



**University of Dundee**

## **Myeloperoxidase inhibitor AZD5904 enhances human sperm function in vitro**

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1 **Title: Myeloperoxidase inhibitor AZD5904 enhances human sperm function in**  
2 **vitro**

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4

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18

19 **Abstract**

20 **Study question:** Does AZD5904, a myeloperoxidase inhibitor (MPOi), have any  
21 effect on human sperm function in vitro?

22 **Summary answer:** AZD5904 improves sperm function in an in-vitro model of  
23 oxidative stress (OS) and potentially offers a novel treatment approach for male  
24 infertility.

25 **What is known already:** Male infertility is an underlying or contributory cause in half  
26 of all couples experiencing difficulties conceiving, yet there is currently no effective  
27 treatment or cure. OS is a common pathology in a significant proportion of infertile  
28 men. It can negatively affect sperm motility and the ability to fertilise a mature oocyte  
29 as well as DNA integrity and therefore represents an attractive target for therapeutic  
30 intervention.

31 **Study design, size, duration:** This study included population-based samples from  
32 men (23 - 50 years) attending Ninewells Assisted Conception Unit, Dundee for  
33 diagnostic semen analysis, July 2017 - September 2018. Semen samples (n=47) from  
34 45 patients were used.

35 **Participants/materials, setting, methods:** Neutrophils activated using zymosan  
36 were incubated with prepared human spermatozoa for 2 hours (T2) and 24 hours (T24)  
37 to create an in-vitro model of OS. Parallel samples were co-incubated with AZD5904,  
38 a myeloperoxidase inhibitor, to examine its effects. Sperm motility was assessed by  
39 computer-assisted sperm analysis (CASA) at T2 and T24. Functional motility was  
40 assessed by sperm penetration assay. Statistical analysis was performed using  
41 GraphPad Prism.

42 **Main results and the role of chance:** There was no significant difference in total or  
43 progressive sperm motility between any treatment and control groups at T2 or T24.

44 Nonetheless, significant positive effects on sperm function were observed with  
45 AZD5904, with 16/45 (35.6%) samples (with both normal and abnormal baseline  
46 semen analysis characteristics) displaying a  $\geq 20\%$  increase in sperm penetrated  
47 through viscous media ( $P < 0.003$ ).

48 **Limitations, reasons for caution:** This was an in-vitro study.

49 **Wider implications of the findings:** Treatment with AZD5904 resulted in significant  
50 increased sperm penetration in one of three samples treated, which is likely to  
51 represent improvement in sperm function required for fertilisation. We are now  
52 planning a clinical trial to validate these results and hope that this could represent a  
53 new treatment for male infertility.

54 **Study funding/competing interest(s):** AZD5904 was shared through the  
55 AstraZeneca Open Innovation program. The study was funded by AstraZeneca and  
56 sponsored by the University of Dundee. Additional funding was provided by Chief  
57 Scientist Office/NHS Research Scotland (SMDS). AW and HJS are employed by  
58 AstraZeneca Open Innovation. SMDS is Associate Editor of Human Reproduction  
59 and Editorial Board member of Reproduction & Fertility. CLRB is Editor of RBMO  
60 and has received lecturing fees from Merck and Ferring and is on the Scientific  
61 Advisory Panel for Ohana BioSciences. CLRB was chair of the World Health  
62 Organization Expert Synthesis Group on Diagnosis of Male infertility (2012–2016).  
63 The other authors declare no conflict of interest.

64 **Trial registration number:** N/A

65

66 **Key words**

67 male infertility, myeloperoxidase inhibitor, oxidative stress, sperm, sperm function

68

## 69 **Introduction**

70 Infertility is a common issue that affects 1 in 7 couples of reproductive age (Slama, et  
71 al., 2012). Male factor is an underlying or contributory cause in half of all cases of  
72 infertility (Agarwal, et al., 2015) yet there is no effective treatment or cure. Currently,  
73 the only option for couples is to use assisted reproduction technologies (ART),  
74 although this is an expensive and invasive approach without guarantee of success.  
75 There is both a clinical demand and an unmet need for an effective treatment to  
76 improve sperm count or quality in infertile men to enable their partners to conceive  
77 naturally.

78 Reactive oxygen species (ROS) are by-products of normal cellular metabolism, and  
79 are required for key events in sperm, including fertilisation. However, oxidative stress  
80 (OS) arises when levels of ROS overwhelm antioxidant defences (Agarwal, et al.,  
81 2006), leading to a spectrum of cellular damage and dysfunction, and ultimately cell  
82 death. Whilst male infertility is unexplained in the majority of cases (Wu, et al., 2010),  
83 OS is acknowledged to be a common pathology in a significant proportion of infertile  
84 men (Aitken, et al., 2010, Tremellen, 2008). The main contributors of ROS in the  
85 ejaculate are white blood cells (WBC), mostly neutrophils (Aitken and Fisher, 1994,  
86 Plante, et al., 1994). Current theories propose prostate, epididymis or seminal glands  
87 as possible origins of WBC, however further studies are needed (Wolff, 1995).

88 Nonetheless, neutrophils are found in the male genital tract and in virtually every  
89 ejaculate and can have detrimental effects on human sperm function both in vitro  
90 (Aitken and Baker, 2013, Henkel, et al., 2005) and in vivo (Barraud-Lange, et al.,  
91 2011).

92 Myeloperoxidase (MPO) is a peroxidase enzyme produced mainly by neutrophils. It  
93 plays a major role in host defence and kills microbes by catalysing the formation of

94 reactive oxygen intermediates, including hypochlorous acid. It also plays a role in  
95 inflammation, generating OS and mediating tissue damage. Inhibition of MPO and its  
96 downstream inflammatory pathways represents an attractive and logical target for  
97 therapeutic intervention for male infertility. AZD5904 is a potent (IC<sub>50</sub> 140 nM)  
98 irreversible inhibitor of MPO (shared by AstraZeneca through their Open Innovation  
99 program for clinical and preclinical research). In this study, we investigated the effect  
100 of AZD5904 on spermatozoa incubated in the presence of activated neutrophils to  
101 create an in-vitro model of OS.

102

### 103 **Materials and Methods**

104 This study included 45 unselected male patients (age 23 – 50 years) attending  
105 Ninewells Assisted Conception Unit (ACU), Dundee for diagnostic semen analysis  
106 between July 2017 and September 2018. Samples were excluded where sperm  
107 concentration  $\leq 2$  million/ml as patients with severe oligozoospermia are known to  
108 have up to 10-fold higher incidence of genetic abnormalities compared to the general  
109 population (Clementini, et al., 2005, Vincent, et al., 2002) and are therefore less  
110 likely to have correctable male infertility. Research consent was in accordance with  
111 the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version  
112 8), under local ethical approval (13/ES/0091) from East of Scotland Research Ethics  
113 Service (EoSRES) REC 1. The methodology was as per Björndahl guidelines  
114 (Björndahl, et al., 2016).

### 115 **Semen Sample Preparation**

116 Semen samples (n=45 from 45 patients) were collected by masturbation after 2 to 7  
117 days abstinence. Following diagnostic semen analysis, surplus samples were

118 allocated for research. One patient returned on two further occasions to submit a  
119 sample for research, hence 47 samples were included in the study in total.

120 Slides (two per sample) were first prepared by smearing with 10 $\mu$ l raw semen and  
121 stored at -20°C for later analysis. Semen samples were then prepared by density  
122 gradient centrifugation (DGC; 300 xg for 20 minutes) as described previously (Tardif,  
123 et al., 2014). The uppermost layer was collected into an Eppendorf tube and  
124 centrifuged (17,000 xg; 20 minutes). The supernatant, which comprised seminal  
125 fluid, was centrifuged (17,000 xg; 20 minutes), and the resulting supernatant was  
126 stored at -20°C. The 80% DGC fraction cellular pellet was washed in non-  
127 capacitating media (NCM; Supplementary table SI) at 300 xg for 10 minutes,  
128 resuspended in 1ml NCM and incubated at 37°C for immediate use in experiments.

### 129 **Neutrophil Isolation**

130 Blood was collected from volunteers under local ethical approval (13/ES/0091) from  
131 East of Scotland Research Ethics Service (EoSRES) REC 1. Blood was mixed with  
132 Histopaque 1119 (Sigma, UK) at a ratio of 1:1.2 and centrifuged (800 xg; 20  
133 minutes) as previously described (Zambrano, et al., 2016). The cellular phase was  
134 resuspended in phosphate-buffered saline (PBS), centrifuged (300 xg; 10 minutes)  
135 and the pellet was resuspended in PBS. Percoll density gradients were prepared by  
136 sequentially overlaying 2ml 85%, 80%, 75%, 70% and 65%. 2ml of blood suspension  
137 was overlaid and centrifuged (800 xg; 20 minutes) to separate cells by  
138 isopycnic densities. The upper and 65% layers containing mononuclear  
139 peripheral blood cells (monocytes, lymphocytes) were discarded. The 70%, 75% and  
140 80% layers (containing neutrophils) were washed in PBS (10 minutes; 300 g). The  
141 resulting pellet was washed repeatedly in red cell lysis buffer until colourless then

142 resuspended in 1ml supplemented Earle's balanced salt solution (sEBSS) and  
143 incubated at 37°C.

#### 144 **Incubation with AZD5904**

145 Spermatozoa were resuspended in sEBSS solution (25mM sodium bicarbonate,  
146 0.3% BSA; pH 7.4). Three different test conditions were assessed: spermatozoa (S),  
147 spermatozoa and neutrophils (SN; an in-vitro model of OS) and spermatozoa,  
148 neutrophils and 3µM AZD5904 drug (SND). The concentration of AZD5904 was  
149 selected as this represents the expected exposure from therapeutic dose studies  
150 and has been demonstrated to be efficacious in other pre-clinical models  
151 (<https://openinnovation.astrazeneca.com/azd5904.html>). Dimethylsulphoxide  
152 (DMSO; 1% final concentration) was used as vehicle control and had no  
153 independent effect on sperm motility, as previously reported (Martins da Silva, et al.,  
154 2017). Neutrophils were added to spermatozoa at a ratio of 1 ~~neutrophil:~~neutrophil:  
155 3 spermatozoa and activated using 1µg/ml (final concentration) Zymosan  
156 (Invitrogen) as previously described (Munoz-Caro, et al., 2015).

#### 157 **Motility and Kinematics Assessment**

158 Sperm motility (n=45) was assessed using computer assisted sperm assessment  
159 (CASA; Hamilton-Thorne, Beverly, MA, USA). Motility parameters were assessed at  
160 the start of the co-incubation with neutrophils (time 0; T0), after 2 hours (T2) and after  
161 24 hours (T24) for three test conditions, S, SN and SND.

#### 162 **Sperm Penetration Test**

163 The sperm penetration test (n=45) was performed as previously described (Alasmari,  
164 et al., 2013). Briefly, flat capillary tubes (Rectangle Boro Tubing, CM Scientific) were  
165 filled with 1% methylcellulose dissolved in capacitating media (CM; Supplementary



166 Table SI) and one end was blocked using plasticine. The open end was placed into  
167 an Eppendorf containing cells following 2-hour incubation under each experimental  
168 condition (S, SN, SND) and incubated for 1 hour at 37°C, 5% CO<sub>2</sub>. The number of  
169 spermatozoa penetrated to 1cm were counted manually and results were expressed  
170 as a ratio to the control (S). One participant (R3111) submitted two further samples,  
171 which were similarly subjected to experimental conditions and subsequent sperm  
172 penetration test to demonstrate reproducibility of the drug effect. The sperm  
173 penetration test was also performed on a limited number of samples (n=11) where  
174 cells were exposed to extended experimental conditions (S, SN, SND) for 24 hours.  
175 Similar to that described above, the number of spermatozoa penetrated to 1cm after  
176 1 hour incubation at 37°C and 5% CO<sub>2</sub> were counted ~~manually~~manually, and results  
177 were expressed as a ratio to the control (S).

### 178 **Oxygen radical absorbance capacity (ORAC) Assay**

179 Antioxidant capacity in seminal plasma was measured using the ORAC assay.  
180 Trolox (6-hydroxyl-2, 5, 7, 8-tetramethyl-chromane-2-carboxylic acid), a water-  
181 soluble analogue of vitamin E, was used as the antioxidant assay standard. The  
182 assay results are expressed in mmol Trolox equivalent/L. Unfortunately, it was not  
183 technically possible to perform the ORAC assay for all samples in the study. Seminal  
184 plasma samples (n=36) were serially diluted in PBS (1:200 to 1:2000) then added to  
185 a 96-well microplate (Greiner Bio-One Ltd, UK). Fluorescein Sodium (10 nM) was  
186 added to each well containing either Trolox or seminal plasma. FLUOstar OPTIMA  
187 (BMG Labtech) microplate reader was used to assess fluorescence. After 10 cycles,  
188 AAPH (2,2-Azobis(2-amidinopropane) dihydrochloride), a free radical generator, was  
189 added to all wells except those of the negative controls. The run was restarted and  
190 continued for 240 cycles.

## 191 **White Blood Cell Count**

192 Semen slides were stained with May-Grünwald Giemsa (MGG) solution and allowed  
193 to air dry. Two counts were performed, with 200 spermatozoa counted per replicate.  
194 WBC were also counted, and the concentration was calculated for each sample using  
195 the formula (number of WBC/400) x sperm concentration.

## 196 **Flow Cytometry**

197 Malondialdehyde (MDA) was measured as a marker of lipid peroxidation using flow  
198 cytometry. Four conditions were assessed for each sample (n=45): spermatozoa (S),  
199 spermatozoa and neutrophils (SN), spermatozoa, neutrophils and 3 $\mu$ M AZD5904  
200 (SND) and spermatozoa and 4mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; SH) Aliquots were  
201 incubated for 2 hours. An additional aliquot containing only spermatozoa (S) was  
202 used as a secondary antibody control. Aliquots were then incubated (30 minutes;  
203 37°C) with 1:50 anti-MDA antibody (ab27642, Abcam, Cambridge) as previously  
204 described (Moazamian, et al., 2015). The tubes were centrifuged (300 xg; 5 minutes)  
205 and the supernatant discarded. The cells were washed twice with sEBSS and 1:50  
206 fluorescent labelled goat anti-rabbit secondary antibody (Thermofisher, UK) was  
207 added (10 minutes; 37°C). The cells were washed twice further, resuspended in  
208 sEBSS solution, and assessed using flow cytometry (BD LSR Fortessa cell analyser,  
209 BD Biosciences).

## 210 **Statistical Analysis**

211 Statistical analysis was conducted using GraphPad Prism version 8.0.0 (GraphPad  
212 software, San Diego, CA USA).

213

## 214 **Results**

### 215 **Motility and CASA Kinematics**

216 Motility and kinematics were measured at three different time points, time zero (T0), 2  
217 hours (T2) and 24 hours (T24) for each sample (n=45) under three conditions:  
218 spermatozoa only (S), spermatozoa with neutrophils (ratio 3:1) (SN) and spermatozoa  
219 with neutrophils (ration 3:1) plus 3 $\mu$ M AZD5904 (SND). As expected, motility declined  
220 over time, however, there was no significant difference in total and progressive motility  
221 between the experimental and control groups at any time point (Figure 1A and B). The  
222 findings were similar for CASA kinematics (VAP, VSL, VCL, ALH) (data not shown).  
223 According to WHO recommendations (2010), at least 200 cells should be assessed to  
224 allow for an accurate motility count. However, it was not possible to count 200 cells in  
225 some patient samples, either due to low sperm concentration or because the sample  
226 prepared poorly following DGC (n=14). We therefore also present results for samples  
227 with  $\geq$ 200 cells counted (n=31; Supplementary Figure 1A and 1C) and those where  
228  $<$ 200 cells counted (n=14; Supplementary Figure 1B and 1D), although similarly there  
229 was no significant difference in total or progressive motility, or kinematic parameters,  
230 between experimental conditions in these subgroups.

### 231 **Sperm Penetration Test**

232 The sperm penetration test was used to investigate the effect of AZD5904 treatment  
233 on functional motility and ability to penetrate viscous media. Aliquots from each test  
234 condition were measured after 2 hours (n=45) and, where possible, 24 hours  
235 incubation (n=11). Figure 2A shows results after 2 hours incubation. Cells counted are  
236 expressed as a ratio of experimental conditions (neutrophils (SN) or neutrophils and  
237 3 $\mu$ M AZD5904 (SND)) to control (sperm only; S). A difference of 20% was arbitrarily

238 used to indicate a positive or negative effect, based on experience using sperm  
239 penetration assays for drug discovery studies within our research group (Martins da  
240 Silva, et al., 2017, McBrinn, et al., 2019, Tardif, et al., 2014). 3 $\mu$ M AZD5904 had a  
241 positive effect and enhanced sperm penetration in 16/45 (35.6%) samples, whereas  
242 22/45 (48.9%) samples showed no response at 2 hours and 7/45 (15.6%) samples  
243 showed reduced sperm penetration. Overall 3 $\mu$ M AZD5904 resulted in a significant  
244 increase in sperm penetration (Figure 2B and 2C; P=0.003). After 24-hour incubation,  
245 3 $\mu$ M AZD5904 increased sperm penetration in 4/11 (36.4%) samples, whereas 7/11  
246 (63.4%) showed no response at 24 hours, but none showed a negative effect (Figure  
247 2D). A significant overall increase in sperm function was also seen (Figure 2E and 2F;  
248 P=0.039). The results also appeared to be reproducible. One patient submitted a  
249 sample for research on three separate occasions. Although semen characteristics  
250 results were variable, each sample consistently showed improved sperm function with  
251 3 $\mu$ M AZD5904 (Supplementary Figure 2).

252 Andrology and CASA characteristics for patient samples are shown in Table I (n=45).  
253 Clinical data for patients who subsequently attended the fertility clinic for assessment  
254 are shown in Table II (n=28).

### 255 **Oxygen Radical Absorbance Capacity (ORAC) Assay**

256 Results of the ORAC assay are expressed as Trolox equivalents (n=36; Figure 3).  
257 There was no obvious correlation between seminal plasma antioxidant capacity and  
258 sperm penetration assay results.

### 259 **Flow Cytometry**

260 Malondialdehyde (MDA), a metabolite of lipid oxidative damage, is regarded as a  
261 biomarker for OS and was assessed using flow cytometry (n=45; Figure 4). Sperm  
262 treated with 4mM H<sub>2</sub>O<sub>2</sub> (SH) showed significantly higher levels of MDA than all other  
263 conditions tested (P<0.05). Incubation with 3μM AZD5904 (SND) for 2 hours did not  
264 reduce lipid peroxidation compared to the control (S) and MDA was also not  
265 significantly different between the control (S) or neutrophil treatment (SN) groups.

## 266 **Discussion**

267 This pre-clinical study investigated the effect of AZD5904, an MPO inhibitor, on human  
268 sperm. Myeloperoxidase (MPO) converts ROS into other highly reactive oxidants,  
269 including hypochlorous acid (van der Veen, et al., 2009). Although these substances  
270 have potent microbicidal properties, they can also unintentionally damage host cells,  
271 including spermatozoa if present in the ejaculate (Pullar, et al., 2017). We  
272 hypothesised that inhibition of MPO would reduce ROS production and therefore have  
273 beneficial effects on sperm motility and function. Semen samples were surplus to  
274 requirements for clinical andrology and were subject to experimental conditions on the  
275 same day as diagnostic semen analysis. Both normal (n=7; 15.6%) and abnormal  
276 (according to WHO (2010) reference values) sperm samples were included in the  
277 study.

278 Despite prolonged exposure to seminal plasma, DGC preparation resulted in selection  
279 of sperm with improved total and progressive motility prior to exposure to experimental  
280 conditions. As expected, sperm motility in vitro declined over time but there were no  
281 significant differences in total or progressive motility, or kinematics, between control  
282 (S) and spermatozoa with activated neutrophils (SN; an in vitro model of OS) or  
283 activated neutrophils and AZD5904 (SND). However, the sperm penetration assay is

284 arguably a more relevant test compared to in-vitro assessment of sperm motility in  
285 prepared samples. By using 1% ~~methlycellulose~~methylcellulose as a surrogate for  
286 viscous challenges encountered in vivo, including cervical mucus, this assay  
287 evaluates sperm function required for fertilisation and is therefore predictive of  
288 behaviour in vivo (Ola, et al., 2003, Tardif, et al., 2014). After 2 hours, 36% (16/45)  
289 samples showed  $\geq 20\%$  increase in sperm penetration following in-vitro treatment with  
290 AZD5904. Similarly, 36% (4/11) samples showed  $\geq 20\%$  improvement in sperm  
291 penetration following in-vitro treatment with AZD5904 for 24 hours. Importantly, no  
292 samples showed significant negative effects at 24 hours. Given the positive effects on  
293 sperm function following a relatively short exposure to AZD5904, we hypothesise that  
294 there could be more substantial benefits with MPO inhibition throughout  
295 spermatogenesis as neutrophils are widely present in the epididymis, are a source of  
296 OS, and are detrimental to sperm function.

297 Although one third of sperm samples showed an improvement in functional motility,  
298 there was no correlation between baseline diagnostic semen analysis or clinical data  
299 to predict response to AZD5904. However, individual responses appeared to be  
300 consistent. One patient provided a sample for research on three separate occasions.  
301 Despite considerable heterogeneity in his samples, all showed a positive improvement  
302 ( $\geq 20\%$ ) in sperm penetration assay with AZD5904.

303 The ORAC assay was used to assess the antioxidant capacity of seminal plasma for  
304 samples studied. We hypothesised that higher antioxidant capacity would protect  
305 against oxidative stress, and that these samples would be less likely to show a  
306 beneficial effect from MPO inhibition. However, there was no correlation between  
307 ORAC assay results and response to AZD5904.

308 In comparison to other cells, the plasma membrane of spermatozoa has notably high  
309 levels of lipids. The high lipid content is important for bilayer fluidity, which is required  
310 for sperm motility and function, but it is particularly vulnerable to ROS mediated  
311 damage (Alvarez and Storey, 1995, Sanocka and Kurpisz, 2004). Although MDA, a  
312 product of lipid peroxidation, was elevated with exposure to H<sub>2</sub>O<sub>2</sub> (SH) compared to  
313 control (S), there was no significant increase when sperm were co-incubated with  
314 activated neutrophils (SN) for 2 hours, nor a decrease when sperm were co-incubated  
315 with activated neutrophils and AZD5904 (SND) for 2 hours. This was in keeping with  
316 data that showed no significant effect of experimental conditions on sperm motility in  
317 vitro.

318 Many samples in this study had leukocytospermia (n=13, 28.9%) yet there was no  
319 correlation with sperm motility, nor response to AZD5904. This was unexpected. MPO-  
320 mediated conversion of H<sub>2</sub>O<sub>2</sub> triggers a defence strategy in neutrophils: the formation  
321 of neutrophil extracellular traps (NETs) (Sollberger, et al., 2018). Human spermatozoa  
322 trigger the formation of NETs in a dose dependent manner. Sperm motility is  
323 negatively affected by this interaction and NETs are therefore thought to also reduce  
324 fertilisation capability (Zambrano, et al., 2016). Intriguingly, NETs do not form in  
325 patients who are unable to produce MPO (Amulic and Hayes, 2011). Inhibition of MPO  
326 can also significantly decrease spermatozoa-triggered NET formation (Zambrano, at  
327 al., 2016).

328 An alternative mechanism of action could be explained by the role that MPO plays in  
329 nitric oxide (NO) oxidase activity and NO bioavailability. Several data suggest a  
330 relevant role of NO in sperm cell pathophysiology. It is plausible that AZD5904  
331 suppresses NO oxidase activity and increases NO bioavailability, which may be  
332 beneficial to sperm function (Lewis, et al., 1996). Nonetheless, data regarding the

333 role of NO in spermatozoa motility is conflicting, with the suggestion that higher  
334 concentrations are detrimental to sperm motility (Rosselli, et al., 1995). Certainly,  
335 normozoospermic fertile men have been reported to have significantly lower NO  
336 concentrations than those of asthenozoospermic infertile men (Balercia, et al.,  
337 2004). A biphasic effect of NO on sperm motility and function may explain the  
338 variability seen in our results.

339 In conclusion, our results indicate a positive effect of AZD5904 on human sperm  
340 function in vitro. Overall one third of samples showed improvement in sperm function  
341 following drug treatment, although it was not possible to predict which samples would  
342 respond using clinical, andrology or research laboratory data. These results represent  
343 an exciting first step towards a novel therapeutic intervention for male fertility, however  
344 further studies are needed to determine the efficacy of AZD5904 in vivo.

345

#### 346 **Authors' roles**

347 CLRB, AW, HJS and SMDS designed the study and obtained funding. SMDS recruited  
348 and received consent from patients. MC and IES performed the experiments and  
349 statistical analysis of data. SMDS, CLRB, AW and HJS analysed and interpreted the  
350 data. The manuscript was drafted by MC, IES and SMDS. All authors contributed to  
351 the construction, writing and approval of the final manuscript.

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358 (SMDS).

359 **Conflict of interest**

360 AW and HJS are employed by AstraZeneca Open Innovation. SMDS is Associate  
361 Editor of Human Reproduction and Editorial Board member of Reproduction &  
362 Fertility. CLRB is Editor of RBMO and has received lecturing fees from Merck and  
363 Ferring and is on the Scientific Advisory Panel for Ohana BioSciences. CLRB was  
364 chair of the World Health Organization Expert Synthesis Group on Diagnosis of Male  
365 infertility (2012–2016). The other authors declare no conflict of interest.

366 **Data availability**

367 Data available on request from researchers

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370

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373 **Figure Legends**

374 **Figure 1**

375 **Total and progressive motility at time 0 (T0), 2 hours (T2) and 24 hours (T24)**

376 **assessed using CASA.** No significant differences were seen between experimental  
377 conditions: sperm with neutrophils (ratio 3:1) (SN), sperm with neutrophils and 3 $\mu$ M  
378 AZD5904 (SND) and sperm only (S; control). N=45. **A** Total motility (%)  $\pm$  SEM. T0 S  
379 78.5  $\pm$  2.56; SN 75.3  $\pm$  2.73; SND 76.6  $\pm$  2.56. T24 S 47.1  $\pm$  3.95; SN 48.8  $\pm$  3.92;  
380 SND 55.0  $\pm$  3.62. **B** Progressive motility (%)  $\pm$  SEM. T0 S 61.5  $\pm$  3.30; SN 61.6  $\pm$   
381 3.18; SND 59.71  $\pm$  3.21. T24 S 27.0  $\pm$  3.03; SN 30.9  $\pm$  3.19; SND 35.6  $\pm$  3.25.

382 **Figure 2**

383 **Sperm penetration indices and individual sperm penetration results. A** Sperm  
384 penetration index for andrology samples (n=45). Penetration of 1% methylcellulose  
385 (cervical mucus substitute) was assessed following incubation under experimental  
386 conditions for two hours. Number of sperm penetrated to 1cm for paired samples  
387 (sperm with neutrophils (3:1) (SN), sperm with neutrophils and 3 $\mu$ M AZD5904  
388 (SND)) were counted after one hour and expressed as a ratio to control (S). Samples  
389 shaded green showed  $\geq$  20% increase in sperm penetrated in samples treated with  
390 AZD5904 compared to sperm and neutrophils alone (16/45; 35.6%). Samples  
391 shaded pink showed  $\geq$  20% decrease in sperm penetrated in samples treated with  
392 AZD5904 compared to sperm and neutrophils alone (7/45; 15.6%). **B** Individual  
393 sperm penetration results by experimental condition (n=45). Overall, treatment with  
394 AZD5904 (SND) for 2 hours showed significant increase in sperm penetration (\*\*  
395 P=0.003). **C** Linked sperm penetration results for individual semen samples (2  
396 hours). **D** Sperm Penetration Index for andrology samples assessed following

397 incubation for 24 hours (n=11). Number of sperm penetrated to 1cm for paired  
398 samples following 2-hour incubation under experimental conditions (sperm with  
399 neutrophils (3:1) (SN), sperm with neutrophils and 3 $\mu$ M AZD5904 (SND)) were  
400 counted and expressed as a ratio to control (S). Samples shaded green showed  $\geq$   
401 20% increase in sperm penetrated in samples treated with AZD5904 compared to  
402 sperm and neutrophils alone (4/11; 36.4%). **E** Individual sperm penetration results  
403 following 24-hour incubation under experimental conditions (n=11). Treatment with  
404 AZD5904 (SND) for 24 hours showed significant increase in sperm penetration (\*\*  
405 P=0.039). **F** Linked sperm penetration results for individual semen samples (24  
406 hours).

#### 407 **Figure 3**

408 **Measurement of seminal fluid antioxidant capacity using the oxygen radical**  
409 **absorbance capacity (ORAC) assay.** N=36. Trolox was used as antioxidant assay  
410 control and results are expressed as a ~~trolox~~Trolox equivalents. Green bars indicate  
411 samples where AZD5904 had a positive ( $\geq$ 20%) effect on sperm function  
412 (penetration assay). Red bars indicate samples where AZD5904 had a negative ( $\geq$  -  
413 20%) effect on sperm function. Black bars indicate samples where AZD5904 had no  
414 effect on sperm function. There was no apparent correlation between seminal  
415 antioxidant capacity and response to AZD5904 in vitro.

#### 416 **Figure 4**

417 **MDA fluorescence for experimental conditions S, SN, SND and S + 4mM H<sub>2</sub>O<sub>2</sub>**  
418 **(SH).** N=45. Exposure to 4mM H<sub>2</sub>O<sub>2</sub> resulted in significantly higher lipid peroxidation  
419 than other conditions (\*\*P < 0.001). The error bars represent the standard error of  
420 mean (SEM).

421 **Supplementary Figure 1**

422 **Total and progressive motility at time 0 (T0), 2 hours (T2) and 24 hours (T24).**

423 **A** Total motility (%)  $\pm$  SEM where  $\geq 200$  cells were counted (n=31). T0 S  $84.9 \pm 2.00$ ;  
424 SN  $80.5 \pm 2.71$ ; SND  $81.9 \pm 2.03$ . T24 S  $56.5 \pm 4.24$ ; SN  $57.3 \pm 4.56$ ; SND  $60.4 \pm$   
425  $3.88$ . **B** Total motility (%)  $\pm$  SEM where  $< 200$  cells were counted (n=14). T0 S  $64.4 \pm$   
426  $5.26$ ; SN  $63.8 \pm 5.20$ ; SND  $64.8 \pm 5.73$ . T24 S  $26.4 \pm 5.36$ ; SN  $30.2 \pm 4.58$ ; SND  
427  $43.2 \pm 6.86$ . **C** Progressive motility (%)  $\pm$  SEM where  $\geq 200$  cells were counted. T0 S  
428  $66.2 \pm 3.67$ ; SN  $65.0 \pm 3.93$ ; SND  $62.7 \pm 3.77$ . T24 S  $32.1 \pm 3.54$ ; SN  $37.4 \pm 3.84$ ;  
429 SND  $38.6 \pm 3.70$ . **D** Progressive motility (%)  $\pm$  SEM where  $< 200$  cells were counted.  
430 T0 S  $51.1 \pm 5.94$ ; SN  $54.0 \pm 4.79$ ; SND  $53.1 \pm 5.64$ . T24 S  $15.5 \pm 4.46$ ; SN  $16.7 \pm$   
431  $3.41$ ; SND  $28.9 \pm 6.10$ .

432 **Supplementary Figure 2**

433 **Results of sperm penetration assay (2-hour incubation under experimental**  
434 **conditions) from patient R3111, tested on three separate occasions within**  
435 **same spermatogenic cycle.** Although baseline motility and kinematic  
436 characteristics differed for each of the three samples submitted, AZD5904  
437 consistently elicited a positive response ( $\geq 20\%$  increase in sperm penetrated).

438 **Table I**

439 **Diagnostic semen analysis and CASA data for study samples.** N=45. Each  
440 sample was allocated a study research code. Samples that showed increase in  
441 functional motility (sperm penetration) following treatment with AZD5904 are  
442 indicated green. Samples that showed a decrease in functional motility following  
443 treatment with AZD5904 are indicated pink.

444 **Table II**

445 **Clinical data for subset of patients subsequently attending for fertility clinic**  
446 **assessment, including pregnancy outcome after 12 months.** N=28. Samples  
447 that showed increase in functional motility (sperm penetration) following treatment  
448 with AZD5904 are indicated green. Samples that showed a decrease in functional  
449 motility following treatment with AZD5904 are indicated pink.

450 **Supplementary Table I**

451 **Composition of media used in experiments**

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