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Evaluating human sperm DNA integrity: relationship between 8-hydroxydeoxyguanosine quantification and the sperm chromatin structure assay

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Summary

In our work, we have used 8-hydroxy-deoxyguanosine (8-OH-dG), one of the major oxidative products of sperm DNA, in a population of patients consulting for infertility. We found an inverse relationship between sperm concentration and the log of the ratio of 8-OH-dG to dG ($P < 0.01$). On the same patients' sperm samples, the sperm chromatin structure assay (SCSA) was performed. An inverse relationship was observed between the DNA fragmentation index and sperm concentration ($P < 0.001$). There was also a positive relationship between SCSA and log 8-OH-dG/dG. This indicates that DNA fragmentation measured by the SCSA originates in part from oxidative products. In a few patients, antioxidant treatment decreased the DNA fragmentation index below the threshold of 30% that is crucial for subfertility.

Keywords: Sperm DNA fragmentation, Reactive oxygen species, 8-Oxodeoxyguanosine, Sperm chromatin structure assay

Introduction

The impact of male subfertility on overall reproduction is still a matter of debate. It can be estimated that infertility is shared equally between male and female partners: at least one-third of couples' infertility is strictly related to male factors. Sperm analysis, although informative in a first approach, has shown strength and limits: morphology has demonstrated its strong impact not only on the fertilization process but also on early embryonic development (Yovitch *et al*., 1984; Janny & Menezo, 1994; Menezo & Dale, 1995). A range of methods have been developed to evaluate male infertility in relation to paternal DNA integrity. On the basis of individual sperm, it is clear that DNA integrity might be totally independent of all semen parameters, including sperm morphology, concentration and motility (Evenson *et al*., 1991).

There is still a need for homogeneity in the methods used to measure DNA integrity and in the evaluation of the relationship among these methods. Sperm chromatin abnormalities have been attributed to abnormal apoptotic processes that lead to double-stranded DNA breaks. However, DNA strand breaks and apoptosis need not be linked (Sakkas *et al*., 2002). Reactive oxygen species (ROS) affect not only lipid structure (peroxidation) but also DNA structure through oxidative processes (Kodama *et al*., 1997; Ni *et al*., 1997; Shen *et al*., 1999), leading to DNA strand breaks. ROS-related DNA oxidation products are associated with potentially mutagenic changes in DNA structure. Nevertheless, there are some uncertainties (Moller *et al*., 1998) concerning methodological aspects of the quantitative evaluation of 8-hydroxydeoxyguanosine (8-OH-dG), which is the major product of DNA oxidation; 8-OH-dG has a tautomeric form called 8-oxodeoxyguanosine. (Fig. 1).

Our first objective was to improve the reproducibility of the extraction and dosage sensitivity of 8-OH-dG; high-performance liquid chromatography coupled

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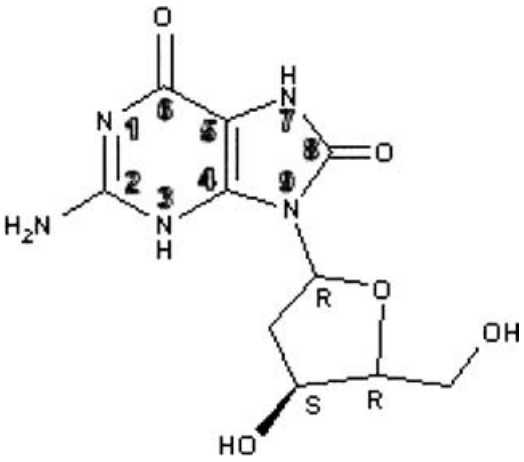

Figure 1 The structure of 8-OH-dG.

with electrochemical determination is the method of choice for the analysis (Beckman *et al*. 1997). Our second objective was to compare the 8-OH-dG results with sperm chromatin structure assay (SCSA) results. The SCSA test was chosen for comparison because it is strongly correlated with COMET and TUNEL analyses (Aravindan *et al*., 1997) and has the advantage of a clear-cut clinical threshold for the success of assisted reproduction techniques (Evenson *et al*. 2002). Moreover, ROS have recently been suggested to have a negative impact on sperm DNA fragmentation (Muratori *et al*. 2003).

Materials and methods

Sperm samples

The 64 sperm samples were collected from a fertility centre (Institut Rhonalpin pour l'étude de la Reproduction humaine), from patients coming for sperm analysis. The samples included in this study had between 1 million and 100 million spermatozoa per ml and $< 200{,}000$ round cells per ml. The study was made on raw sperm.

Sperm collection and storage

Sperm samples are divided into two parts. One part is stored as raw material in liquid nitrogen for SCSA (Evenson *et al*., 1991, 2002). The second part was stored frozen before 8-OH-dG quantification.

DNA extraction methods

The DNA extraction methods developed by Ward & Coffey (1989) and Kao *et al*. (1995) were compared at pH 7.4 and pH 8.3. Following a double extraction with phenol–chloroform and isoamylic acid, DNA extracts were treated with proteinase K in Tris medium plus dithiothreitol. The yields were calculated based on 2 pg DNA per spermatozoon (Fraga *et al*., 1991).

High-performance liquid chromatography and electrochemical detection

High-performance liquid chromatography (HPLC) is performed with a Waters apparatus including a pump (Waters 600), an injector (Waters 717), an electrochemical detector Chromosystem and an ultraviolet-light detector (Waters 2487). The column is a 5 μm silica CI8 grafted Uptisphere (Interchim). The mobile phase is 50 mM CH_3COONa, 12.5% methanol, pH 5.3. The whole system is computerized using Waters Millennium 32 software.

The potential of oxidation is set at +0.65 V and the sensitivity level is 5 nA.

The standard solution of 8-OH-dG is prepared at a concentration of 353 μM in an aqueous solution of sodium acetate (50 mM). All the calibration curves are realized by dilution of this stock solution. Deoxyguanosine quantification is made by ultraviolet absorption starting with a stock solution at a concentration of 3 mM.

Sperm chromatin structure assay

The technique used is the one described by Evenson *et al*. (2002) using acridine orange and flow cytometry. In this case, the raw sample is diluted if necessary to a concentration of 1 million to 2 million spermatozoa per ml, treated in acidic conditions. Analysis is based on 5000–10,000 spermatozoa. Calculation of the DNA fragmentation index (DFI) is computerized using the SCSAsoft program. The range of DFI for the samples varied from 1% to 80%.

Statistical analysis

All the calculations were performed using Student's *t* test for linear regression.

Results

8-OH-dG determination and relationship to sperm counts

The protocol developed by Kao *et al*. (1995) had a significantly greater DNA yield. The experiments made on samples with a concentration of 60 milllion spermatozoa give a mean yield of 75% for the protocol of Kao *et al*. at pH 8.3. Moreover, the repeatability for the same sample is over 90%. The yields obtained for other protocols (13) at pH 7.4 and pH 8.3 give at best 19% and 15%, respectively, of the theoretical value (based on 15 estimates: 2 pg DNA per spermatozoon and 0.62 nanomoles dG per μg DNA). The range for DNA concentration is $1.3 \times 10^{-4} - 2 \times 10^{-6}$ μg per sample. The values for 8-OH-dG were $1 \times 10^{-10} - 5 \times 10^{-14}$ moles

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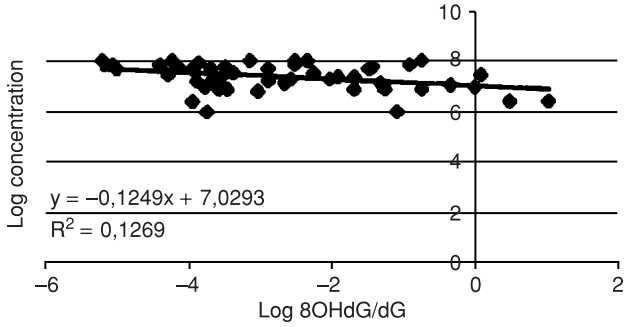

Figure 2 Sperm concentration and 8-OH-dG.

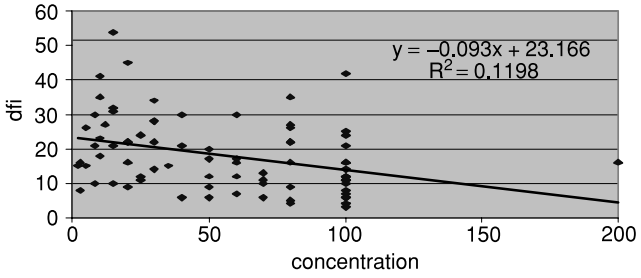

Figure 3 Sperm concentration and DFI.

per sample. The range observed for the ratio 8-OH-dG/dG is wide: from 1.2×10^{-2} to 6×10^{-6}. Owing to the wide range of values obtained, even for 8-OH-dG/dG, all the calculations are made as log values. There is an inverse relationship between sperm count and the ratio 8-OH-dG/dG.

If y is the log of sperm count and x is log 8-OH-dG/dG, there is a relationship between them: $y = -0.125x + 7.03$, with correlation coefficient $r = 0.356$, $r^2 = 0.127$, $P < 0.01$ (Fig. 2).

SCSA and sperm count

The DFI gives the value of the DNA fragmentation; it includes the moderate and high fragmentation.

If y is the DFI and x is the sperm count (in millions in the sample), there is a relationship between them: $y = -0.093x + 23.17$, with $r^2 = 0.12$, $r = 0.34$, $r^2 = 0.12$, $P < 0.001$ (Fig. 3).

Relation between SCSA (DFI) and 8-OH-dG

There is a relationship between DFI and 8-OH-dG. If y is log 8-OH-dG/dG and x is DFI then $y = 0.0267x - 3.08$, with $r = 0.32$ and $r^2 = 0.104$, with $P < 0.01$ (Fig. 4).

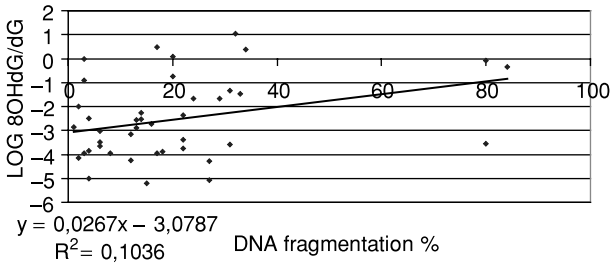

Figure 4 DFI and 8-OH-dG.

Discussion

A certain level of ROS is beneficial for some aspects of sperm physiology (De Lamirande & Gagnon 1995) but genomic DNA is susceptible to decay caused by ROS. The resulting oxidative DNA damages have been implicated in degenerative diseases and cancer. They are well known to affect male fertility (Kodama *et al*., 1997; Loft *et al*., 2003) and are induced by various external agents. In a first approach, it was important to evaluate the extraction methods for quantification of 8-OH-dG in sperm. By nature, the DNA matrix is very compact in spermatozoa, unlike other eukaryotic cells moreover, sperm samples are heterogeneous. There is an inverse relationship between 8-OH-dG and sperm count; this confirms the results of Ni *et al*. (1997). In addition, the correlation coefficient found here ($r = -0.356$) is very similar to that obtained by Ni *et al*. ($r = -0.389$). This is obviously related to the fact that poor sperm count usually corresponds to poor quality spermatozoa generating ROS by themselves. In order to determine DNA and chromatin structure, there are several possibilities: the Comet assay (Hughes *et al*., 1996), the TUNEL assay (Sailer *et al*., 1995), '*in situ*' translation assay (Manicardi *et al*., 1995) and the SCSA (Evenson *et al*., 1991, 2002). In addition, the SCSA correlates strongly with other tests measuring DNA fragmentation and strand breaks (Aravindan *et al*., 1997). Moreover, this method offers clinically significant results. Pregnancy rates are significantly lower in men with a DFI of > 30% (Evenson *et al*., 2002). This is also our experience: there was only one ongoing pregnancy out of more than 120 cycles including 51 patients with a sperm DFI > 30%. There is a weak relationship between 8-OH-dG and the SCSA. This confirms that ROS are involved, at least partly, in the three sources of sperm chromatin abnormalities (apoptosis, necrosis (Darzynkievicz *et al*., 1997) and DNA topoisomerase II), in agreement with the findings of Muratori *et al*. (2003). Several mechanisms are supposed to prevent these oxidative damages in the genital tract; glutathione synthetase, peroxidase, superoxide dismutase and catalase are the main enzymes involved in the prevention of

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ROS formation. Some of these might be present in the female genital tract and in oocytes (Guerin *et al*. 2001). In the oocyte, 8-OH-dG might be removed from oxidatively damaged DNA by base excision repair (Bessho *et al*., 1993; Rosenquist *et al*., 1997). Over a certain threshold of decays, DNA cannot be repaired any longer. Low molecular weight reducing substances such as vitamins (C and E) and sulfur-derived compounds (hypotaurine, glutathione) are also efficient in preventing ROS formation both *in vivo* and *in vitro* (Comhaire *et al*., 2000; Donelly *et al*., 1999). Interestingly, we started to treat patients with a mixture of vitamins (C, E and β-carotene) and zinc and selenium for 90 days to 'cover' a full spermatogenetic cycle. Only four out 23 patients involved who started with a DFI of at least 30% (initially 39%, 41%, 30% and 36%) fell below this critical threshold (19%, 21%, 22% and 23%, respectively). One of the four couples, the one whose DFI fell from 30% to 22%, started a pregnancy, after 5 years of unexplained infertility. This decrease in DFI following antioxidant treatment gives further evidence that ROS might negatively affect sperm chromatin quality as measured by the SCSA. Currently, more patients are enrolling in this antioxidant study to determine how the decrease in DFI might impact ART.

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