

Malonaldehyde formation and DNA fragmentation: two independent sperm decays linked to reactive oxygen species

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Summary

Malondialdehyde (MDA), a product involved in membrane lipid peroxidation, was dosed in the sperm of 163 patients who had consulted the clinic regarding hypofertility. We attempted to determine if there was correlation between MDA content, sperm World Health Organization parameters and DNA fragmentation that results mainly from reactive oxygen species assaults. We found that no correlation could be established; however MDA and sperm decondensation were shown to be significantly linked. The impact of membrane polyunsaturated fatty acids and the role of phospholipid hydroperoxide glutathione peroxidase are discussed.

Keywords: DNA fragmentation, Fatty acids, Lipid peroxidation, Malondialdehyde, Reactive oxygen species

Introduction

Reactive oxygen species (ROS: the superoxide anion radical, hydrogen peroxide and hydroxyl radical), are released as byproducts of aerobic metabolism, activated by small molecules such as xenobiotics and iron. ROS alter most types of cellular molecules, in particular those that affect membrane lipids and DNA. Oxidative stress, originating from the environment and from mitochondrial activity, is involved in defective sperm quality and is strongly related to male infertility. It has been shown that, despite acceptable fertilization rates, severe defects in sperm quality are associated

with poor embryo quality and compromised outcomes. Measurement of 8-hydroxydeoxyguanosine/deoxyguanosine (8-OH-dG/dG) shows that oxidative products are an important cause of embryo fragmentation (Oger *et al.*, 2003, De Iulii *et al.*, 2009) and these oxidative products may have a mutagenic effect per se. During and immediately after the fertilization process, the oocyte cytoplasm should theoretically repair any decay in the integrity of sperm DNA; when ICSI is used, this repair can only partially improve embryo developmental potential. In fact, the human oocyte has a complete, but finite, capacity for DNA repair (Ménézo *et al.*, 2007), even in the best maternal conditions (Frydman *et al.*, 2007). Sperm DNA fragmentation leads to reduced fertility (Henkel *et al.*, 2004; Guerin *et al.*, 2005) and deleterious effects in the offspring, including childhood cancers (Aitken & Baker, 2006; Fernández-Gonzalez *et al.*, 2008) can be expected when the DNA repair capacity of the oocyte is overwhelmed. Human sperm has a high content of polyunsaturated fatty acids (PUFA): they can be an important source of ROS generation (Aitken *et al.*, 2006) and an excessive level of these PUFA could be a mark of sperm immaturity. A common by-product of lipid peroxidation is malondialdehyde (MDA). Its level is elevated in oligoasthenozoospermic

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and asthenozoospermic sperm (Ben Abdallah *et al.*, 2009). MDA can form protein and DNA adducts: we recently found the potentially mutagenic adducts ethenoadenosine and ethenoguanosine in human sperm (Badouard *et al.*, 2007). In this study we have tried to determine if a correlation can be established between DNA fragmentation and tertiary structure, linked to maturity and malonaldehyde formation.

Material and methods

The study was performed in 163 patients consulting for subfertility. The work received approval from the ethical committees of the three clinics and the two laboratories involved. Semen samples were collected by masturbation after a sexual abstinence of 3–5 days as routinely requested. After liquefaction, sperm was analysed according to WHO guidelines. Length of abstinence, total number of spermatozoa in the whole ejaculate, viability, motility, detailed morphology, and survival rate after a 24-h incubation period were recorded. Movement characteristics of sperm were assessed by CASA, using the Hamilton Thorne Integrated Visual Optical System.

As computer analysis may overestimate sperm counts, these were confirmed by diluting the samples in toluidine blue and counting the spermatozoa on THOMA slides. Sperm motility was verified by dropping 10 μ l of homogenized sperm on a THOMA slide with glass coverslip.

The remainder of the sample was aliquoted and frozen for further analysis, which included sperm DNA fragmentation, decondensation and malondialdehyde levels. With this approach we can have a simultaneous instant evaluation of the oxidative damage links to ROS *in vivo*.

DNA damage analysis

The detection of DNA strand breaks was performed by a flow cytometry-based terminal transferase dUTP nick end labelling (TUNEL) assay, as previously published (Cohen-Bacrie *et al.*, 2008; Epics[®] XL-MCL[™] cytometer, Beckman Coulter). The samples were resuspended and washed twice in 5 ml of PBS and centrifuged 10 min at 1200 rpm; 100 μ l of washed spermatozoa were fixed in a solution containing PBS + 1% PFA (37%) for 1 h at room temperature. The samples were vortexed, and then centrifuged for 10 min at 1200 rpm, the supernatants were discarded. The pellets were washed in 300 μ l of PBS and centrifuged 10 min, then resuspended in 100 μ l of permeabilization solution (1% sodium citrate + 1% Triton X) and cooled for 3 min at 4°C. The samples were again vortexed and centrifuged to remove the supernatants. The pellets

were washed once in 300 μ l of PBS and stored overnight at 4°C.

Positive control samples were prepared by adding 1 μ l of DNase, 1 μ l of buffer and 98 μ l of *in situ* cell (In Situ Cell Death Detection Kit, Fluorescein, Roche) to the pellet of a normospermic patient's semen sample. A negative control of 50 μ l of buffer only was included. The sperm samples were vortexed and centrifuged to remove the supernatant and re-centrifuged 10 min after the addition of 300 μ l of PBS; 5 μ l of enzyme and 45 μ l of buffer were added to the pellet. All the tubes were placed at 37°C for 45 min, and then washed twice in 300 μ l of PBS and centrifuged 10 min to remove the supernatant. The pellets were resuspended in 750 μ l of PBS and transferred to tubes specially designed for the cytometer; at least 5000 cells per patient were analysed.

Lipid peroxidation

Measurement of thiobarbituric acid reactive substances (TBARS) is a commonly employed and reliable method for assay of oxidative stress. Sperm lipid peroxidation level was assessed using the Oxitek kit (ZeptoMetrix Corporation) according to the manufacturer's instructions. In brief, after DNA damage analysis, whole homogenates of sperm were sonicated for 5-s intervals at 40 V setting over ice. A total of 100 μ l of SDS and then 2.5 ml TBA/buffer reagent were added forcefully to the samples, which were then incubated at 95°C for 1 h. The samples were cooled to room temperature on ice and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatants at 532 nm was quantified using a Spectra Plus 384 spectrophotometer (Molecular Devices).

Chromatin decondensation analysis (SDI, sperm decondensation index)

The aniline blue technique (Dadoune *et al.*, 1988; Auger *et al.*, 1990; Hamadeh *et al.*, 2001) was used in this study, following our observations that this technique showed better specificity (Belloc *et al.*, 2009). Briefly, after sperm preparation, the pellet was permeabilized by incubation with sodium dodecylsulphate (1% SDS) in sodium citrate. Processed spermatozoa were spread onto a glass slide, and allowed to dry. The smears were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate-buffered saline (PBS; pH 7.2) for 30 min. The slides were then stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 15 min at ambient temperature. Two hundred sperm cells were evaluated and the percentage of blue stained sperm heads was calculated. Two classes of staining intensities were distinguished: unstained and completely or partially stained sperm heads.

Table 1 DNA fragmentation, chromatin decondensation and malonaldehyde (MDA)

	MDA (ng/ml) (\pm SD)
DNA fragmentation (%)	
<20	6.4 (2.3)
20–30	6.8 (2.5)
30–40	7 (2.7)
>40	6.7 (1.9)
Chromatin decondensation (%)	
<15	6.6 (2.5)
15–25	6.5 (1.5)
>25	9.3 (1.5)

Statistics

The relationship between MDA and other sperm markers was analysed by correlation coefficients. Moreover, a variance analysis was conducted to analyse the evolution of MDA according to fragmentation in four classes (<20, 20–29, 30–39 and \geq 40) and to decondensation in three classes (<15, 15–24 and \geq 25). Statistical significance was established for a p -value of <0.05, as usual. Analysis was carried out using SAS software, release 9.

Results

In our experiment involving 163 patients, we were able to find the classical correlations indicating that our sample was not biased (Tables 1 and 2): (i) age and sperm DNA fragmentation, $p < 0.001$; (ii) type A motility and fragmentation (negative): $p < 0.001$; (iii) sperm concentration and immaturity (decondensation), $p < 0.04$.

Thiobarbituric acid reactive substances, which reflect lipid peroxidation, are expressed in terms of MDA equivalent (mg/ml). The mean values are between 6.5 and 7.5 ng/ml. For MDA, no correlation was found between the age of the patient ($p < 0.89$), DNA fragmentation ($p = 0.88$) and the sperm parameters of concentration ($p < 0.75$), total motility

($p < 0.26$) and type A motility ($p < 0.97$). MDA formation and sperm DNA decondensation are correlated ($p < 0.05$).

Conclusions

The metabolism of molecular oxygen in the environment of spermatozoa is important. In our study we found the classical increase in DNA fragmentation with paternal age, and a correlation between fragmentation and motility (Guerin *et al.*, 2005; Evenson & Wixon, 2006). Two estimated types of DNA decay can be induced by oxidative stress linked to reactive oxygen species in sperm and sites of action are membrane lipids and DNA. In terms of lipids, 4-hydroxy-2-nonenal (HNE) and MDA are well-known toxic products of lipid peroxidation. For DNA, 8-OH-dG is the main ROS-derived adduct, but not the only one, others include Fapy A and G, C and T glycol etc.). It is now clearly established that 8-OH-dG formation is a major cause of DNA fragmentation (Oger *et al.*, 2003; de Iuliis *et al.*, 2009). The only additional process so far described is the production of DNA adducts (EthenoG and A), which originate from condensation of the oxidized lipid fragment on the bases (Badouard *et al.*, 2007). In this study we deliberately did not separate sperm and seminal plasma in order to avoid any oxidative interference that classically occurs during sperm processing (i.e. centrifugation in high oxygen tension).

Our data show no correlation between DNA fragmentation and lipid peroxidation, which suggests that there are two independent steps. Data from Aitken & Clakson (1987), suggest that the DNA decays are from endogenous (mitochondrial) origin whereas lipid peroxidation could originate from the environment. However, lipid peroxidation can be also the result of an abnormal spermatogenic process during testicular transit (Aitken *et al.*, 2006). There is a correlation between MDA formation and decondensation. This also fits with the observations of Aitken & Clarkson (1987), who suggest that anomalies in lipid transformation in sperm membranes in the

Table 2 Statistical analysis, significance between analytical sperm parameters

	MDA	Age	Concentration	Total motility	Type A motility	Fragmentation	Decondensation
MDA	–	0.89	0.75	0.71	0.97	0.88	0.05
Age		–	0.02	0.26	0.28	0.001	0.11
Concentration			–	0.001	0.001	0.16	0.04
Total motility				–	0.001	0.01	0.04
Type A motility					–	0.001	0.09
Fragmentation						–	0.96
Decondensation							–

Significant correlations: bold characters. MDA, malonaldehyde.

testis could indicate sperm immaturity. This oxidation would be caused by an excessively high level of membrane *cis*-unsaturated fatty acids, which are highly sensitive to oxidation. In addition, phospholipids-dependent glutathione peroxidase (PI-GPX), has a dual role: prevention of ROS decays and also oxidation of the cysteine moieties, which lock and compact the DNA (Ursini, 1997). An excessive amount of this enzyme: (i) by a too high level of ROS (age related); or (ii) by a too high number of sperm cells; will both increase peroxidation and decrease compaction.

In conclusion, there is no direct correlation between the two different types of ROS-linked damage, to lipids and to DNA. Both types of damage affect DNA condensation and fragmentation; they are not necessarily dependent (Wyrobek *et al.*, 2005; Belloc *et al.*, 2009) but both of these factors affect embryonic development after fertilization (Henkel *et al.*, 2004; Carrell, 2008; Rousseaux *et al.*, 2008). The two types of damage can have cumulative effects by the formation of potentially mutagenic adducts. This aspect should not be overlooked in assisted reproductive treatments.

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