LOW AMOUNTS OF MITOCHONDRIAL REACTIVE OXYGEN SPECIES
DEFINE HUMAN SPERM QUALITY

Running title: Mitochondrial ROS and human sperm function

Summary Sentence: Characterization of human sperm ejaculates with a mitochondrial-specific ROS probe reveals distinct subpopulations of gametes, with the cells with the lowest amount of ROS possessing the highest functional attributes.

Keywords: sperm, human, reactive oxygen species, mitochondria, fertility

Mónica Marques\(^1\), Ana Paula Sousa\(^1\), Artur Paiva\(^3\), Teresa Almeida-Santos\(^2\), João Ramalho-Santos\(^1\)*

\(^1\)Biology of Reproduction and Stem Cell Group, CNC- Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal,
\(^2\)Human Reproduction Service, University Hospitals of Coimbra, Pr. Mota Pinto, Ed. São Jerónimo 3000-075 Coimbra, Portugal
\(^3\)Portuguese Institute for Blood and Transplantation, Coimbra, Portugal.
\(^4\)Faculty of Medicine, University of Coimbra, Portugal
\(^5\)Department of Life Sciences, University of Coimbra, 3001-401 Coimbra, Portugal

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* Correspondence: João Ramalho-Santos, Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal, Tel: 351239-855 760, Fax: +351(239)-855 789, E-mail: jramalho@ci.uc.pt
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, comprised of sperm that produce increasing amounts of mROS (MitoSOX−, MitoSOX+, 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 works therefore not only describes discreet 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.

PORTUGUESE ABSTRACT
O presente estudo baseia-se na marcação de espermatozoides humanos com um marcador específico, o MitoSOX™ Red (MitoSOX), para espécies reativas de oxigénio mitocondriais (mROS) e posterior análise da marcação por citometria de fluxo. Numa primeira análise mostrámos que o ejaculado humano é heterogéneo no
que respeita à produção de mROS, dado que obtivemos três subpopulações distintas
de espermatozoides que diferem no conteúdo em mROS, designadas por MitoSOX-,
MitoSOX+ e MitoSOX++. Ao estudarmos estas subpopulações concluímos que a
subpopulação de espermatozoides com menor produção de mROS é a que está
correlacionada com gâmetas mais funcionais, nomeadamente com a viabilidade, com
a percentagem de espermatozoides não apoptóticos, com melhores parâmetros
seminais, e com a seleção de gâmetas mais funcionais realizada através de
processamento das amostras para aplicação em técnicas de procriação medicamente
assistida (PMA); nomeadamente centrifugação por gradiente de densidade e swim-up.
Verificámos também que nas amostras que deram origem a gravidez a maior
percentagem de espermatozoides pertence à subpopulação MitoSOX-. Por
conseguinte, o nosso estudo não só demonstra a heterogeneidade do ejaculado
humano em termos de mROS, mas também mostra que estes níveis de mROS podem
ser utilizados para avaliar as amostras de esperma, e para isolar subpopulações de
espermatozoides com melhor qualidade.
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 the male gamete.

ROS are important in term of both cell signaling and pathology, and mitochondria-produced ROS (mROS) are especially known to have major roles (reviewed by (Sena and 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 and Lamothe, 2009; Aitken and Koppers, 2011; Tvrda 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, Agarwal et al. 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 if ROS-related properties vary within the population. In fact, mitochondrial function, specifically mitochondrial membrane potential (MMP), 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.
MATERIAL AND METHODS

Reagents & Media

All reagents were from Sigma-Aldrich (St. Louis, Missouri, USA) and all probes were from Molecular Probes/Invitrogen (Eugene, Oregon, USA), unless otherwise stated. Several sperm suspension media were used: PBS-Glucose-BSA, sperm preparation medium, simple phosphate buffered saline (PBS) and HEPES buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl$_2$, pH=7.4)

Sperm preparation medium (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 CaCl$_2$, 5mM glucose, 0.5mM MgCl$_2$, 3% (w/v) bovine serum albumin (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 to 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 800g for 5 minutes) as described (WHO 2010). This pellet was then re-suspended 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 minutes centrifugation at 800g, using a specific medium (ISolate®, Irvine Scientific, Saint Ana, California, USA), and the isolated fraction was re-suspended 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 minutes 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 for the native semen samples we performed a PBS wash only. Regarding the processed samples, for those after DGC the only treatment done was DGC and for those obtained by the swim-up method, we performed the DGC and the swim-up method, 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 of sperm suspensions were sequentially mixed with 10μL of 1% (w/v) eosin for 30 seconds and then with 10μL of 10% (w/v) nigrosin for another 30 seconds. 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 300µL) was analyzed using a BD FACS Canto II (Becton Dickinson, New Jersey, USA) flow cytometer. 50 000 cells were acquired in each experimental condition using the FACS DIVA (Becton Dickinson, New Jersey, USA) software and the results were processed in the Infinicyt V1.5 (Infinicyt, Cytognos, Salamanca, Spain) software. Control experiments were carried out using cell sorting to ensure that only sperm (and not debris, leucocytes or other round cells; Figure 1) was being gated and analyzed in all assays, as described previously (Sousa et al. 2011).

To detect mitochondria-produced reactive oxygen species (ROS) MitoSOX™ Red (MitoSOX), a specific fluorescent probe for superoxide produced by mitochondria, was used, as previously described (Amaral et al. 2013b, Sousa et al. 2013). The MitoSOX™ Red probe is composed of dihydroethidium, that 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 800g for 5 minutes. Then they were incubated with MitoSOX™ Red at a final concentration of 3 µM and in a final volume of 300µL of medium (according to different experimental conditions different media were used), for 15 minutes 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 determined using the blue aragon laser, and emitted fluorescence was detected with a bandpass filter of 585/42.

To determine the percentage of viable cells in flow cytometry experiments the fluorescent dye SYTOX® Green was used, as previously described (Varum et al. 2007). This probe stains (non-viable) sperm with a damaged plasma membrane. In this case 0.1 µL of SYTOX® Green diluted with HEPES buffer was added for each 100µL of sperm suspension (final concentration 0.5 nM). Samples were incubated at room temperature for 15 minutes in the dark. Before and after the incubation with the probe the sample was washed with PBS and centrifuged at 800g for 5 minutes. 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 minutes. SYTOX® Green fluorescence was detected using a 488nm excitation laser, to detect the Fluorescein isothiocyanate fluorophore (FITC), and with a bandpass filter of 530/30nm.

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 know 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 100µL of cell suspension 5µL of annexin V solution were added and the mixture was incubated for 15 minutes at room temperature in the dark, as noted in the manufacturers instructions. Sperm were then washed with HEPES buffer (800g for 5 minutes) 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°C, for 1 hour) prior to annexin V labeling [Varum et al., 2007].

Statistical Analysis

Statistical analysis was performed using the IBM SPSS® 20 software (Chicago, IL). 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 non-parametric test, respectively. Pearson’s coefficient was calculated to correlate different sets of experiments when data had a normal distribution, while Spearman’s was calculated in all other cases. To analyze data related with patients we used Fisher’s exact test. Statistical significance was considered when $p \leq 0.05$. All data are presented as mean $\pm$ standard deviation (SD).
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++; Figure 2A). Of all 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 mitochondrial-generated ROS, led to an increase in the MitoSOX-positive subpopulations, with the concomitant disappearance of the MitoSOX negative subpopulation (Figure 2B).

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 (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). This data seems 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 towards ROS formation. To determine if 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 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 differential gradient centrifugation (DGC; data not shown).

MitoSOX subpopulations in human sperm samples with distinct initial quality

To determine if MitoSOX subpopulations are related to distinct sperm quality parameters we first decided to determine if 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 (Figure 3A), but normozoospermic had a higher percentage of the MitoSOX- subpopulation (and a lower percentage of the MitoSOX++ subpopulation) after sperm processing for Assisted Reproductive Technologies (ART; Figure 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 (Figure 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.

**Correlation of pregnancy with sperm MitoSOX subpopulations**

Finally we attempted to determine if the different MitoSOX subpopulations might be correlated with ART success, and thus with the achievement of pregnancy. As shown in Figure 5 a positive immunological pregnancy test result (IPT+) was significantly associated with a higher MitoSOX- subpopulation, and with lower amounts of MitoSOX++ subpopulation. Patient data is 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 1 to 3 embryos were transferred, with an average of 2 embryos for IPT+ and 1.88 for IPT-. For all the parameters described above we did not find any statistically significant differences between populations. We also checked if there was any difference according the age of the patient (female
or male) that could be related with 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 if endogenous ROS levels, and particularly mROS, could identify sperm subpopulations with distinct characteristics.

We determined that most human sperm samples are comprised of 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. 2012c), 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 (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 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. 2012a, 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 both to the centrifugation itself, as well as to the removal of putative semen antioxidants (Agarwal et al. 1994a, Thilagavathi et al. 2012). We show here 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 of 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 tell 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 with 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 here 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 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 if distinct populations are related with 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 with sperm quality and ART outcomes.
ACKNOWLEDGMENTS

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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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FIGURE LEGENDS

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.

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.

Figure 3- MitoSOX subpopulations in normozoospermic and non-normozoospermic human sperm samples. Sperm subpopulations (MitoSOX-, MitoSOX+, 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 differential gradient centrifugation (DGC, B; One-sample Kolmogorov-Smirnov test and t test were performed). Data are presented as mean ± SD. ** represents p≤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+, MitoSOX++) were quantified in native semen samples, and in those same samples following processing by A) differential 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 ± SD. * represents p ≤ 0.05, ** represents p≤0.01, *** represents p≤0.001.

**Figure 5-** MitoSOX subpopulations in sperm samples and pregnancy results. Sperm subpopulations (MitoSOX-, MitoSOX+, 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 ± SD. * represents p ≤ 0.05, ** represents p≤0.01. One-sample Kolmogorov-Smirnov test and t test were performed.
**Table 1** - Correlations obtained between sperm subpopulations based on their mROS content and viable and apoptotic cells assayed in the same samples.

<table>
<thead>
<tr>
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<th>MitoSOX-</th>
<th>MitoSOX+</th>
<th>MitoSOX++</th>
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<tbody>
<tr>
<td><strong>Live cells</strong></td>
<td><strong>r = 0.758</strong></td>
<td>-</td>
<td><strong>r = -0.775</strong></td>
</tr>
<tr>
<td><strong>n=23</strong></td>
<td>*<strong>p&lt;0.001</strong></td>
<td><strong>n=23</strong></td>
<td>*<strong>p&lt;0.001</strong></td>
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<tr>
<td><strong>r = -0.843</strong></td>
<td><strong>r = 0.436</strong></td>
<td><strong>r = 0.554</strong></td>
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</tr>
<tr>
<td><strong>Apoptotic cells</strong></td>
<td>***<strong>p&lt;0.001</strong></td>
<td><em>p&lt;0.05</em>*</td>
<td><strong>p&lt;0.01</strong></td>
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<tr>
<td><strong>n=26</strong></td>
<td><strong>n=26</strong></td>
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* MitoSOX, annexin V (apoptotic sperm) and SYTOX Green (non-viable sperm) assays were carried out by flow cytometry, as described in Materials & Methods.
Table 2 – Pregnancy data for samples used in this study: Patient age, number of embryos transferred (mean ± standard deviation), cause of infertility cause, primary or secondary sterility and technique performed (number of cases).

<table>
<thead>
<tr>
<th></th>
<th>IPT+</th>
<th>IPT-</th>
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<td>17</td>
<td>-</td>
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<td>Female age</td>
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<tr>
<td>Number of transferred embryo</td>
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<td>1.88 ± 0,70</td>
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<td>Female factor</td>
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<tr>
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<tr>
<td>ICSI</td>
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IPT+ - Immunological pregnancy test positive; IPT- - Immunological pregnancy test negative; ART - Assisted reproductive technique; ICSI - Intracytoplasmic sperm injection; IVF – in vitro fertilization
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.

70x62mm (300 x 300 DPI)
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.

130x82mm (300 x 300 DPI)
Figure 3D MitoSOX subpopulations in normozoospermic and non-normozoospermic human sperm samples. Sperm subpopulations (MitoSOX-, MitoSOX+, 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 differential gradient centrifugation (DGC, B; One-sample Kolmogorov-Smirnov test and t test were performed). Data are presented as mean ± SD. ** represents p≤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+, MitoSOX++) were quantified in native semen samples, and in those same samples following processing by A) differential 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 ± SD. * represents p ≤ 0.05, ** represents p≤0.01, *** represents p≤0.001.
Figure 5- MitoSOX subpopulations in sperm samples and pregnancy results. Sperm subpopulations (MitoSOX-, MitoSOX+, 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 ± SD. * represents p ≤ 0.05, ** represents p≤0.01. One-sample Kolmogorov-Smirnov test and t test were performed.