Low amounts of mitochondrial reactive oxygen species define human sperm quality

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Abstract

We have applied the mitochondria-specific superoxide fluorescent probe MitoSOX Red (MitoSOX) to detect mitochondria-specific reactive oxygen species (mROS) production in human sperm samples using flow cytometry. We show that human ejaculates are heterogeneous in terms of mROS production, with three subpopulations clearly detectable, comprising sperm that produce increasing amounts of mROS (MitoSOX–, MitoSOX+, and MitoSOX++). The sperm subpopulation producing the lowest amount of mROS represented the most functional subset of male gametes within the ejaculate, as it was correlated with the highest amount of live and non-apoptotic sperm and increased both in samples with better semen parameters and in samples processed by both density-gradient centrifugation and swim-up, both known to select for higher quality sperm. Importantly, the MitoSOX– subpopulation was clearly more prevalent in samples that gave rise to pregnancies following assisted reproduction. Our work, therefore, not only describes human sperm heterogeneity at the mROS level but also suggests that mROS may represent a strategy to both evaluate sperm samples and isolate the most functional gametes for assisted reproduction.

Free Portuguese abstract

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Introduction

The human ejaculate is a very heterogeneous biological mixture containing subpopulations of sperm with distinct properties and functional abilities (Sousa *et al.* 2011). Several properties are thought to be needed for a functional mammalian sperm to achieve fertilization, including a morphologically normal structure, motility or the ability to undergo important events such as capacitation and the acrosome reaction, for example (Amaral *et al.* 2013a). Discrete levels of reactive oxygen species (ROS) have also been proposed as players in the activation of male gamete.

ROS are important in terms of both cell signaling and pathology, and mitochondria-produced ROS (mROS) are especially known to have major roles (reviewed by Sena & Chandel (2012)). In mammalian sperm, the endogenous production of mROS has been linked with the activity of mitochondrial respiratory chain complexes I and III (Koppers *et al.* 2008), although the

male gamete may have other ROS-producing sources such as NADPH oxidase (Musset *et al.* 2012). Furthermore, the presence of excessive amounts of leukocytes in the semen seems to be responsible for sperm contact with exogenous (leukocyte-produced) ROS (Henkel 2011). In general, both intracellular and extracellular ROS have been associated not only with sperm damage, such as apoptosis, but also with a positive role in terms of cell functionality (i.e. capacitation; de Lamirande & Lamothe 2009, Aitken & Koppers 2011, Tvrdá *et al.* 2011). This difference depends on issues of timing and amount of ROS production.

ROS levels can be correlated with human semen quality, as non-normozoospermic samples seem to have higher amounts than normozoospermic samples (Li *et al.* 2012), and there is a relationship between poor semen parameters and ROS levels (Wang *et al.* 2003, Allamaneni *et al.* 2005, Ramya *et al.* 2010). However, other studies failed to confirm these findings

(Zorn *et al.* 2010). Moreover, there is an association between semen ROS levels and male infertility (Agarwal *et al.* 2003, 2006) and ROS levels could be correlated with idiopathic male infertility, suggesting a possible diagnostic application (Dohle *et al.* 2005, Agarwal *et al.* 2006). Concomitantly, other studies have shown a negative relationship between semen ROS levels and embryo development/pregnancy (Zorn *et al.* 2003).

Given the heterogeneity of human sperm samples, it would be of interest to determine whether ROS-related properties vary within the population. In fact, mitochondrial function, specifically mitochondrial membrane potential, has been proposed to distinguish better-quality gametes (Sousa *et al.* 2011). Considering that mitochondria are also the main source of cellular ROS, we thus attempted to identify and characterize distinct human sperm subpopulations based on mROS content and to correlate this data with sperm functionality.

Materials and methods

Reagents and media

All reagents were from Sigma–Aldrich and all probes were from Molecular Probes/Invitrogen (Eugene, OR, USA), unless otherwise stated. Several sperm suspension media were used: PBS–glucose–BSA, sperm preparation medium (SPM), simple PBS, and HEPES buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH=7.4).

SPM (Medicult-Origio, Jyllinge, Denmark) is a commercial medium routinely used for sperm preparation prior to its use in assisted reproductive technologies (ART). PBS–glucose–BSA is PBS supplemented with 0.9 mM CaCl₂, 5 mM glucose, 0.5 mM MgCl₂, 3% (w/v) BSA, and 1% (v/v) Gibco penicillin/streptomycin, pH=7.2 (Amaral *et al.* 2011).

Sperm sample collection and processing

Patients undergoing routine semen analysis or fertility treatment were recruited from the Fertility Clinic (University Hospitals of Coimbra, Portugal). All 100 patients signed informed consent forms, and all human material was used in accordance with the appropriate ethical and Internal Review Board (IRB) guidelines provided by the University Hospitals of Coimbra. Fresh semen samples were obtained by masturbation after 3–5 days of sexual abstinence and seminal analysis was carried out in conformity to the World Health Organization Guidelines (WHO 2010). All samples used were analyzed as described by WHO guidelines (WHO 2010), including parameters such as pH, volume, concentration, motility, morphology, and presence or absence of leukocytes. As detailed later on, both samples that had and that lacked leukocytes were used, and analyses were performed on distinct stages of sperm preparation.

In some experiments, the initial ejaculate, or native semen sample (without any conventional treatment), was directly used after two washes with PBS (centrifugation at 800 *g* for 5 min) as described (WHO 2010). This pellet was then resuspended in SPM at room temperature for all other experiments. In other assays,

native semen samples (without any PBS wash) were further processed by density-gradient centrifugation (DGC), with a 10-min centrifugation at 800 *g*, using a specific medium (ISolate, Irvine Scientific, Saint Ana, CA, USA) and the isolated fraction was resuspended in SPM. In addition, in some experiments, swim-up was performed following DGC. In this case, the pellet obtained by DGC was gently covered with SPM and incubated at 37 °C for at least 30 min to allow motile sperm to spontaneously migrate into the overlaying media, which was then carefully removed, leaving behind non-migrated cells (Amaral *et al.* 2007, Sousa *et al.* 2011, Baptista *et al.* 2013). In experiments with native semen samples we performed a PBS wash only. Regarding the processed samples, for those after DGC the only treatment carried out was DGC; and for those obtained by the swim-up method we performed DGC and swim-up, sequentially (processed samples were not washed with PBS). We used a total of 44 unprocessed samples and 99 processed samples (84 after density-gradient centrifugation and 15 after swim-up). To control for possible differences in sperm viability following different sperm preparation techniques, the eosin/nigrosin assay was used (WHO 2010). Briefly, 10 µl sperm suspensions were sequentially mixed with 10 µl of 1% (w/v) eosin for 30 s and then with 10 µl of 10% (w/v) nigrosin for another 30 s. A smear was then prepared and 200 sperm were counted using a bright field microscope and the percentages of non-viable (eosin-stained) and viable sperm were assessed.

Flow cytometry

For all flow cytometry experiments, a cell suspension with 5 million sperm/ml (final volume of 300 µl) was analyzed using a BD FACS Canto II (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Fifty thousand cells were acquired in each experimental condition using the FACS DIVA (Becton Dickinson) Software and the results were processed in the Infinicyt V1.5 (Infinicyt, CytoSgnos, Salamanca, Spain) Software. Control experiments were carried out using cell sorting to ensure that only sperm (and not debris, leucocytes or other round cells; Fig. 1) was being gated and analyzed in all assays, as described previously (Sousa *et al.* 2011).

To detect mROS, MitoSOX Red (MitoSOX), a specific fluorescent probe for superoxide produced by mitochondria, was used as described previously (Amaral *et al.* 2013b, Sousa *et al.* 2013). The MitoSOX Red probe is composed of dihydroethidium, which only reacts with superoxide anion, coupled with and triphenylphosphonium cation that directs the probe to mitochondria. The reaction product exhibits fluorescence after binding DNA (Aitken *et al.* 2013). All samples were washed with PBS and centrifuged at 800 *g* for 5 min. Then, they were incubated with MitoSOX Red at a final concentration of 3 µM and in a final volume of 300 µl medium (according to different experimental conditions different media were used), for 15 min at 37 °C in the dark. At the end of this incubation the cell suspension was washed and analyzed. As a positive control, antimycin A (80 µM final concentration) was incubated simultaneously with MitoSOX Red. This condition is known to greatly increase the production of mitochondrial ROS (Amaral *et al.* 2013b). MitoSOX Red fluorescence was

Figure 1 Flow cytometry sperm gating. A representative dot plot obtained to gate sperm using logarithm of side and forward scatter. Round cells such as leukocytes and other debris were excluded.

determined using the blue argon laser and emitted fluorescence was detected with a bandpass filter of 585/42 nm.

To determine the percentage of viable cells in flow cytometry experiments, the fluorescent dye SYTOX Green was used, as described previously (Varum *et al.* 2007). This probe stains (non-viable) sperm with a damaged plasma membrane. In this case, 0.1 μ l of SYTOX Green diluted with HEPES buffer was added for each 100 μ l sperm suspension (final concentration 0.5 nM). Samples were incubated at room temperature for 15 min in the dark. Before and after the incubation with the probe, the sample was washed with PBS and centrifuged at 800 *g* for 5 min. As a positive control, samples were fixed to induce cell death, using a solution composed of PBS with formaldehyde (2% v/v; pH = 7.2), for 40 min. SYTOX Green fluorescence was detected using a 488 nm excitation laser to detect the FITC fluorophore, and with a bandpass filter of 530/30 nm.

Sperm apoptosis was assessed using FITC-labeled annexin V (Immunostep, Salamanca, Spain), a calcium-dependent protein with affinity for phosphatidylserine. In intact cells, this probe thus detects the externalization of phosphatidylserine to the outer leaflet of the sperm plasma membrane, a well-known apoptotic event. As previously described (Varum *et al.* 2007), samples were washed twice with PBS and the pellet was resuspended in HEPES buffer. For each 100 μ l cell suspension, 5 μ l annexin V solution were added and the mixture was incubated for 15 min at room temperature in the dark, as noted in the manufacturer's instructions. Sperm were then washed with HEPES buffer (800 *g* for 5 min) and the pellet resuspended in HEPES buffer before flow cytometry analysis, using the same settings as described above for SYTOX Green. As a positive control, apoptosis was induced with hydrogen peroxide (1 mM, at 37 $^{\circ}$ C, for 1 h) prior to annexin V labeling (Varum *et al.* 2007).

Statistical analysis

Statistical analysis was performed using the IBM SPSS 20 Software. All variables were checked for normal distribution through the one-sample Kolmogorov–Smirnov test. For two group comparisons, and depending on whether the data had a normal or non-normal distribution, we used either the *t*-test or the Mann–Whitney *U* non-parametric test respectively. Pearson's coefficient was calculated to correlate different sets of experiments when data had a normal distribution, while Spearman's coefficient was calculated in all other cases. To analyze data related to patients, we used Fisher's exact test. Statistical significance was considered when $P \leq 0.05$. All data are presented as mean $\pm$ s.d.

Results

MitoSOX subpopulations in human sperm: relationship with apoptotic and viable cells

In most human sperm samples (both native and prepared), flow cytometry MitoSOX analysis suggested the existence of three clearly distinct subpopulations, a major subpopulation composed of sperm that did not bind the probe (MitoSOX $^{-}$), and two with increasing amounts of fluorescence (MitoSOX $^{+}$ and MitoSOX $^{++}$; Fig. 2A). Of all the samples analyzed, 68% followed this pattern, while in 14% only two subpopulations were obvious (MitoSOX $^{-}$ and MitoSOX $^{+}$), and in 18%, a minor MitoSOX $^{+++}$ subpopulation was detected. As predicted, positive control experiments using antimycin A, a mitochondrial inhibitor of complex III known to cause an increase in mROS, led to an increase in the MitoSOX $^{+}$ subpopulations, with the concomitant disappearance of the MitoSOX $^{-}$ subpopulation (Fig. 2B).

Figure 2 MitoSOX subpopulations in human sperm. Representative examples of histograms obtained after flow cytometry analysis of samples incubated with MitoSOX Red (A) and with MitoSOX Red plus antimycin A (positive control; B). Three different subpopulations are clearly obtained: MitoSOX $^{-}$ (1), MitoSOX $^{+}$ (2), and MitoSOX $^{++}$ (3). Adding antimycin A results in the disappearance of the MitoSOX $^{-}$ subpopulation, with concomitant increases in the other two subpopulations.

Table 1 Correlations obtained between sperm subpopulations based on their mROS content and viable and apoptotic cells assayed in the same samples^a.

MitoSOX –	MitoSOX +	MitoSOX ++
Live cells		
$r=0.758$	–	$r=-0.775$
*** $P<0.001$		*** $P<0.001$
$n=23$		$n=23$
Apoptotic cells		
$r=-0.843$	$r=0.436$	$r=0.554$
*** $P<0.001$	* $P<0.05$	** $P<0.01$
$n=26$	$n=26$	$n=26$

^aMitoSOX, annexin V (apoptotic sperm), and SYTOX Green (non-viable sperm) assays were carried out by flow cytometry, as described in the 'Materials and methods' section.

When correlating the different amounts of sperm (after DGC) belonging to the distinct MitoSOX subpopulations with the number of apoptotic (annexin V-positive) and viable cells (SYTOX Green-negative) in the same samples, clear relationships were evident (Table 1). Indeed, the presence of MitoSOX – sperm was correlated with both the presence of live and especially of non-apoptotic sperm, while correlations were reversed for the MitoSOX ++ subpopulation and weak or non-existent for the MitoSOX + subpopulation (Table 1). These data seem to suggest that the MitoSOX – subpopulation represents more functional male gametes in a given sample.

MitoSOX subpopulations and leukocytospermia

In human semen, both the male gamete and leukocytes present in the ejaculate can contribute toward ROS formation. To determine whether the presence of leukocytes in the ejaculate changed sperm ROS production as monitored by MitoSOX, we compared normozoospermic samples (i.e. sperm samples classified as normal using WHO criteria) containing large quantities of leukocytes (leukocytospermia, $n=30$) with normozoospermic samples lacking leukocytes ($n=61$). Importantly, as stated in the 'Materials and methods' section, only sperm were gated and analyzed in all these experiments, and no differences were found between samples with or without leukocytes, i.e. the presence of leukocytes in the ejaculate did not change the number of sperm in the three distinct MitoSOX subpopulations detected ($P>0.05$, data not shown). This was true whether the initial native semen samples were considered, or if samples were analyzed after density-gradient centrifugation (DGC; data not shown).

MitoSOX subpopulations in human sperm samples with distinct initial quality

To determine whether MitoSOX subpopulations are related to distinct sperm quality parameters, we first decided to determine whether there were differences

between normozoospermic samples and samples that would not be classified as normal using WHO criteria. Interestingly, normozoospermic and non-normozoospermic semen samples were not statistically distinct if the initial unprocessed semen samples were considered (Fig. 3A), but normozoospermic samples had a higher percentage of the MitoSOX – subpopulation (and a lower percentage of the MitoSOX ++ subpopulation) after sperm processing for ART (Fig. 3B).

MitoSOX subpopulations in native and prepared human sperm samples

To further determine the possible relevance of MitoSOX subpopulations within normal samples, we evaluated their prevalence in both native normozoospermic semen samples, and in the same samples following processing by DGC and swim-up, as usually carried out to isolate more functional male gametes for ART. As shown (Fig. 4). The MitoSOX – subpopulation was very significantly increased following both sperm preparation techniques, at the expense of either the MitoSOX ++ (DGC) or MitoSOX + (swim-up) subpopulations.

Figure 3 MitoSOX subpopulations in normozoospermic and non-normozoospermic human sperm samples. Sperm subpopulations (MitoSOX –, MitoSOX +, and MitoSOX ++) were quantified in both normozoospermic ($n=61$) and non-normozoospermic ($n=20$) human sperm samples, both in native semen samples (A) one-sample Kolmogorov–Smirnov test and t -test were performed, and in samples following processing by density-gradient centrifugation (DGC) (B) one-sample Kolmogorov–Smirnov test and t -test were performed. Data are presented as mean $\pm$ s.d. ** $P\leq 0.01$.

Figure 4 MitoSOX subpopulations in native and processed human sperm. The three different sperm subpopulations previously identified according to MitoSOX reactivity (MitoSOX⁻, MitoSOX⁺, and MitoSOX⁺⁺) were quantified in native semen samples, and in those same samples following processing by (A) density-gradient centrifugation (DGC, $n=13$; one-sample Kolmogorov–Smirnov test and t -test were performed) and (B) swim-up ($n=15$; one-sample Kolmogorov–Smirnov test and t -test were performed). Data are presented as mean $\pm$ s.d. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

Correlation of pregnancy with sperm MitoSOX subpopulations

Finally, we attempted to determine whether the different MitoSOX subpopulations might be correlated with ART success, and thus with the achievement of pregnancy. As shown in Fig. 5, a positive immunological pregnancy test (IPT⁺) result was significantly associated with a higher MitoSOX⁻ subpopulation, and with lower amounts of MitoSOX⁺⁺ subpopulation. Patient data are presented in Table 2, and it is noteworthy that the pregnant and non-pregnant groups were otherwise statistically indistinguishable when clinically relevant parameters were considered. We considered a total of 26 patients for which the infertility cause could be female, male, both, or idiopathic. In our population, the most prominent female infertility cause was tubal factor and, after the female factor, idiopathic infertility is the most common cause listed. In these patients, IVF was performed more often than ICSI, but this is true for both IPT⁻ and IPT⁺. In our population one to three embryos were transferred, with an average of two embryos for IPT⁺ and 1.88 for IPT⁻. For all the parameters described above, we found no statistically significant differences between populations. We also checked whether there was any

difference according to the age of the patient (female or male) that could be related to pregnancy success, and again this was not the case (Table 2).

Discussion

Both pathological and physiological effects have been described for ROS in sperm. In this study, the goal was to determine whether endogenous ROS levels, and particularly mROS, could identify sperm subpopulations with distinct characteristics.

We determined that most human sperm samples comprised three subpopulations, as defined using the fluorescent probe for mROS MitoSOX Red and flow cytometry analysis. When simultaneously considering apoptotic markers and viability, the MitoSOX⁻ subpopulation (sperm that showed little or no probe staining) included better-quality sperm, while the increase in mROS levels in the MitoSOX⁺ and MitoSOX⁺⁺ subpopulations resulted in progressively worse sperm characteristics. The relationship between sperm mROS levels and apoptosis is known (Koppers *et al.* 2011, Aitken *et al.* 2012a), as is the connection between ROS levels and sperm viability (Marchetti *et al.* 2002, Espinoza *et al.* 2009). Furthermore, the link between mROS production and apoptosis is recognized in other cell types (Sinha *et al.* 2013), although the particular compartmentalization of sperm cell might result in different types of cellular damage (Aitken *et al.* 2012b).

The presence of leukocytes in a semen sample has been correlated with sperm damage, and this is thought to be caused by ROS production in the white blood cells

Figure 5 MitoSOX subpopulations in sperm samples and pregnancy results. Sperm subpopulations (MitoSOX⁻, MitoSOX⁺, and MitoSOX⁺⁺) were quantified in human sperm samples used in assisted reproduction technologies (ART) that gave rise to a positive immunological pregnancy test (IPT⁺, $n=9$) and that did not result in pregnancy (IPT⁻, $n=17$). Data are presented as mean $\pm$ s.d. * $P \leq 0.05$ and ** $P \leq 0.01$. One-sample Kolmogorov–Smirnov test and t -test were performed.

Table 2 Pregnancy data for samples used in this study: patient age, number of embryos transferred (mean $\pm$ s.d.), cause of infertility, primary or secondary sterility and technique performed (number of cases).

	IPT+	IPT–	P value
Number of patients	9	17	–
Age			
Female	35.22 $\pm$ 3.23	35.23 $\pm$ 2.84	0.922
Male	38.66 $\pm$ 5.36	35.58 $\pm$ 3.82	0.102
Number of transferred embryo	2 $\pm$ 0.60	1.88 $\pm$ 0.70	0.659
Infertility cause			0.574
Male factor	1	2	–
Female factor	2	8	–
Male and female factor	1	2	–
Idiopathic	5	5	–
Type of infertility			0.357
Primary	1	11	–
Secondary	8	6	–
ART type			0.664
IVF	7	11	–
ICSI	2	6	–

IPT+, immunological pregnancy test positive; IPT–, immunological pregnancy test negative; ART, assisted reproductive technique.

(Henkel 2011). Although some groups reported that there is a relationship between endogenous sperm ROS levels and an excess of leukocytes (Henkel *et al.* 2005, Mupfiga *et al.* 2013), we found no difference between leukocytospermic samples and non-leukocytospermic samples regarding mROS levels both in the native sample and after density gradient centrifugation, as also reported by others (Henkel *et al.* 2010), suggesting that the presence of leukocytes in the semen does not seem to influence endogenous sperm production of mROS.

In terms of classic sperm parameters (using WHO standards), non-normozoospermic samples had a lower percentage of sperm in the MitoSOX– subpopulation than normozoospermic samples, if analysis was carried out following density gradient centrifugation. Conversely, the MitoSOX++ subpopulation was increased in non-normozoospermic samples. Other groups have found relationships between ROS levels and sperm quality or alterations in sperm parameters, including motility, morphology, and concentration (Wang *et al.* 2003, Henkel *et al.* 2005, Cocuzza *et al.* 2008, Aitken *et al.* 2012c, Li *et al.* 2012, Ferramosca *et al.* 2013). Conventional processing techniques used to prepare sperm for ART are often associated with an induction of sperm ROS, likely due to both the centrifugation itself and the removal of putative semen antioxidants (Agarwal *et al.* 1994a, Thilagavathi *et al.* 2012). We here show that prepared samples had a higher percentage of sperm in the MitoSOX– subpopulation and a lower percentage of MitoSOX++ sperm. These differences, both between normozoospermic and non-normozoospermic samples, and samples before and after centrifugation, might result from the removal of dead cells (Larson *et al.* 1999).

Indeed, others have also described a decrease in superoxide anion using this procedure, although an increase in hydrogen peroxide levels was also found (Mahfouz *et al.* 2010).

On the other hand, when comparing native sample with the migrated subpopulation obtained after swim-up, which we have previously shown to be beneficial to isolate better-quality sperm in terms of capacitation and chromatin integrity (Sousa *et al.* 2011, Tavares *et al.* 2013), we also detect a higher percentage of sperm in the MitoSOX– subpopulation relative to the native sample. Interestingly, with this methodology the subpopulation affected in the opposite direction was the MitoSOX+ subpopulation, which tells us that the swim-up method is suitable for more subtly isolating a subpopulation with lower ROS, given that the MitoSOX++ subpopulation is already greatly decreased prior to performing swim-up separation (Agarwal *et al.* 1994b). Migrated samples also predictably had higher viability than native samples, although there was no statistically significant difference in terms of apoptosis.

Finally, sperm ROS levels have been suggested to play a role in infertility (Pasqualotto *et al.* 2001, Agarwal *et al.* 2003, Venkatesh *et al.* 2011), and we obtained statistically significant differences between samples that did or did not give rise to pregnancy following ART in terms of mROS. In agreement with the data discussed so far, successful outcomes could be linked to samples with a higher percentage of sperm with lower mROS. Although others have reported no difference in ROS levels between samples that give rise to pregnancy and samples that did not, but differences were found if a pre-established ROS level cut-off value was disregarded (Zorn *et al.* 2003). To exclude other parameters that could be related to pregnancy success, we also evaluated male and female age, the type and cause of infertility, the technique performed, and the number of embryos transferred. We did not find any association between these parameters and pregnancy results, suggesting that in this case of the parameters monitored only sperm mROS are influencing pregnancy.

In summary, we show that human sperm samples are heterogeneous in terms of mROS generated and can be divided into discreet subpopulations. Moreover, the subpopulation with lower amounts of mROS (MitoSOX–) consistently seemed to include better-quality sperm, whatever the criteria used, from functional markers to issues related to sample preparation and ultimately including the establishment of pregnancy following ART. Given that ROS are known to play a role in sperm function, it seems odd that the subpopulation with better quality sperm is the one negative for mROS. However, it is possible that the chosen marker is not sensitive enough to detect basal levels of mitochondrial superoxide required or that basal ROS are obtained via other sources, such as NADPH oxidase, with increasing levels of mROS (as monitored with this particular

probe/assay) representing the pathological amounts known to adversely affect sperm function. More studies with a larger patient cohort and distinct methodologies are required to clarify the role of ROS in sperm (both mitochondrial and non-mitochondrial, both endogenous and exogenous), for example determining whether distinct populations are related to distinct levels of sperm capacitation. The establishment of possible cut-off values that may allow for the distinction between basal physiological levels from pathological levels might also be possible. Regardless, this study shows that mROS can identify a better-quality sperm subpopulation within a human ejaculate and that the endogenous mROS levels are related to sperm quality and ART outcomes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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