosomes still augmented a $[Ca^{2+}]_i$ signal after the progesterone induced-calcium
389 signal reached a maximum threshold (Figure S5), suggesting that exosomes exerted a
390 distinct and/or complex activation pathway that in part differed from the
391 progesterone-induced open CatSper channel. These findings may be attributed to the
392 combined effect of multiple cargos in exosomes.

393 Considering the CatSper-mediated hyperactivation of sperm [34], which could be
394 triggered by exosomes, we speculated that exosomes may enhance sperm motility
395 parameters, especially hyperactivation. Our results demonstrated that exosomes
396 facilitated sperm hyperactivation and augmented sperm penetration into a
397 methylcellulose medium, suggesting that exosomes may enhance the ability of sperm
398 to pass through the viscous female reproductive tract. Furthermore, our data indicated
399 that physiological concentrations of exosomes promoted sperm total and progressive
400 motility, reflecting physiological conditions at the early stage of ejaculation,
401 confirming previous reports showing exosomes promoted sperm motility in an
402 artificial buffer [15, 45]. A recent study reported that exosomes derived from
403 normozoospermic but not from asthenozoospermic individuals improved spermatozoa
404 motility and triggered capacitation, suggesting that semen quality was affected by
405 male tract exosomes [18]. In this study, the authors proposed that exosomes carried
406 proteins, such as CRISP1, involved in sperm motility regulation. Other exosome
407 proteins, CD38 and cSrc kinases, also were thought to confer sperm hypermotility [46,
408 47]. In this study, we confirmed that protein components in exosomes regulate sperm

409 motility, especially hyperactivation, because the hyperactivated sperm count and the
410 sperm's ability to pass through a viscous medium were impaired after exosome
411 proteins were eliminated by Pro K or heat treatment.

412 Finally, we found that exosomes improved the motility of sperm from patients
413 with asthenospermia, as determined by the elevated progressive motility and ability to
414 penetrate viscous medium. This finding was consistent with previous work indicating
415 that sperm motility can benefit from exosomes [28]. Nevertheless, the physiological
416 significances of exosomes for sperm function regulation remain an intriguing and
417 challenging topic. Exosomes with other seminal plasma and female reproductive tract
418 components may together modify sperm function required for fertilization. We also
419 found that that exosomes isolated from severe asthenozoospermic samples (PR < 15%)
420 have a markedly weaker effect on $[Ca^{2+}]_i$ activation than normal exosomes. This
421 result, combined with the recent report, suggests the dysfunction and dyssecretosis of
422 exosomes may contribute to low sperm motility and infertility [18]. Calcium
423 regulation is crucial for sperm motility. Spermatozoa from some asthenozoospermic
424 patients exhibited a reduced responsiveness to progesterone [48]. Hence, we propose
425 that high quality endogenous or engineering-modified exosomes may be developed as
426 a therapeutic agent to treat the lowered sperm motility caused by calcium signal
427 response disorders.

428 In conclusion, SPE activated CatSper to induce $[Ca^{2+}]_i$ in human sperm via
429 extracellular Ca^{2+} influx, which contributed to exosome-induced hyperactivation.
430 Exosomes provide cargos that allow sperm to mobilize and manage a

431 CatSper-regulated Ca^{2+} signaling mechanism in sperm. The improvement of
432 asthenozoospermic sperm motility exerted by exosomes provides a new potential
433 clinical opportunity. This study provides insight into the role and mechanism of
434 human SPE in sperm function, and reveals a novel CatSper regulator for further
435 examination in cases of sperm dysfunction and male infertility caused by $[\text{Ca}^{2+}]_i$
436 dysregulation.

437 **Supplementary data**

438 Figure S1. Exosome Cargos induced- $[\text{Ca}^{2+}]_i$ increase via extracellular Ca^{2+} influx.
439 Figure S2. Contents of PGE1 and PGE2 in exosomes and effect of them on sperm
440 $[\text{Ca}^{2+}]_i$.
441 Figure S3. Effect of exosomes on sperm motility.
442 Figure S4. Calcium contained in exosomes could be monitored by Flou-4 AM.
443 Figure S5. Synergistic effect of progesterone with exosomes on sperm $[\text{Ca}^{2+}]_i$
444 response.

445 **Acknowledgments**

446 The authors thank all the participants who took part in this study, as well as all
447 medical staff who helped with human semen collection in Nanchang Reproductive
448 Hospital and Jiangxi Maternal and Child Health Hospital. We thank Charles Allan,
449 PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the
450 English text of a draft of this manuscript.

451 **Author Contributions**

452 X.H.Z., D.D.S. and X.N.Z. designed the study and wrote the manuscript with

453 feedback from the other authors. H. K. performed sperm patch-clamp recordings. D.D.
454 S. and W.W. Z. participated in single cell calcium imaging, the penetration of artificial
455 viscous media assay and CASA, hyperactivation, WB and the detailed data analysis.
456 H.Y.C. and H. K. acquired and processed all patient samples. X.H.Z. and X.N.Z. were
457 responsible for primary study oversight, data analysis, interpretation and manuscript
458 revision. All authors read and approved the final version of the manuscript for
459 submission.

460 **Funding**

461 This research was supported by the National Natural Science Foundation of China
462 (81760283, 31801238, 81871201 and 81871207), the National Key Research and
463 Development Program of China (2018YFC1003500) and the China Scholarship
464 Council (201906825011).

465 **Conflict of interest**

466 All authors have declared no conflict of interest.

467 **References**

- 468 [1] Mathieu MMartin-Jaular L, Specificities of secretion and uptake of exosomes and other
469 extracellular vesicles for cell-to-cell communication, 21 (2019) 9-17.
- 470 [2] Singh A, Fedele C, Lu H, Nevalainen M T, Keen J HLanguino L R, Exosome-mediated Transfer
471 of alphavbeta3 Integrin from Tumorigenic to Nontumorigenic Cells Promotes a Migratory
472 Phenotype, Mol Cancer Res 14 (2016) 1136-1146.
- 473 [3] Chen L, Feng Z, Yue H, Bazdar D, Mbonye U, Zender CHarding C V, Exosomes derived from
474 HIV-1-infected cells promote growth and progression of cancer via HIV TAR RNA, 9 (2018)

475 4585.

476 [4] Arvidson G, Ronquist G, Wikander GOjteg A C, Human prostates membranes exhibit very
477 high cholesterol/phospholipid ratios yielding high molecular ordering, *Biochim Biophys Acta*
478 984 (1989) 167-73.

479 [5] Thimon V, Frenette G, Saez F, Thabet MSullivan R, Protein composition of human
480 epididymosomes collected during surgical vasectomy reversal: a proteomic and genomic
481 approach, *Hum Reprod* 23 (2008) 1698-707.

482 [6] Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud R P, Strobl J, Westerberg K,
483 Gottardo R, Tewari M, Hladik F, Exosomes in human semen carry a distinctive repertoire of
484 small non-coding RNAs with potential regulatory functions, *Nucleic Acids Res* 42 (2014)
485 7290-304.

486 [7] Ronquist G K, Larsson A, Stavreus-Evers A, Ronquist G, Prostates are heterogeneous
487 regarding size and appearance but affiliated to one DNA-containing exosome family, *Prostate*
488 72 (2012) 1736-45.

489 [8] Ronquist G, Prostates are mediators of intercellular communication: from basic research to
490 clinical implications, *J Intern Med* 271 (2012) 400-13.

491 [9] Noda T, Ikawa M, Physiological function of seminal vesicle secretions on male fecundity,
492 *Reproductive medicine and biology* 18 (2019) 241-246.

493 [10] Saez F, Motta C, Boucher D, Grizard G, Antioxidant capacity of prostates in human semen,
494 *Mol Hum Reprod* 4 (1998) 667-72.

495 [11] Park K H, Kim B J, Kang J, Nam T S, Lim J M, Kim H T, Park J K, Kim Y G, Chae S W, Kim U
496 H, Ca²⁺ signaling tools acquired from prostates are required for progesterone-induced

497 sperm motility, *Sci Signal* 4 (2011) ra31.

498 [12] Machtinger R, Laurent L CBaccarelli A A, Extracellular vesicles: roles in gamete maturation,
499 fertilization and embryo implantation, *Hum Reprod Update* 22 (2016) 182-93.

500 [13] Du J, Shen J, Wang Y, Pan C, Pang W, Diao HDong W, Boar seminal plasma exosomes
501 maintain sperm function by infiltrating into the sperm membrane, *Oncotarget* 7 (2016)
502 58832-58847.

503 [14] Bechoua S, Rieu I, Sion BGrizard G, Prostatomes as potential modulators of tyrosine
504 phosphorylation in human spermatozoa, *Syst Biol Reprod Med* 57 (2011) 139-48.

505 [15] Saez FSullivan R, Prostatomes, post-testicular sperm maturation and fertility, *Front Biosci*
506 (Landmark Ed) 21 (2016) 1464-73.

507 [16] Lin Y, Liang A, He Y, Li Z, Li Z, Wang GSun F, Proteomic analysis of seminal extracellular
508 vesicle proteins involved in asthenozoospermia by iTRAQ, *Mol Reprod Dev* (2019).

509 [17] Abu-Halima M, Ludwig N, Hart M, Leidinger P, Backes C, Keller A, Hammadeh MMeese E,
510 Altered micro-ribonucleic acid expression profiles of extracellular microvesicles in the
511 seminal plasma of patients with oligoasthenozoospermia, *Fertil Steril* 106 (2016)
512 1061-1069.e3.

513 [18] Murdica V, Giacomini E, Alteri A, Bartolacci A, Cermisoni G C, Zarovni N, Papaleo E,
514 Montorsi F, Salonia A, Viganò PVago R, Seminal plasma of men with severe
515 asthenozoospermia contain exosomes that affect spermatozoa motility and capacitation,
516 *Fertility and Sterility* 111 (2019) 897-908.e2.

517 [19] Alasmari W, Costello S, Correia J, Oxenham S K, Morris J, Fernandes L, Ramalho-Santos J,
518 Kirkman-Brown J, Michelangeli F, Publicover SBarratt C L, Ca²⁺ signals generated by

519 CatSper and Ca²⁺ stores regulate different behaviors in human sperm, J Biol Chem 288 (2013)
520 6248-58.

521 [20] Pereira R, Sa R, Barros ASousa M, Major regulatory mechanisms involved in sperm motility,
522 Asian J Androl 19 (2017) 5-14.

523 [21] Navarrete F A, Garcia-Vazquez F A, Alvau A, Escoffier J, Krapf D, Sanchez-Cardenas C,
524 Salicioni A M, Darszon AVisconti P E, Biphasic role of calcium in mouse sperm capacitation
525 signaling pathways, J Cell Physiol 230 (2015) 1758-1769.

526 [22] Darszon A, Nishigaki T, Wood C, Trevino C L, Felix RBeltran C, Calcium channels and Ca²⁺
527 fluctuations in sperm physiology, Int Rev Cytol 243 (2005) 79-172.

528 [23] Palmerini C A, Carlini E, Nicolucci AArienti G, Increase of human spermatozoa intracellular
529 Ca²⁺concentration after fusion with prostasomes, Cell Calcium 25 (1999) 291-296.

530 [24] Park K-H, Kim B-J, Kang J, Nam T-S, Lim J M, Kim H T, Park J K, Kim Y G, Chae S-WKim
531 U-H, Ca²⁺ Signaling Tools Acquired from Prostrasomes Are Required for
532 Progesterone-Induced Sperm Motility, Science Signaling 4 (2011) ra31-ra31.

533 [25] Chung J J, Miki K, Kim D, Shim S HShi H F, CatSperzeta regulates the structural continuity of
534 sperm Ca(2+) signaling domains and is required for normal fertility, 6 (2017).

535 [26] Lishko P VMannowetz N, CatSper: A Unique Calcium Channel of the Sperm Flagellum, Curr
536 Opin Physiol 2 (2018) 109-113.

537 [27] Mannowetz N, Miller M RLishko P V, Regulation of the sperm calcium channel CatSper by
538 endogenous steroids and plant triterpenoids, 114 (2017) 5743-5748.

539 [28] Brenker C, Goodwin N, Weyand I, Kashikar N D, Naruse M, Krahling M, Muller A, Kaupp U
540 BStrunker T, The CatSper channel: a polymodal chemosensor in human sperm, Embo j 31

(2012) 1654-65.

[29] Zhang X, Zhang P, Song D, Xiong S, Zhang H, Fu J, Gao F, Chen HZeng X, Expression profiles and characteristics of human lncRNA in normal and asthenozoospermia sperm, Biol Reprod (2018).

[30] Brenker C, Rehfeld A, Schiffer C, Kierzek M, Kaupp U B, Skakkebaek N EStrunker T, Synergistic activation of CatSper Ca²⁺ channels in human sperm by oviductal ligands and endocrine disrupting chemicals, Hum Reprod 33 (2018) 1915-1923.

[31] Palmerini C A, Carlini E, Nicolucci AARienti G, Increase of human spermatozoa intracellular Ca²⁺ concentration after fusion with prostasomes, Cell Calcium 25 (1999) 291-6.

[32] Oliw E H, Fabiani R, Johansson LRonquist G, Arachidonic acid 15-lipoxygenase and traces of E prostaglandins in purified human prostasomes, J Reprod Fertil 99 (1993) 195-9.

[33] Shimizu Y, Yorimitsu A, Maruyama Y, Kubota T, Aso TBronson R A, Prostaglandins induce calcium influx in human spermatozoa, Mol Hum Reprod 4 (1998) 555-61.

[34] Luo T, Chen H Y, Zou Q X, Wang T, Cheng Y M, Wang H F, Wang F, Jin Z L, Chen Y, Weng S QZeng X H, A novel copy number variation in CATSPER2 causes idiopathic male infertility with normal semen parameters, Hum Reprod (2019).

[35] Aalberts M, Sostaric E, Wubbolts R, Wauben M W, Nolte-'t Hoen E N, Gadella B M, Stout T AStoorvogel W, Spermatozoa recruit prostasomes in response to capacitation induction, Biochim Biophys Acta 1834 (2013) 2326-35.

[36] Jeppesen D K, Fenix A M, Franklin J L, Higginbotham J N, Zhang Q, Zimmerman L J, Liebler D C, Ping J, Liu Q, Evans R, Fissell W H, Patton J G, Rome L H, Burnette D TCoffey R J, Reassessment of Exosome Composition, Cell 177 (2019) 428-445.e18.

[37] Höög J LLötvall J, Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy, *Journal of Extracellular Vesicles* 4 (2015) 28680.

[38] Alvarez□Rodriguez M, Ntzouni M, Wright D, Khan K I, López□Béjar M, Martinez C ARodriguez□Martinez H, Chicken seminal fluid lacks CD9□And CD44□bearing extracellular vesicles, *Reproduction in Domestic Animals* (2020).

[39] Sullivan RSaez F, Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology, *Reproduction* 146 (2013) R21-35.

[40] Ronquist G K, Larsson A, Ronquist G, Isaksson A, Hreinsson J, Carlsson LStavreus-Evers A, Prostatosomal DNA characterization and transfer into human sperm, *Mol Reprod Dev* 78 (2011) 467-76.

[41] Ivanova V, Garbuzenko O B, Reuhl K R, Reimer D C, Pozharov V PMinko T, Inhalation treatment of pulmonary fibrosis by liposomal prostaglandin E2, *Eur J Pharm Biopharm* 84 (2013) 335-44.

[42] Davidson S M, Cabral-Lilly D, Maurio F P, Franklin J C, Minchey S R, Ahl P LJanoff A S, Association and release of prostaglandin E1 from liposomes, *Biochim Biophys Acta* 1327 (1997) 97-106.

[43] Lishko P V, Botchkina I LKirichok Y, Progesterone activates the principal Ca²⁺ channel of human sperm, *Nature* 471 (2011) 387-91.

[44] Miller M R, Mannowetz N, Iavarone A T, Safavi R, Gracheva E O, Smith J F, Hill R Z, Bautista D M, Kirichok YLishko P V, Unconventional endocannabinoid signaling governs sperm activation via the sex hormone progesterone, *Science* 352 (2016) 555-9.

[45] Stegmayr BRonquist G, Promotive effect on human sperm progressive motility by prostasomes,

585 Urol Res 10 (1982) 253-7.

586 [46] Kim B J, Park D R, Nam T S, Lee S H, Kim U H, Seminal CD38 Enhances Human Sperm
587 Capacitation through Its Interaction with CD31, PLoS One 10 (2015) e0139110.

588 [47] Krapf D, Ruan Y C, Wertheimer E V, Battistone M A, Pawlak J B, Sanjay A, Pilder S H,
589 Cuasnicu P, Breton S, Visconti P E, cSrc is necessary for epididymal development and is
590 incorporated into sperm during epididymal transit, Dev Biol 369 (2012) 43-53.

591 [48] Espino J, Mediero M, Lozano G M, Bejarano I, Ortiz Á, García J F, Pariente J A, Rodríguez A B,
592 Reduced levels of intracellular calcium releasing in spermatozoa from asthenozoospermic
593 patients, Reproductive Biology and Endocrinology 7 (2009) 11.

594

595

596 **Figure legends**

597 Figure 1. Characteristic of SPE. (a) The diameter range of exosomes. After
598 ultracentrifugation, the exosomes were analyzed with Flow NanoAnalyzer. (b) (c) The
599 characteristic and concentration of particles. Intact exosomes and triton X-100-treated
600 (1%, 30 min) exosomes were detected by the Flow NanoAnalyzer. (d) (e) Expression
601 of CD63 in SPE. Exosomes were subjected to immunofluorescence staining with
602 antibodies directed against CD63 and then measured with Flow NanoAnalyzer. (f)
603 Expression of CD63 and CD9, exosome markers, in SPE. Exosomes from two
604 different donors were subjected to SDS-PAGE and immunoblot analysis with an
605 antibody directed against CD63 and CD9. Western blots are representative of N = 3
606 experiments. (g) Morphology of exosomes by transmission electron microscope. It
607 showed that there is a typical feature of exosomes with intact lipid bilayers and
608 saucer-like structure of particles.

609 Figure 2. Exosomes induced $[Ca^{2+}]_i$ increase via extracellular Ca^{2+} influx. (a)
610 Examples of $[Ca^{2+}]_i$ change was illustrated with the time frame. (b) The statistical
611 analysis of the effects of different concentrations of exosomes isolated by
612 ultracentrifugation on sperm $[Ca^{2+}]_i$. Sperm $[Ca^{2+}]_i$ was monitored after loading cells
613 with Fluo-4 AM-AM (2 μ M) and F127 (0.1% w/v) and the fluorescence intensity of
614 the sperm was detected by microplate reader before and after adding the different
615 concentrations of exosomes. (c) Single-sperm $[Ca^{2+}]_i$ imaging indicated a $[Ca^{2+}]_i$
616 response in sperm after exosome treatment. Sperm $[Ca^{2+}]_i$ was stained with Fluo-4
617 AM-AM (2 μ M) and F127 (0.1% w/v), and the fluorescence intensity of the sperm

618 was visualized and detected before and after adding exosomes. (d) Effect of exosomes
619 on sperm $[Ca^{2+}]_i$ in Ca^{2+} -free buffer. Sperm $[Ca^{2+}]_i$ was determined before and after
620 adding exosomes in the HS buffer with presence or absence of BAPTA (12 mM) or
621 EGTA(5 mM). (e) The statistical analysis of the amplitude $\Delta F/F_0$ of $[Ca^{2+}]_i$ change
622 from (d) time-course traces ($n = 3$). Data were presented as mean \pm SME ($n = 10$). * P
623 < 0.05 , ** $P < 0.01$, *** $P < 0.001$ versus HS group.

624 Figure 3. CatSper mediated exosome-induced $[Ca^{2+}]_i$ signal. (a) The CatSper blocker,
625 Mi, impaired the exosome-activated $[Ca^{2+}]_i$ response. Sperm $[Ca^{2+}]_i$ was monitored
626 after loading sperm with Fluo-4 AM-AM (2 μ M) and F127 (0.1% w/v) and the
627 fluorescence intensity of the sperm was detected by microplate reader after treating
628 sperm with exosomes in the presence or absence of Mi (30 μ M). (b) The statistical
629 analysis of the amplitude $\Delta F/F_0$ of $[Ca^{2+}]_i$ change from (a) time-course traces ($n = 5$).
630 (c) Exosomes activated CatSper current in normal human sperm but not in (e)
631 CatSper-deficient sperm. CatSper currents were examined by whole-cell clamp
632 technique from -100 to $+100$ mV as described in Materials and Methods Section.
633 Different concentrations of exosomes diluted in DVF (divalent free) buffer was
634 perfused to record exosome-induced CatSper currents. DVF solutions were used to
635 record baseline monovalent CatSper currents. (d) Statistical analysis of the mean
636 CatSper currents at $+100$ mV (positive) and -100 mV (negative) for (c). (f) $[Ca^{2+}]_i$ in
637 CatSper-deficient sperm have no response to exosomes. After staining sperm with
638 Fluo-4 AM-AM (2 μ M) and F127 (0.1% w/v) for 30 min, sperm $[Ca^{2+}]_i$ was detected
639 before and after adding exosomes (sperm : exosome = 1:1) and/or P4 (2 μ M). A23187,

640 a Ca^{2+} signal activator with CatSper independent, (1 mM) served as control. Data
641 were presented as mean \pm SME (n = 5). $^{###}\text{P} < 0.001$ versus exosome group. $^{*}\text{P} < 0.05$,
642 $^{**}\text{P} < 0.01$, $^{***}\text{P} < 0.001$ versus DVF group.

643 Figure 4. Exosomes-loading protein and non-protein components provoked $[\text{Ca}^{2+}]_i$
644 signal. (a) Effect of exosome components on sperm $[\text{Ca}^{2+}]_i$. (c) Effect of ultrasonic on
645 exosome-induced Ca^{2+} level burst. (e) Effect of enzymolysis with Pro K (protease K)
646 on exosome-induced Ca^{2+} level burst. (g) Effect of Pro K on exosome
647 supernatant-induced Ca^{2+} burst. (i) Effect of heating on exosome-induced Ca^{2+} level
648 burst. (k) Effect of long-term conservation on exosome-induced Ca^{2+} level burst. (b)
649 (d) (f) (h) (j) (l) Statistical analysis of the amplitude of the Ca^{2+} changes from (a) (c)
650 (e) (g) (i) (k), respectively. Triton X-100 (0.002%)-penetrated exosomes were
651 ultra-centrifuged to obtain supernatants and precipitates. After stimulating sperm with
652 exosomes, supernatants, precipitates, ultrasonic-, heating- and Pro K-treated
653 exosomes, sperm $[\text{Ca}^{2+}]_i$ was monitored by a microplate reader with Fluo-4 AM-AM
654 (2 μM) staining. Data were presented as mean \pm SME (n = 8). $^{#}\text{P} < 0.05$, $^{##}\text{P} < 0.01$
655 versus exosome group. $^{*}\text{P} < 0.05$, $^{**}\text{P} < 0.01$, $^{***}\text{P} < 0.001$ versus HS group.

656 Figure 5. Exosomes and its cargos promoted human sperm hyperactivation. (a) The
657 effect of exosomes on human sperm hyperactivation. (b) Mi impaired
658 exosome-induced the ability of penetration into viscous medium. The exosome
659 supernatant-induced (c) hyperactivation and (d) the ability of sperm to penetrate into
660 artificial viscous media were attenuated by Pro K. The exosome supernatant-induced

(e) hyperactivation and (f) the ability of sperm to penetrate into artificial viscous media were attenuated by heating. Hyperactivation was assessed via the CASA system and 200 spermatozoa were counted at least for each assay. The sperm was capacitated in HTF medium for 3 h and treated with exosomes for 1 h at 37°C. Hyperactivation was defined as those cells with a curvilinear velocity (VCL) ≥ 150 $\mu\text{m/s}$, linearity (LIN) $< 50\%$, and lateral head displacement (ALH) ≥ 7 μm as described in Materials and Methods Section. The ability of sperm to penetrate into artificial viscous media was evaluated as the following protocol. After sperm were selected by a swim-up purification procedure (30 min, 37 °C) and the better vigor sperm in upper fractions were washed twice in HS buffer. Exosomes or treated supernatants were added after capacitation for 2 h. Three h later, 20 μL of 1% methylcellulose was inhaled into the capillary glass tube, and the capillary was placed in the mixed sperm. The number of sperms 3 cm in the capillary from the base of the tube was recorded after 1 h. Data were presented as mean \pm SME (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 versus no treatment group (HTF). ##P < 0.01 versus exosome-treated group.

Figure 6. The effects of exosomes on sperm motility and $[\text{Ca}^{2+}]_i$ signal in asthenozoospermia. (a) Effect of exosomes on sperm $[\text{Ca}^{2+}]_i$. Sperm $[\text{Ca}^{2+}]_i$ was monitored after loading cells with Fluo-4 AM-AM (2 μM) and F127 (0.1% w/v) and the fluorescence intensity of the sperm was detected by microplate reader before and after adding the different concentrations of exosomes extracted from normozoospermic or asthenozoospermic patients. (b) Statistical analysis of the

683 amplitude of the Ca^{2+} changes from (a). (c) The percentage of progressive motility in
 684 spermatozoa derived from donors determined by CASA before or after treatment with
 685 exosomes isolated from normozoospermic or asthenozoospermic patients. HSA
 686 stimulation served as a positive control. (d) The ability of sperm to penetrate into
 687 artificial viscous media was evaluated by above mentioned methods. Data were
 688 presented as mean \pm SME (n = 5-10). *P < 0.05, **P < 0.01, ***P < 0.001 versus no
 689 treatment group (HTF). ##P < 0.01 versus normozoospermic exosome-treated group.

690 Figure S1. The exosome cargos induced- $[\text{Ca}^{2+}]_i$ increase in sperm depended on
 691 extracellular Ca^{2+} influx. Sperm $[\text{Ca}^{2+}]_i$ were recorded according to above mentioned
 692 methods. The data were cumulative of at least three independent experiments. *P <
 693 0.05, **P < 0.01, ***P < 0.001 versus HS. ###P < 0.001 versus exosomes.

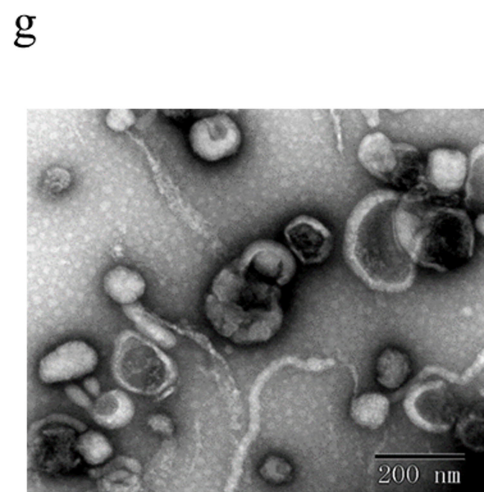
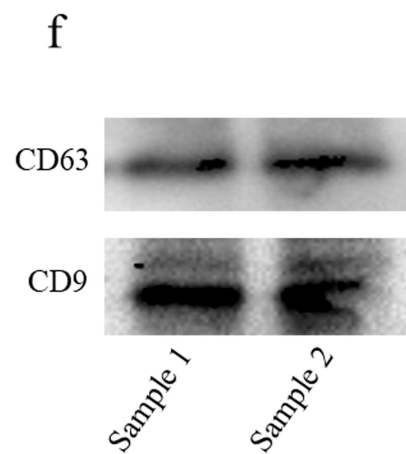
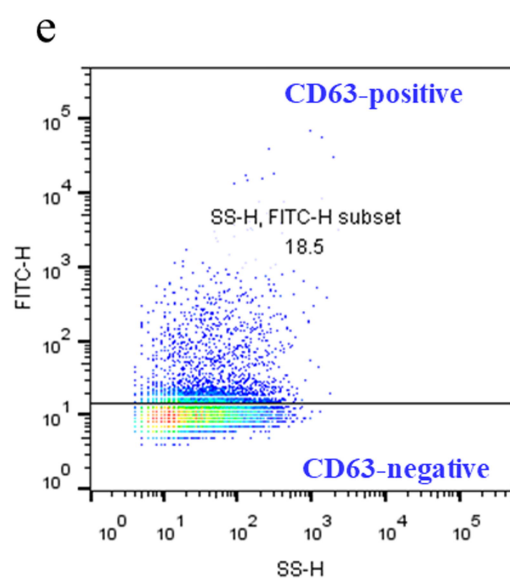
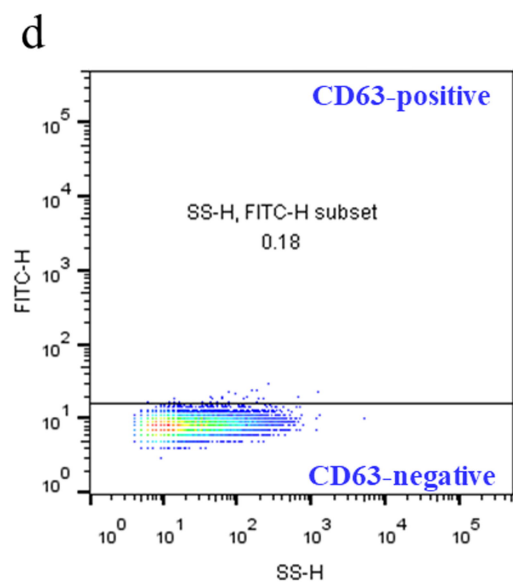
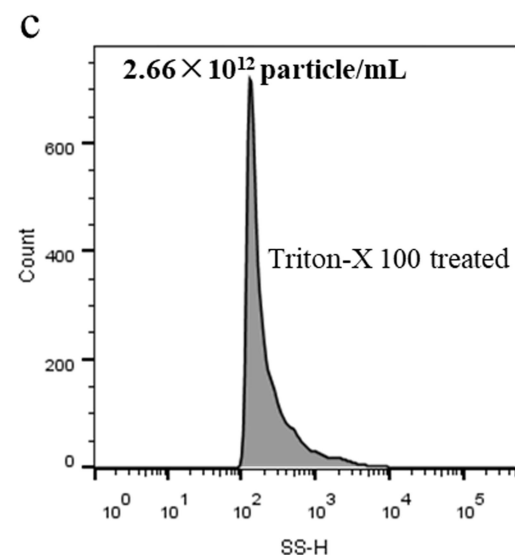
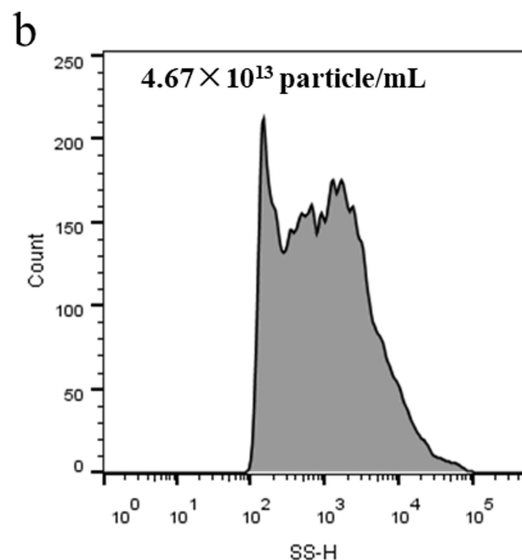
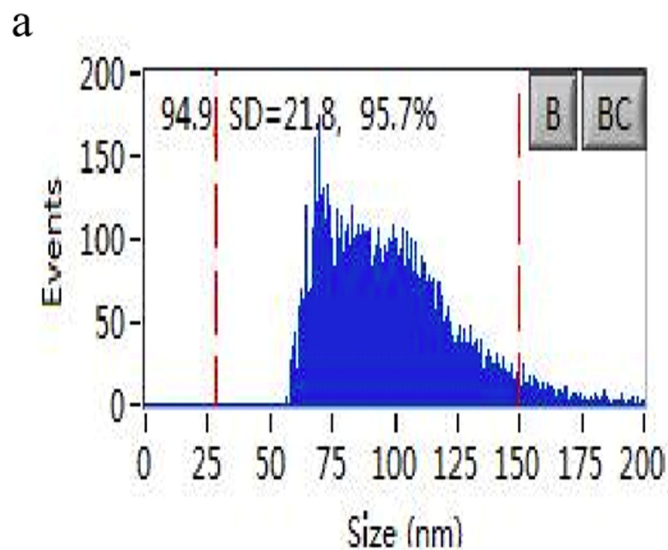
694 Figure S2. Contents of PGE1 and PGE2 in exosomes and effect of them on sperm
 695 $[\text{Ca}^{2+}]_i$. (a) Contents of PGE1 and PGE2 were examined by Elisa Kits. (b)-(e) Sperm
 696 $[\text{Ca}^{2+}]_i$ were recorded after stimulated to PGE1 or PGE2 according to above
 697 mentioned methods. The data were cumulative of at least three independent
 698 experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus HS.

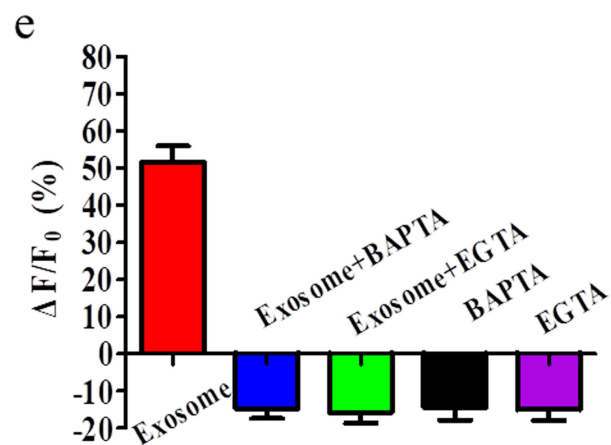
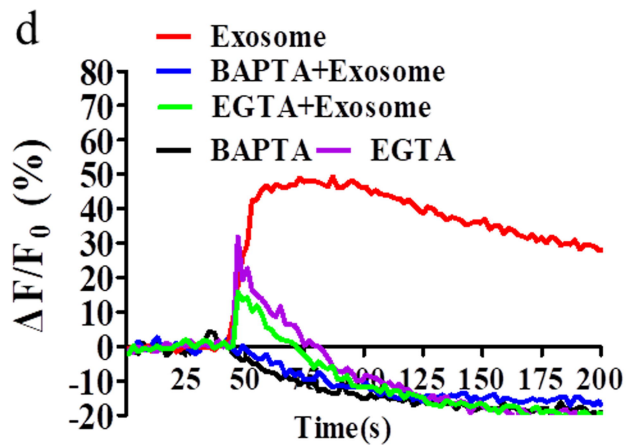
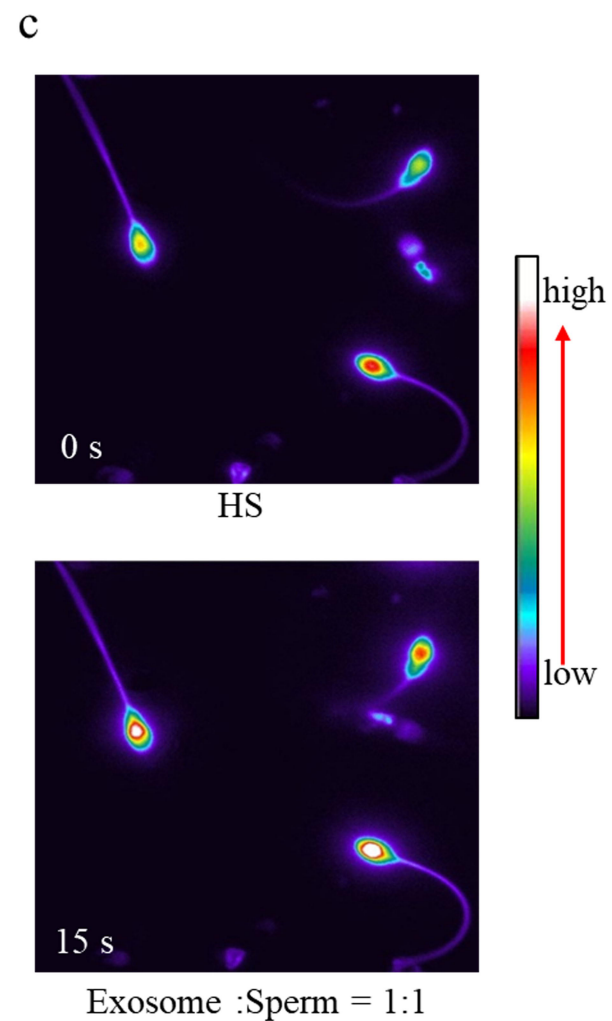
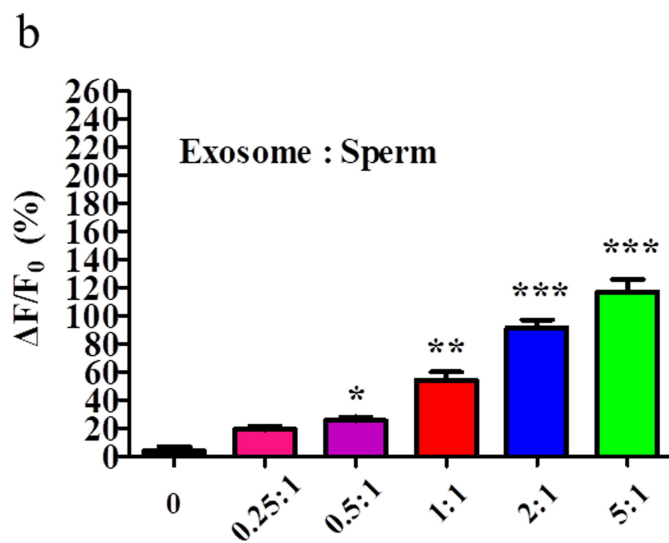
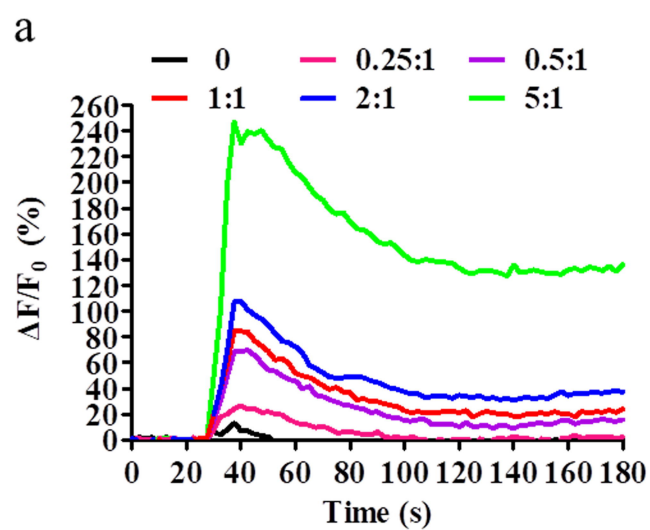
699 Figure S3. Effect of exosomes on sperm motility. Human sperm were incubated in
 700 seminal plasma or exosomes-free seminal plasma at 37 °C and 5% CO_2 incubator for
 701 different time. The motion parameters, (a) total motility, (b) PR, (c) VCL, (d) VSL, (e)
 702 VAP, (f) LIN, were analyzed by CASA. A minimum of 200 sperms were counted for
 703 each assay. The data were cumulative of at least three independent experiments. *P <

704 0.05, **P < 0.01, ***P < 0.001 versus seminal plasma.

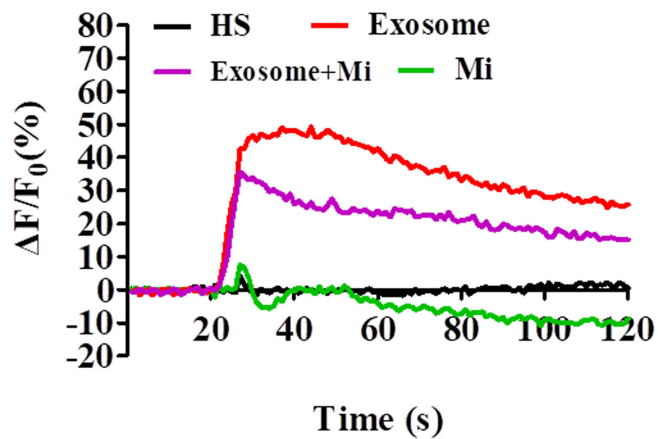
705 Figure S4. Calcium contained in exosomes. The fluorescence intensity of Ca^{2+} in
706 exosomes was detected by Multimode Plate Reader with Fluo 4 (2 μM) staining.

707 Figure S5. Synergistic effect of progesterone with exosomes on sperm $[\text{Ca}^{2+}]_i$
708 response. Sperm $[\text{Ca}^{2+}]_i$ were recorded according to above mentioned methods. The
709 data were cumulative of at least three independent experiments.

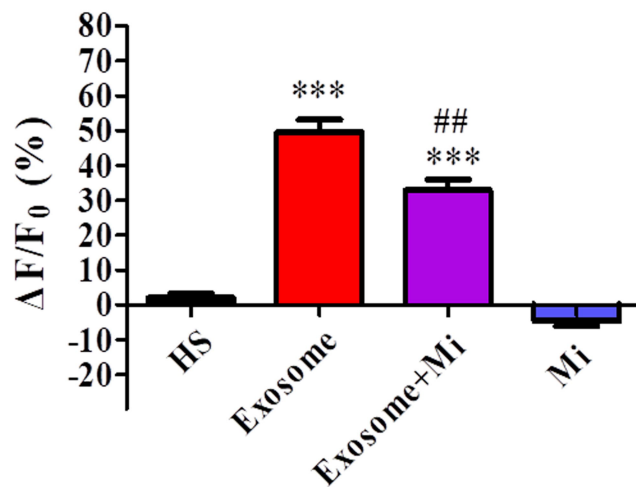




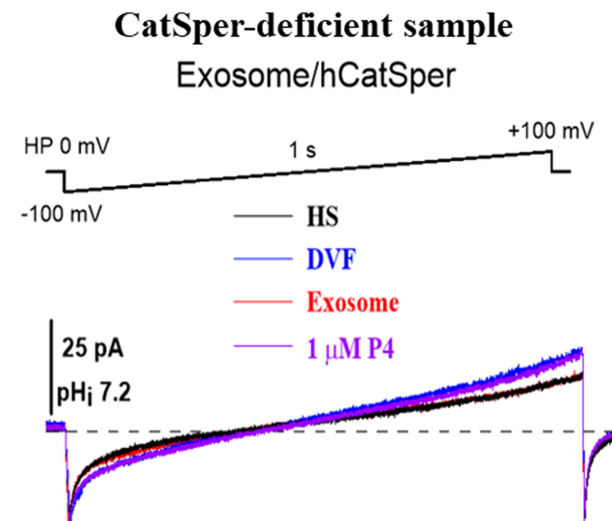
a



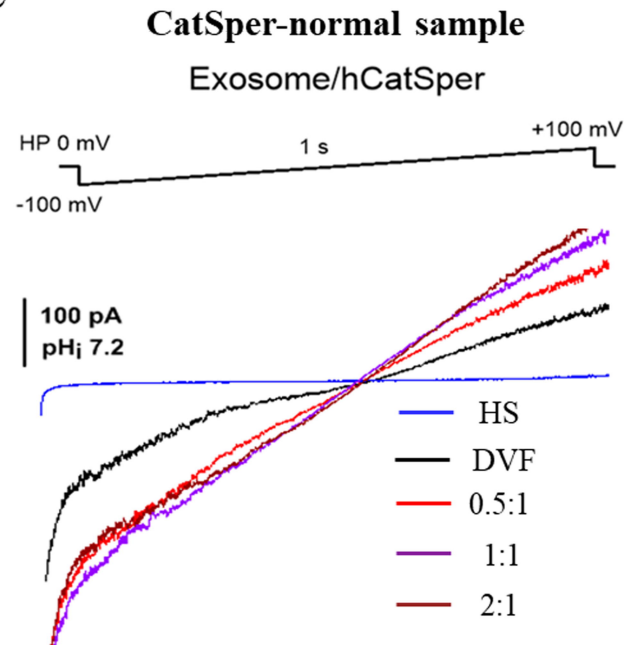
b



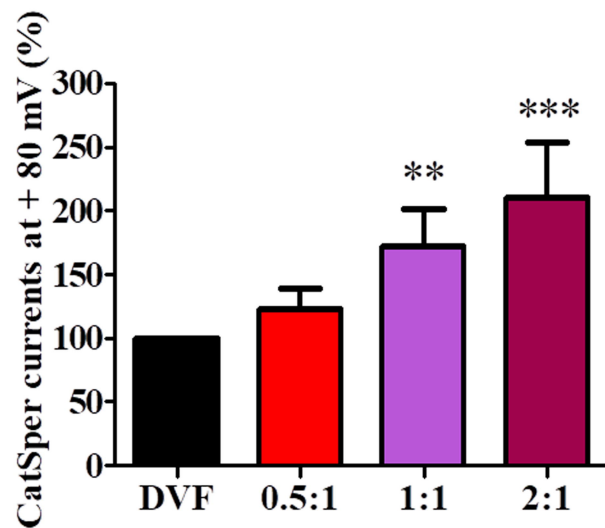
e



c



d



f

