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Article

Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress

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KEY MESSAGE
Semen samples with high levels of oxidative stress as determined by a nitroblue tetrazolium assay, have diminished sperm DNA longevity after ejaculation. Given that seminal plasma was found to be the primary source of oxidative stress, rapid separation of this fraction may improve sperm DNA quality in these patients.

A B S T R A C T

The nitroblue tetrazolium (NBT) reaction as a tracer of oxidative stress was examined in 707 ejaculates from seven clinics. Semen was initially surveyed by classifying the NBT reaction using a pre-established rank for the Oxisperm® test based on three colourimetric levels: L1, low (n = 141 [20%]);

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L2, medium (n = 538 [76%]) and L3, high (n = 28 [4%]). L3 was indicative of a high level of superoxide anions. Halosperm® chromatin dispersion assay was used to analyse samples of ejaculates 30 min after ejaculation; no difference was found in DNA fragmentation of L1 or L3; L3 category semen samples incubated for 24 h at 37°C showed a significantly faster rate ($P < 0.001$) of DNA damage than those in L1. The NBT reaction was further characterized in the ejaculates of 100 patients to determine the relative contribution of seminal plasma, spermatozoa, or both. Seminal plasma was the most significant fraction of $•O_2^-$ localization, whereas sperm fractions generated detectable reactive oxygen species in only 32% of the ejaculates. Formazan precipitates were primarily associated with the sperm mid-piece and seminal leukocytes; however, not all spermatozoa stained positive to formazan and not all leukocytes presented with equivalent production of superoxide anions.

Introduction

Reactive oxygen species (ROS) are commonly known to negatively affect somatic or germ cell lines and are a major cause of cellular damage. Examples of ROS include the superoxide anion ($•O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and the peroxyl radical ($•HO_2^-$); the former being the most abundant ROS in human semen and the precursor of other ROS types. The superoxide anion is primarily produced from mitochondrial electron transport chain complexes I and III (Chen et al., 2009), and there has been significant interest in using it as a marker of oxidative damage given its ubiquity and strong chemical reactivity. All types of ROS can induce cellular damage in biological molecules such as proteins, lipids and DNA (Agarwal et al., 2014; Du Plessis et al., 2015; Kohen and Nyska, 2002). Excessive ROS production in the male reproductive tract is of concern because it leads to oxidative stress, which has potential toxic effects on sperm quality and function. In fact, between 25 and 50% of patients attending infertility clinics have high concentrations of ROS that may be associated with abnormal sperm motility, membrane integrity and DNA quality (Aitken, 1995; Ferramosca et al., 2007, 2013). For example, patients with a varicocele, typically have ROS concentrations higher than those of other infertile patients; in these patients, the affect of ROS is likely to be associated with heat stress affecting developing germ cells (Smith et al., 2006). Nevertheless, the exact prevalence of ROS in the subfertile male population remains poorly understood (Gharagozloo and Aitken, 2011). Part of the problem stems from the complexity of some techniques that are used to measure ROS, which limits the widespread utilization of ROS measurements as a routine procedure in the andrology clinic. Additionally, the plethora of different ROS radicals being assessed and how they are being assessed makes it challenging to draw firm conclusions on the real prevalence and significance of ROS in male infertility.

Various strategies have been used to assess the presence and effects of ROS (Agarwal et al., 2014; Kohen and Nyska, 2002). The NBT assay is a technique that has traditionally been used to determine the production of $•O_2^-$ in somatic and germ cells (Choi et al., 2006; Dimitrova et al., 2013; Sharma and Agarwal, 1996). The yellow NBT molecule is water-soluble, membrane permeable and reduced by $•O_2^-$ to a blue formazan deposit (Halliwell and Gutteridge, 1985). The specificity of this reaction has been demonstrated by the inhibitory effect of superoxide dismutase (SOD) as this enzyme catalyzes the dismutation of two molecules of $•O_2^-$ to form one molecule of oxygen and one molecule of H_2O_2 (Baehner et al., 1975). Therefore, the levels of $•O_2^-$, H_2O_2 and •OH are in constant conversion until equilibrium is established. Although $•O_2^-$ in semen samples has previously been evaluated using NBT (Amarasekara et al., 2014; Esfandiari et al., 2003; Tunc et al., 2010), a clear understanding of the association between positive reactions to NBT and the different fractions of the ejaculate (sperm versus seminal plasma), or of the prevalence of positive reactions in the neat ejaculate within a large cohort of men seeking fertility assistance, is lacking. Moreover, the effect of ROS on sperm DNA quality deserves more detailed attention, especially in establishing direct relationships between both concepts.

We conducted a multinational and multicentre cross-sectional study to determine the prevalence of positive responses to NBT in the ejaculates of a large number of infertile men attending infertility clinics around the world. The following were studied: the relationship of high and low NBT reacting ejaculates on initial sperm DNA quality and after incubation at 37°C for up to 24 h; the relative contribution of seminal plasma, spermatozoa, or both, to the strength of NBT reaction; and which primary region of the sperm cell the signal is being generated from.

Material and methods

Study design

This prospective design was a cross-sectional study that incorporated fresh ejaculates obtained from 707 men seeking fertility evaluation in 2014 and 2015 at seven participating clinics in Australia, Brazil, Germany, Mexico, Poland, South Korea and Spain. All participants provided informed consent to use their semen samples for the analysis. The data obtained by individual centres were compiled and subsequently analysed at the Genetics Unit of the Autónoma University of Madrid. This study was approved by the research committee or internal clinical board of each participant institution and complied with the standards for the reporting of cross-sectional studies (STROBE statement, http://strobe-statement.org). Patients were offered the assay in addition to the standard seminogram and informed that their semen sample would be analysed as part of larger worldwide multicentre study on seminal oxidative stress. No charge was levied for the assay and signed consent for participation was requested.

Participants

Men were included if they were between the ages of 18 and 45 years, seeking fertility evaluation and consented to donate a semen specimen for research. Participants were asked to abstain from ejaculation for 2–3 days before collection. All participants used a collection room located in the same facility as the andrology laboratory. Ejaculates were collected by masturbation into sterile cups. A single ejaculate was obtained from each individual and all specimens were discarded after assessments. Men were excluded if they reported semen spillage or loss during collection, had a history of the following, or

both: medical illness, including fever in the past 3 months, evidence of clinical or subclinical (leukocyte >1 million/ml of semen) genital infection, severe oligozoospermia (<1 million sperm per ml of semen), azoospermia, or use of gonadotoxic medication.

Assessment of $\bullet O_2^-$ levels in neat semen

The presence $\bullet O_2^-$ in the semen specimens was assessed using the Oxisperm® kit (Halotech® DNA, Madrid, Spain); this protocol is based on the nitroblue tetrazolium (NBT) test that produces a stable colourimetric reaction in a biological sample that may contain $\bullet O_2^-$. To perform this assay, a tube containing the reagent gel was placed in a water bath at 90°C for about 5 min to allow the gel to liquefy. After gel equilibration at 37°C, liquefied semen was added according to the kit specifications and the mixture incubated for 45 min at 37°C. Depending on the concentration of superoxide anion and the cellular content of the various oxido-reductases, a colour precipitate that ranged from a light pink to dark purple-black was produced. The NBT reaction gives rise to solid deposits of formazan on the plasma membranes, which are also readily detectable by bright field microscopy. In this study, we only considered three levels of colourimetric reaction: L1, low/absence of colour; L2, medium; and L3, high (**Figure 1**). Although the colour assessment can be carried out using semi-quantitative micro-densitometric approaches or image analysis protocols, results obtained after direct visual observation are not significantly different (de la Casa et al., 2015); therefore, a visual qualitative classification, which is both less expensive and easier to carry out, was used here. This was achieved by visual colour comparison with a pre-established pantone supplied with the kit. A single experienced senior technician performed the Oxisperm® test in each centre; the technician in each centre underwent before training as provided by Halotech® DNA instructions. Each run was carried out with appropriate positive and negative controls. Positive controls included the use of activated leukocytes from peripheral blood that were incubated with tetradecanoylphorbol acetate (TPA; Sigma Aldrich, Madrid, Spain) at a concentration of 1.6×10^{-7} for 30 min at 37°C. Negative controls involved use of non-activated leukocytes from peripheral blood. Positive and negative controls were processed in parallel with the test specimens. Two trained observers independently scoring 60 samples twice in a blinded fashion evaluated the reproducibility of test scores in one of the participating centres (Brazil). The inter-observer variability was calculated by analysing the readings obtained by two observers while the intra-observer variability was analysed by calculating the difference between two readings obtained by the same observer.

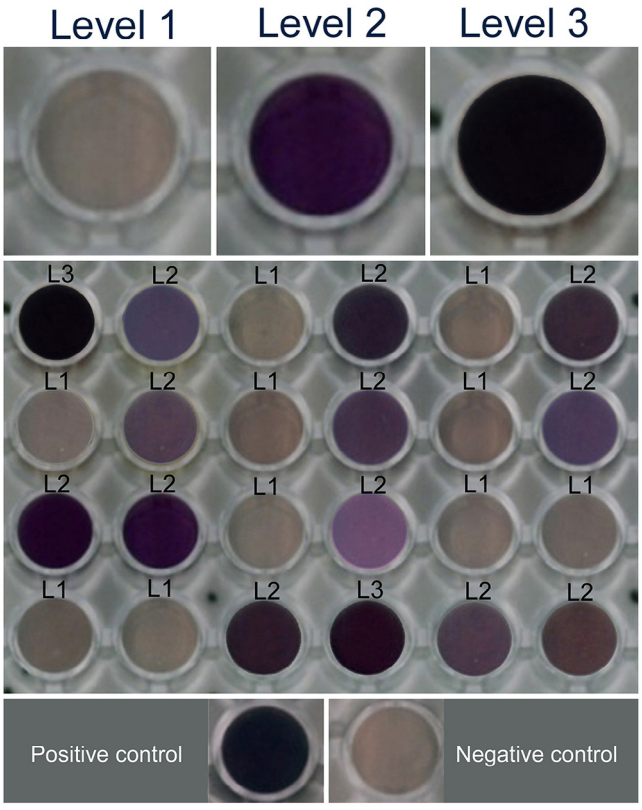

Figure 1 – Visualization of the three pre-established colour levels of reactivity using the Oxisperm® kit to assess $\bullet O_2^-$ content in neat semen samples and visual colour comparison using a pre-established pantone. L1, no detectable reactivity or low reactivity level; L2, Medium reactivity level; L3, high reactivity level. The reaction was conducted in multi-well microtitre plates. Positive and negative controls correspond to leukocytes incubated with and without tetradecanoylphorbol acetate, respectively.

Sperm DNA fragmentation assessment

Initial sperm DNA fragmentation (SDF) was assessed 30 min after ejaculation and was determined from chromatin dispersion patterns using the Halosperm kit (Halotech DNA SL, Madrid, Spain) in 143 samples (25 corresponding to L1, 102 to L2 and 16 to L3). To assess the dynamic behaviour of SDF, 30 semen samples that stained as L1 or L3 after $\bullet O_2^-$ assessment were incubated at 37°C for 0, 2, 6 and 24 h and the SDF assessed at each time interval using the Dyn-Halosperm kit (Halosperm DNA, Madrid, Spain). The morphology of different sized haloes was visualized by epifluorescence microscopy after being stained with the DNA intercalating fluorochrome Gel Red (Biotium, Hayward, CA, USA) and anti-fading agent Vectashield (Vector Laboratories, Burlingame, CA, USA). Image visualization and capture was carried out using a Leica DMLB microscope with epifluorescence light-emitting diode illumination with a charge-coupled device (Leica Microsystems, Barcelona, Spain).

Assessment of $\bullet O_2^-$ levels in semen fractions

In a parallel experiment, each patient's response to the NBT reaction was assessed in the neat ejaculate, seminal plasma and sperm fractions. A total of 100 individuals who had a positive reaction to NBT were included in this analysis. Thirty minutes after ejaculation, semen samples were centrifuged at 400 g for 6 min to produce a sperm pellet (spermatozoa) and supernatant (seminal plasma). The supernatant was directly reacted with NBT by mixing 50 µL of the reactive gel included in the kit with 50 µL of seminal plasma and incubated for 45 min at 37°C; the resulting reaction colour was recorded. The sperm pellet was further diluted using phosphate-buffered saline to achieve a final and homus sperm concentration of 50×10^6 mL^{-1} and, from this dilution, 50 µL of the re-suspended sperm pellet was reacted with 50 µL of the reactive gel following the same protocol as the seminal plasma. Reactivity results were compared in each specimen before and after semen fractionation. Specimens were classified on the basis of presence or absence of reactivity as follows: P1, negative response (L1) to NBT reactivity in the neat ejaculate and in both semen fractions

(frequencies not included in this study); P2, positive response (L2 or L3) to NBT reactivity in the neat ejaculate and in both semen fractions; P3, positive response (L2 or L3) in the neat semen and in the seminal plasma fraction, but negative (L1) in the sperm fraction and P4, positive response (L2 or L3) to NBT reactivity in the neat specimen and in the sperm fraction but negative (L1) in the seminal plasma fraction.

Microscope analysis of $\bullet O_2^-$ assessment in sperm fractions

The semen fractions from the ejaculates of 100 individuals were assessed under bright-field microscopy after the completion of the NBT reaction to determine the location of the positive NBT signals. Once the reaction was completed in the gel, a smear was produced using a 20 µL aliquot of sperm suspension on a clean microscope glass slide. The slides were dried at room temperature and counterstained using methyl green 2% (Sigma Aldrich, Madrid, Spain). A standard bright field microscope equipped with a Jenoptik ProgRes CCD (Jenoptik Optical System, GmbH) was used for image capture; a non-oil-immersion 63x plan-achromatic objective provided sufficient resolution.

Statistical analysis

The Statistical Package for Social Sciences (SPSS v.11; Chicago, IL, USA) was used for statistical analysis. $P < 0.05$ was considered statistically significant. Absolute and per cent distributions of the different NBT reaction categories were calculated for each participant centre and a contingency table chi-squared test was used to compare results among the different centres. For intra- and inter-observer variability, absolute and per cent differences between individual measurements, and the final recorded value with the cumulative percentages, were calculated. The non-parametric Mann–Whitney U statistical test was used to compare initial SDF values between samples from individuals in the two groups whereas the dynamic loss of DNA quality was assessed using the non-parametric maximum likelihood Kaplan–Meier estimator. To compare the survival distributions of the sperm samples from each group, a Log Rank (Mantel–Cox) test was used (SPSS v.16.0 for Windows, SPSS Inc. 233 S. Wacker Drive. 60606 Chicago, IL). Sperm DNA accumulated survival, in this case was represented as a varying accumulated frequency over time for sperm that were not affected by fragmentation at each time period of the incubation.

Results

Prevalence of a positive NBT reaction in the neat ejaculate

Assessment of the $\bullet O_2^-$ levels within the same clinic revealed an intra-class correlation coefficient between observers of 0.94 and an inter-class correlation coefficient between two readings by the same observer of 0.96. The pooled distribution of the different reactivity levels from all clinics is shown in **Figure 2**. Most patients (n = 538 [76%]) were categorized as having medium (L2) reactivity. In contrast, only 4% (n = 28) of the cohort exhibited high reactivity (L3) whereas 20% (n = 141) showed absence/low reactivity (L1). Overall, 80.1% of the studied population showed semen reactivity to the NBT test, indicating the presence of superoxide anion in the ejaculate. The distribution of reactivity levels by participating centres is shown in **Table 1**. In general, a similar trend was observed among centres in the clustering of specimens, and indicated a large proportion of patients with a medium (L2) level of reactivity and a low proportion with high reactivity (L3). Nevertheless, significant difference (contingency, chi-square = 66.93; df = 12; $P_{\alpha\ 0.05} = 0.000$) was found between the values reported by the different clinics for the levels of NBT reactivity observed.

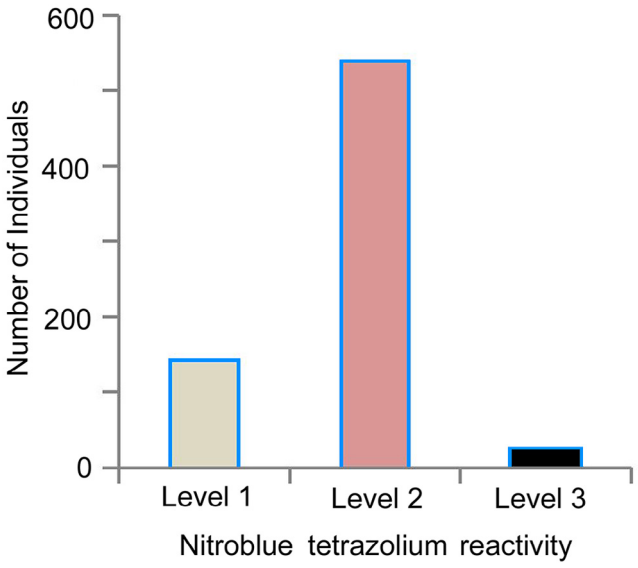

Figure 2 – Distribution of colour levels (L1–L3) (Figure 1) from all participating centres after assessment of $\bullet O_2^-$ content, using the Oxisperm® assay, in test neat semen specimens (n = 707).

Influence of superoxide presence on sperm DNA fragmentation

To compare the effect on sperm DNA fragmentation (SDF) with the different levels of superoxide in the ejaculate, SDF was compared 30 min after ejaculation (T0 – static assessment) and after a period of incubation at 37°C (T0, T2, T6 and T24h – dynamic assessment) (**Figure 3**). With static assessment (T0), 143 individuals were included in the analysis (25 corresponding to L1, 102 to L2 and 16 to L3). The mean ± SD values of SDF obtained in the different groups were 23.3 ± 13.7, 23 0 ± 14.4 and 21.3 ± 10.8 for L1, L2 and L3, respectively; no significant difference was observed between the different groups (Kruskal–Wallis, Chi-Square 0.174; df). With dynamic assessment of SDF, 30 individuals were scored for SDF after incubation of the neat ejaculate at T0, T2, T6 and T24 h at 37°C. Fifteen of initial ejaculates were classified as L1 whereas the remainder (n = 15) were classified as L3. Our analysis revealed that within this selected semen sample population we could observe no statistical differences in the initial T0 value for sperm DNA (**Figure 3A**); the mean ± SD SDF for the L1 and L3 groups was 17.8 ± 10.5 and 21.1 ± 11.2 (Mann–Whitney U = 94.0; Z = –0.768), respectively. After comparing the DNA dynamics between the L1 and L3 groups, however, significant differences in SDF were observed. Individuals characterized as L3 showed a faster rate of DNA damage than those categorized in the L1 group (**Figure 3B**) (Kaplan–Meier; Log-Rank; [Mantel–Cox]: 242.1; df 1; $P < 0.001$). Differences in SDF dynamics were also observed when the different individuals in each NBT reactivity category were compared (L1; Kaplan–Meier; Log–Rank; [Mantel–Cox]: 116.7; df 14; $P < 0.001$) (**Figure 3C**) and (L3; Kaplan–Meier; Log–Rank; [Mantel–Cox]: 148.0; df 14; $P < 0.001$) (**Figure 3D**).

Table 1 – Prevalence of nitroblue tetrazolium reactivity levels (L1–L3) in neat semen specimens (*n* = 707) according to centre.

NBT	Centre 1	Centre 2	Centre 3	Centre 4	Centre 5	Centre 6	Centre 7
Level 1, n (%)	15 (15)	6 (5.6)	33 (33.3)	41 (24.6)	11 (35.5)	20 (19.4)	16 (16.2)
Level 2, n (%)	83 (83)	102 (94.4)	65 (65.7)	116 (69.4)	20 (64.5)	71 (68.9)	82 (82.8)
Level 3, n (%)	2 (2)	0 (0.0)	1 (1.0)	10 (6.0)	0 (0.0)	12 (11.7)	1 (1.0)
Total, n (%)	100 (100)	108 (100)	99 (100)	167 (100)	31 (100)	103 (100)	99 (100)

[a] See **Figure 1**.
NBT, nitroblue tetrazolium.

NBT reactivity in neat and fractioned semen

After assessing 100 individuals in which the neat ejaculate was positive to NBT, 22% of these samples were assigned to P2, 68% to P3 and 10% to P4 categories (**Figure 4**); once a positive colour reaction was confirmed in the neat ejaculate, a positive signal was further identified in the sperm and seminal plasma fractions in 32% and 90% of the cases, respectively. The colour intensity of sperm samples in P4

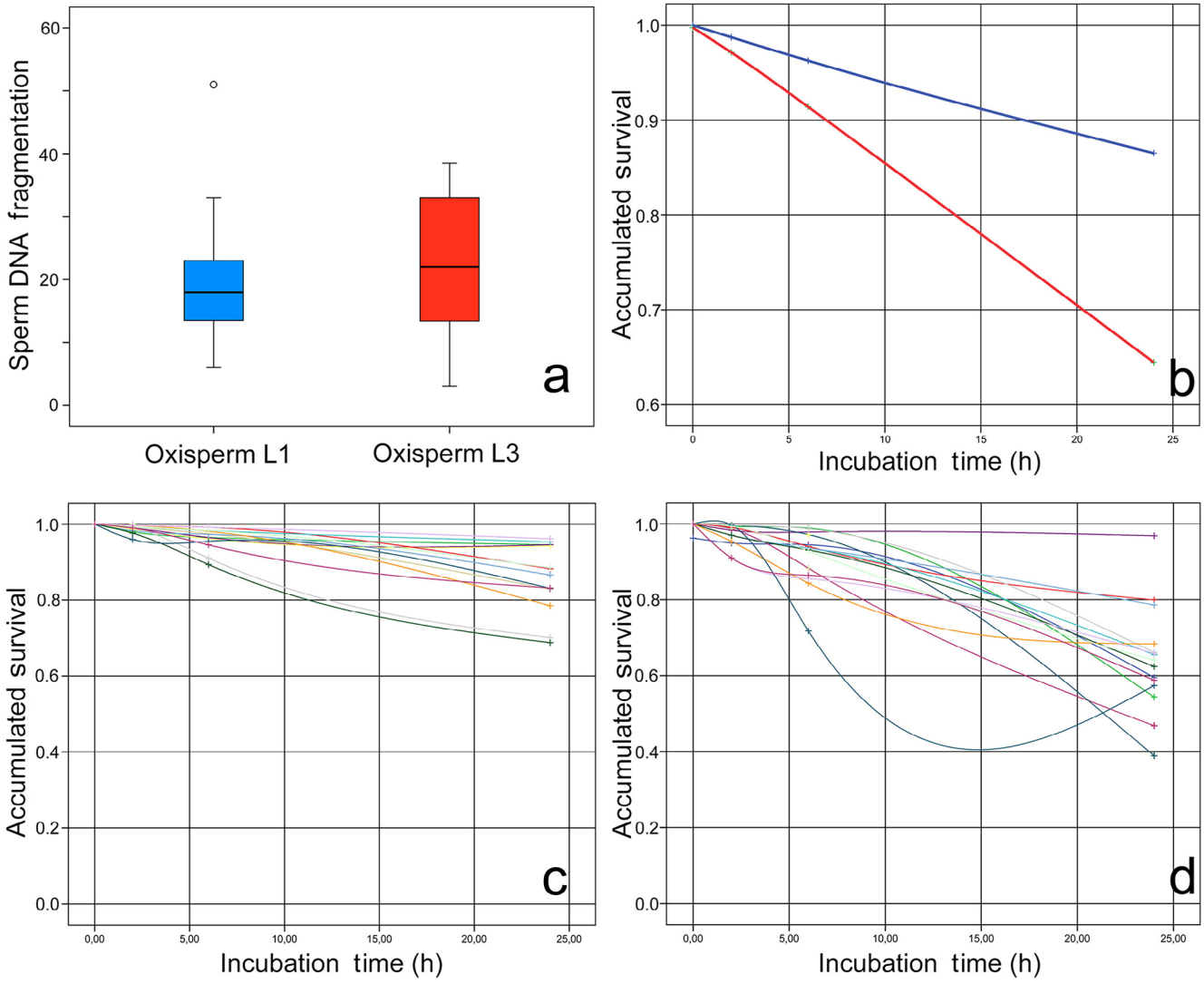

Figure 3 – The effect of L1 and L3 levels of nitroblue tetrazolium reactivity on the static and dynamic values of sperm DNA fragmentation (SDF). (a) Box-whisker plot showing static values for SDF at T0 (after ejaculation) in L1 (*n* = 15) and L3 (*n* = 15) groups created according to the level of nitroblue tetrazolium reactivity. Dynamic values of sperm DNA fragmentation are expressed as sperm DNA accumulated survival defined as accumulated frequency over time of those sperm not affected by DNA fragmentation at each time period of the incubation. Dynamic behaviours have been normalized to the SDF at t0, set as '1' on the Y axis; (b) mean values of sperm DNA dynamics in Oxisperm®-L1 (*n* = 15; blue) and Oxisperm®-L3 (*n* = 15; red) designated spermatozoa, Kaplan–Meier; Log–Rank; Mantel–Cox; 242.1; df 1; $P < 0.001$ for L1 versus L3; (c) intra-group variation of sperm DNA dynamics in group L1 (*n* = 15, Kaplan–Meier; Log–Rank; Mantel–Cox: 116.7; df 14; $P < 0.001$ for the comparison between individuals; (d) intra-group variation of sperm DNA dynamics in group L3 (*n* = 15), Kaplan–Meier; Log–Rank; Mantel–Cox: 148.0; df 14; $P < 0.001$ for the comparison between individuals.

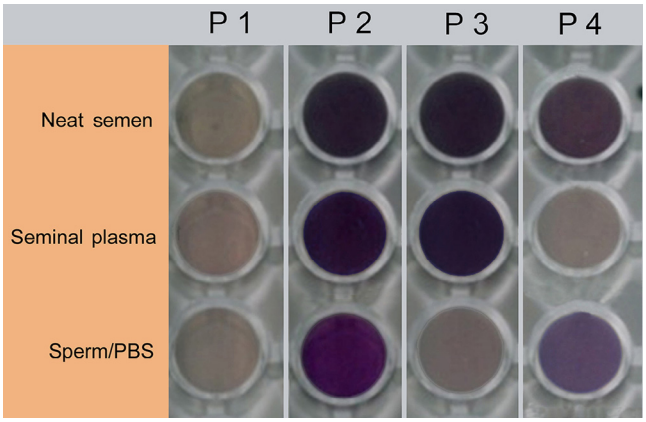

Figure 4 – Colourimetric characterization of different reaction patterns according to the reactivity to nitroblue tetrazolium (NBT) in the neat ejaculate, seminal plasma and sperm fractions.
P1 - negative response to NBT assay in the neat ejaculate, seminal plasma and sperm fractions; P2, positive response (L2 or L3) (Figure 1) in the neat ejaculate and in both seminal plasma and sperm fractions; P3, positive reaction in the neat semen and in the seminal plasma, but negative in the sperm fraction; P4, positive reaction to NBT in the neat specimen and in the sperm fraction, but negative in the seminal plasma. PBS, phosphate-buffered saline.

was slightly less than those sperm registered in both the neat ejaculate and seminal plasma fractions (P2) (**Figure 4**). None of the patients presenting with a negative response in the neat ejaculate showed a positive reaction in the seminal plasma or in sperm fractions.

Signal localization in NBT reactive sperm fractions

In those patient ejaculates that showed NBT reactivity in both the neat specimen and sperm fractions (irrespective of the response in seminal plasma), the signal was localized to spermatozoa, leukocytes, or both. Staining of the sperm cell was preferentially located in the mid-piece and post-acrosomal regions, but rarely covered the entire surface of the sperm head (**Figure 5B and 5C**). The intensity of labelling also varied among cells within the same ejaculate (**Figure 5E**). The proportion of labelled spermatozoa also varied from one individual to another; in 10 randomly selected positive staining ejaculates, the proportion of labeled sperm varied from 2–60% after counting 300 cells. The NBT staining of spermatozoa was present in all patients who were identified as having polymorphonuclear leukocytes (PMN) in the ejaculate (**Figure 5F**). Similarly, the labelling intensity of leukocytes also varied (compare **Figure 5G** bottom with **Figure 5F** top). No patients with a negative response in the neat semen showed traces of formazan precipitate in the sperm fraction (**Figure 5A and 5D**).

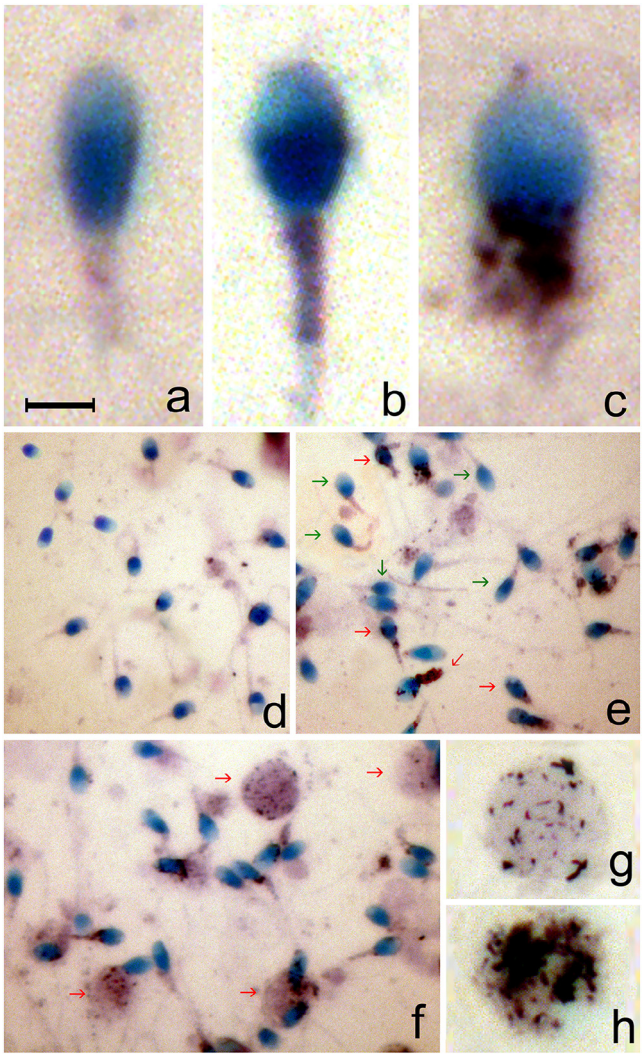

Figure 5 – Visualization of formazan precipitates after reacting the sperm pellet with Oxisperm®. (a) Spermatozoa showing absence of any precipitate; (b) spermatozoa showing positive signal in the mid-piece; (c) spermatozoa showing intense precipitation at the post-acrosomal region; (d) overview of a sperm sample that had a negative reaction; (e) overview of a sperm sample that positively reacted – note spermatozoa with (red arrows) and without precipitates (green arrows) in the head; (f) overview of a sperm sample in which polymorphonuclear leukocytes leukocytes were labelled (red arrows); (g,h) two selected leukocytes showing positive low (g) and high (h) levels of formazan precipitation. Scale bar: a–c: 2 μm; d–f: 10 μm; g–h: 5 μm.

Discussion

Prevalence of NBT reactivity in the ejaculate

The NBT reaction provides information about the levels of •02– in a range of biological samples (including semen) by means of a simple colourimetric reaction (Amarasekara et al., 2014; Esfandiari et al., 2003; Tunc et al., 2010). In this survey examining over 700 patients evaluated across seven different infertility clinics in four continents, 80% of the studied population showed semen reactivity to NBT, indicating the presence of the superoxide anion in the ejaculate. Despite some individual variation in relative proportions of colourimetric categories across the different clinics, most participants evaluated (76%) presented with a medium level of NBT reactivity. Only one in 25 individuals produced ejaculates with NBT high reactivity.

Significant variation in the prevalence of NBT reactivity levels (L1–L3) across the seven centres was found, particularly in levels L1 and L2. For example, centres 1, 2 and 7 all reported high levels of L2 and may have possibly overestimated the incidence of this category or been unable to differentiate it sufficiently from L1. We attempted to account for this variability, however, by having samples analysed by two different observers (inter) in the same centre and twice by the same observer (intra) and determined a correlation coefficient of 0.94 and 0.96, respectively. It was impossible to compare the same ejaculate between centres, as the NBT reaction typically occurs within 45 min, so that the time spent transporting the semen sample between different laboratories made this comparison impractical. Although some of the variability may also be attributed to the technician's individual perception of the colour change, we found in an earlier preliminary study that, although simple visual colour determination can result in a slightly lower colour discrimination ability compared with spectrophotometric and image analysis, these three methods were all still highly correlated (de la Casa et al., 2015). Despite our best efforts to standardize our patient cohorts by excluding those with medical illness, it is also possible that the reported variation in the distribution of NBT reactivity levels was simply a reflection of the relative difference in patient cohorts at each centre. For example, patients in Mexico were younger than those attending the Spanish clinic. Such specific differences associated to NBT reactivity in patient cohorts are topics for future investigation.

The cellular contribution to NBT reactivity

A high level of NBT reactivity in the neat ejaculate of some patients was attributable to spermatozoa, leukocytes, or both. Although an association between high levels of NBT reactivity and sperm DNA fragmentation was found in this study, this association was also time dependent in that the incidence of sperm SDF in high and low NBT staining ejaculates increased over time; the rate of SDF was also significantly higher in L3 ejaculates. This finding was not surprising, given the undisputed association between oxidative stress and DNA damage in spermatozoa (Wright et al., 2014); however, it is interesting that the baseline level of SDF observed in the neat ejaculate 30 min after ejaculation did not seem to be affected by the corresponding level of •O2– reactivity. This observation suggests that a high level of NBT reaction in the neat ejaculate was not initially and directly associated with DNA damage linked to innate sperm production in the patient, but rather is more likely to be associated with prolonged semen exposure to oxidative stress post ejaculation. A prudent strategy in these patients to reduce DNA damage before insemination would be to separate the spermatozoa rapidly from the seminal plasma or from exposure to leukocytes.

The fact that some patients presented with a high level of NBT labelling in both the neat ejaculate and spermatoza, whereas in others the spermatoza were free of such labelling, suggests that spermatozoa affected by the overproduction of superoxide may not be suitable for IVF or intracytoplasmic sperm injection (ICSI). Given that it is still currently problematic to detect compromised sperm cells for ICSI, the possibility exists that embryologists may unintentionally select spermatoza with oxidative damage. Incubation of oocytes with spermatozoa originating from semen samples with high NBT reactivity, and in which sperm DNA fragmentation increases as a function of incubation time, are likely to be sub-optimal and this might help to explain the poorer assisted reproduction technique outcomes observed when conventional IVF is used in couples whose male partner has high SDF (Agarwal et al., 2016). Conversely, we might expect that sperm not staining positive for NBT might have better reproductive outcomes, because sperm are microinjected into oocytes shortly after processing. Nevertheless, it is important to consider that a negative result to NBT does not preclude these spermatozoa from resulting in a higher risk of miscarriage after ICSI, if they also have damaged DNA (Agarwal et al., 2016). The most obvious adverse effect would be oxidative damage combined with compromised sperm DNA integrity. This phenomenon has already been well described, and having direct implications to clinical aspects of reproduction, has been variously addressed (Agarwal et al., 2005; Aitken and Baker, 2006; Cho et al., 2016; Esteves et al., 2014, 2015; Evenson et al., 2002; Henkel et al., 2005).

Nicotinamide adenine dinucleotide phosphate-dependent oxidoreductase, localized at the level of mitochondria within the sperm midpiece, has been shown to contribute ROS and is a major site of oxidative stress (Marchetti et al., 2002; Sabeti et al., 2016) Background ROS, therefore, would normally be expected to be associated with sperm mitochondria but this level of activity is not usually sufficient to reduce NBT resulting in visible formazan precipitates; rather, these precipitates only seem to be associated with increased local ROS production and probably not compensated by antioxidant capacity. In addition to the detrimental effect on nuclear and mitochondrial sperm DNA, a high level of •O2– can also compromise ICSI outcomes by negatively affecting the oocyte cytoplasm. The role of centrosomes and centrioles, the post-transcriptional modifications of alpha tubulins, and modifications to the carboxy-terminal tyrosine amino acid residue from alpha tubulin, have several potential implications for the manner in which the microtubule array is organized. As a large part of oxidative stress is localized at the mid-piece, where the mitochondria are located, damage of tubulines may putatively affect the first steps of fertilization by disrupting the normal formation of tubulin filaments that are necessary for syngamy after sperm penetration. In fact, it is well known that oxidative stress has the capacity to produce tubulin depolymerization (Lee et al., 2005), and this effect may be associated with the lack of orthodox chromatin-tubulin assembly after sperm injection (Gook et al., 1998).

Direct mapping of the NBT reaction at the level of the sperm cell or leukocyte is relatively straightforward using the Oxisperm® technique (**Figure 4**), but the respective contributions of individual sperm cell damage to overall ROS damage is still not clear and deserves further investigation. The differential production of •O2– depending on the leukocyte observed within the same patient may also be an important factor at the time of evaluating the quantitative effect of •O2– on other cells. The production of •O2– would not only depend on the number of PMN observed in the sample but also on the amount of •O2– that each cell is able to produce (Walrand et al., 2003). At the cellular level it would be important to distinguish between the sources of ROS (PMN leukocytes, sperm or both), as the clinical implications of infiltrating leukocytes are different from the pathological conditions in which sperm are themselves the source of ROS.

Seminal plasma contribution to NBT reactivity

Although the NBT reaction is supposedly based on the generation of ROS by sperm and leukocytes, by analysing the distribution of NBT reactivity in respective sperm and seminal plasma fractions, we observed that the larger contribution •O2– was derived from the seminal plasma, with only 32% of samples showing sperm staining positive to the NBT reaction. Our results suggest that assessing the responses

to NBT in both the neat ejaculate and semen fractions (seminal plasma and spermatozoa) may provide an additional qualitative framework to discriminate the source of oxidative damage in individual patients. The location of labelling in sperm primarily involved the mid-piece region, suggested that most of the •O2– was linked to mitochondrial activity. The ability to differentiate the source of the oxidative damage, whether sperm, leukocyte and/or seminal plasma, could be highly instructive as to the respective causes of these abnormally high levels of ROS, thereby potentially facilitating specific treatments to reduce oxidative damage.

A positive response of the NBT reactant with the seminal plasma after ejaculate fractionation and the absence of any discrete precipitate associated to any cell remains difficult to interpret. The seminal plasma contains natural antioxidant such as vitamins C and E, superoxide dismutase, glutathione, uric acid and free radical scavengers (Henkel et al., 2005; Khosrowbeygi and Zarghami, 2007) but it has also been associated with oxidative stress (Lissak et al., 2004; Palan and Naz, 1996). Recently, it has been demonstrated that variations of the proteome associated with the seminal plasma are also related to levels of oxidative stress (Intasqui et al., 2015), such that the seminal plasma can also be used as an index of ROS imbalance or oxidative stress that may be occurring both in the sperm or even in the prostate.

The presence of a positive signal in the seminal plasma in an individual characterized as P2 (neat ejaculate positive, seminal plasma positive, and sperm positive) can be assumed to be caused by excess free formazan deposits obtained after the reaction of •O2– emanating from spermatozoa or PMNs. On the contrary, the reason why those individuals characterized as P3 (neat ejaculate positive, seminal plasma positive, and spermatozoa negative) showed a positive signal is difficult to ascertain. It is possible that exosome or prostasome could be contributing to the generation of ROS; these subcellular particles are surrounded by lipid membranes so that the oxidative processes associated with these lipids could be a potential source of ROS. In particular, prostasomes are human-specific lipid vesicles originating from the prostate that are present in seminal plasma and have been associated with certain levels of antioxidant capacity in semen samples (Saez et al., 1998). Exosomes have also been reported in the human ejaculate (Sullivan et al., 2005; Vojtech et al., 2014); interestingly, these particles have also been associated with oxidative stress (Eldh et al., 2010; Hedlund et al., 2011).

Oxidative stress can be counteracted with treatments such as oral antioxidant intake and varicocele repair (Esteves and Agarwal, 2011). Uncontrolled intake of so-called 'healthy' supplements, however, can also lead to harmful and significant adverse effects (Gutteridge and Halliwell, 2010; Menezo et al., 2007) on semen quality. Therefore, it would seem prudent to have an initial evaluation of the patient's ROS characteristics and to localize the source of the oxidative damage before any consideration is given to antioxidant therapy. The Oxisperm® assay would also be a relatively straightforward way to evaluate the affect of antioxidant therapy on semen quality during and after treatment.

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