Myeloperoxidase inhibitor AZD5904 enhances human sperm function in vitro

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Abstract

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

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

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

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

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

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

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

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

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

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

Trial registration number: N/A

Key words

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

Introduction

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

Reactive oxygen species (ROS) are by-products of normal cellular metabolism, and are required for key events in sperm, including fertilisation. However, oxidative stress (OS) arises when levels of ROS overwhelm antioxidant defences (Agarwal, et al., 2006), leading to a spectrum of cellular damage and dysfunction, and ultimately cell death. Whilst male infertility is unexplained in the majority of cases (Wu, et al., 2010), OS is acknowledged to be a common pathology in a significant proportion of infertile men (Aitken, et al., 2010, Tremellen, 2008). The main contributors of ROS in the ejaculate are white blood cells (WBC), mostly neutrophils (Aitken and Fisher, 1994, Plante, et al., 1994). Current theories propose prostate, epididymis or seminal glands as possible origins of WBC, however further studies are needed (Wolff, 1995). Nonetheless, neutrophils are found in the male genital tract and in virtually every ejaculate and can have detrimental effects on human sperm function both in vitro (Aitken and Baker, 2013, Henkel, et al., 2005) and in vivo (Barraud-Lange, et al., 2011).

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

reactive oxygen intermediates, including hypochlorous acid. It also plays a role in inflammation, generating OS and mediating tissue damage. Inhibition of MPO and its downstream inflammatory pathways represents an attractive and logical target for therapeutic intervention for male infertility. AZD5904 is a potent (IC50 140 nM) irreversible inhibitor of MPO (shared by AstraZeneca through their Open Innovation program for clinical and preclinical research). In this study, we investigated the effect of AZD5904 on spermatozoa incubated in the presence of activated neutrophils to create an in-vitro model of OS.

Materials and Methods

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

Semen Sample Preparation

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

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

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

Neutrophil Isolation

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

resuspended in 1ml supplemented Earle's balanced salt solution (sEBSS) and incubated at 37ºC.

Incubation with AZD5904

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

Motility and Kinematics Assessment

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

Sperm Penetration Test

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

Table SI) and one end was blocked using plasticine. The open end was placed into an Eppendorf containing cells following 2-hour incubation under each experimental condition (S, SN, SND) and incubated for 1 hour at 37ºC, 5% CO_2. The number of spermatozoa penetrated to 1cm were counted manually and results were expressed as a ratio to the control (S). One participant (R3111) submitted two further samples, which were similarly subjected to experimental conditions and subsequent sperm penetration test to demonstrate reproducibility of the drug effect. The sperm penetration test was also performed on a limited number of samples (n=11) where cells were exposed to extended experimental conditions (S, SN, SND) for 24 hours. Similar to that described above, the number of spermatozoa penetrated to 1cm after 1 hour incubation at 37ºC and 5% CO_2 were counted ~~manually~~manually, and results were expressed as a ratio to the control (S).

Oxygen radical absorbance capacity (ORAC) Assay

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

White Blood Cell Count

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

Flow Cytometry

Malondialdehyde (MDA) was measured as a marker of lipid peroxidation using flow cytometry. Four conditions were assessed for each sample (n=45): spermatozoa (S), spermatozoa and neutrophils (SN), spermatozoa, neutrophils and 3μM AZD5904 (SND) and spermatozoa and 4mM hydrogen peroxide (H_2O_2; SH) Aliquots were incubated for 2 hours. An additional aliquot containing only spermatozoa (S) was used as a secondary antibody control. Aliquots were then incubated (30 minutes; 37ºC) with 1:50 anti-MDA antibody (ab27642, Abcam, Cambridge) as previously described (Moazamian, et al., 2015). The tubes were centrifuged (300 xg; 5 minutes) and the supernatant discarded. The cells were washed twice with sEBSS and 1:50 fluorescent labelled goat anti-rabbit secondary antibody (Thermofisher, UK) was added (10 minutes; 37ºC). The cells were washed twice further, resuspended in sEBSS solution, and assessed using flow cytometry (BD LSR Fortessa cell analyser, BD Biosciences).

Statistical Analysis

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

Results

Motility and CASA Kinematics

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

Sperm Penetration Test

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

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

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

Oxygen Radical Absorbance Capacity (ORAC) Assay

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

Flow Cytometry

Malondialdehyde (MDA), a metabolite of lipid oxidative damage, is regarded as a biomarker for OS and was assessed using flow cytometry (n=45; Figure 4). Sperm treated with 4mM H_2O_2 (SH) showed significantly higher levels of MDA than all other conditions tested ($P<0.05$). Incubation with 3μM AZD5904 (SND) for 2 hours did not reduce lipid peroxidation compared to the control (S) and MDA was also not significantly different between the control (S) or neutrophil treatment (SN) groups.

Discussion

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

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

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

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

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

In comparison to other cells, the plasma membrane of spermatozoa has notably high levels of lipids. The high lipid content is important for bilayer fluidity, which is required for sperm motility and function, but it is particularly vulnerable to ROS mediated damage (Alvarez and Storey, 1995, Sanocka and Kurpisz, 2004). Although MDA, a product of lipid peroxidation, was elevated with exposure to H_2O_2 (SH) compared to control (S), there was no significant increase when sperm were co-incubated with activated neutrophils (SN) for 2 hours, nor a decrease when sperm were co-incubated with activated neutrophils and AZD5904 (SND) for 2 hours. This was in keeping with data that showed no significant effect of experimental conditions on sperm motility in vitro.

Many samples in this study had leukocytospermia (n=13, 28.9%) yet there was no correlation with sperm motility, nor response to AZD5904. This was unexpected. MPO-mediated conversion of H_2O_2 triggers a defence strategy in neutrophils: the formation of neutrophil extracellular traps (NETs) (Sollberger, et al., 2018). Human spermatozoa trigger the formation of NETs in a dose dependent manner. Sperm motility is negatively affected by this interaction and NETs are therefore thought to also reduce fertilisation capability (Zambrano, et al., 2016). Intriguingly, NETs do not form in patients who are unable to produce MPO (Amulic and Hayes, 2011). Inhibition of MPO can also significantly decrease spermatozoa-triggered NET formation (Zambrano, at al., 2016).

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

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

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

Authors' roles

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

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Conflict of interest

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

Data availability

Data available on request from researchers

Figure Legends

Figure 1

Total and progressive motility at time 0 (T0), 2 hours (T2) and 24 hours (T24) assessed using CASA. No significant differences were seen between experimental conditions: sperm with neutrophils (ratio 3:1) (SN), sperm with neutrophils and 3µM AZD5904 (SND) and sperm only (S; control). N=45. **A** Total motility (%) ± SEM. T0 S 78.5 ± 2.56; SN 75.3 ± 2.73; SND 76.6 ± 2.56. T24 S 47.1 ± 3.95; SN 48.8 ± 3.92; SND 55.0 ± 3.62. **B** Progressive motility (%) ± SEM. T0 S 61.5 ± 3.30; SN 61.6 ± 3.18; SND 59.71 ± 3.21. T24 S 27.0 ± 3.03; SN 30.9 ± 3.19; SND 35.6 ± 3.25.

Figure 2

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

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

Figure 3

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

Figure 4

MDA fluorescence for experimental conditions S, SN, SND and S + 4mM H_2O_2 (SH). N=45. Exposure to 4mM H_2O_2 resulted in significantly higher lipid peroxidation than other conditions (***P< 0.001). The error bars represent the standard error of mean (SEM).

Supplementary Figure 1

Total and progressive motility at time 0 (T0), 2 hours (T2) and 24 hours (T24). **A** Total motility (%) ± SEM where ≥ 200 cells were counted (n=31). T0 S 84.9 ± 2.00; SN 80.5 ± 2.71; SND 81.9 ± 2.03. T24 S 56.5 ± 4.24; SN 57.3 ± 4.56; SND 60.4 ± 3.88. **B** Total motility (%) ± SEM where < 200 cells were counted (n=14). T0 S 64.4 ± 5.26; SN 63.8 ± 5.20; SND 64.8 ± 5.73. T24 S 26.4 ± 5.36; SN 30.2 ± 4.58; SND 43.2 ± 6.86. **C** Progressive motility (%) ± SEM where ≥ 200 cells were counted. T0 S 66.2 ± 3.67; SN 65.0 ± 3.93; SND 62.7 ± 3.77. T24 S 32.1 ± 3.54; SN 37.4 ± 3.84; SND 38.6 ± 3.70. **D** Progressive motility (%) ± SEM where < 200 cells were counted. T0 S 51.1 ± 5.94; SN 54.0 ± 4.79; SND 53.1 ± 5.64. T24 S 15.5 ± 4.46; SN 16.7 ± 3.41; SND 28.9 ± 6.10.

Supplementary Figure 2

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

Table I

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

Table II

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

Supplementary Table I

Composition of media used in experiments

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