Quantitative evaluation of p53 as a new indicator of DNA damage in human spermatozoa

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ABSTRACT

BACKGROUND: Sperm DNA integrity is considered an important parameter to assess seminal fluid quality and can be used as a predictive test of potential fertility. Amongst the various tests to determine sperm DNA integrity, one is the Acridine Orange test. Recent studies have demonstrated the importance of p53 in maintaining sperm DNA integrity. The aim of this study was to assess if a p53 ELISA assay could be a new indicator of DNA damage in human spermatozoa. **MATERIALS AND METHODS:** 103 human semen samples were evaluated using both Acridine Orange test and p53 ELISA and results were compared. **RESULTS:** A clear correlation between the values measured by two methods was obtained. **CONCLUSIONS:** If this hypothesis will be confirmed by further studies, the p53 ELISA assay could become a new and more precise indicator of DNA damage in human spermatozoa.

KEY WORDS: Acridine Orange test, P53, sperm DNA damage, sperm DNA fragmentation index

INTRODUCTION

Unlike the structure of somatic cells, which are relatively free of chromatin (DNA and histones), sperm chromatin is compact and stable in the nucleus. The nuclear condensation in sperm cells is due to the replacement of about 85% of lysine-rich histones associated with DNA, with transition proteins that are rich in arginine and protamine.[1,2]

Contrary to histones, which form a ring with DNA (nucleosomes), protamines are linked to the grooves of the DNA helix, wrapping tightly around the strands of DNA (approximately 50 kb of DNA for protamine), to form tight and highly organized loops. Moreover, inter- and intramolecular disulfide bonds between the cysteine-rich protamines are also responsible for the compaction and stabilization of the sperm nucleus.[1-3]

The result of these specific associations is an extreme nuclear condensation with the reduction of approximately 10% of the nucleus size.[2]

The key protein that mediates the chromatin compaction is the BRDT (Bromo Domain Testisspecific) protein which is able to promote nuclear remodeling, thus ensuring the transition between the histone chromatin organization, or somatic, and the protaminic nucleus, typical of mature sperm.[4]

The specific nuclear compaction is important to protect the sperm genome from external stress. In fact, physiological and environmental stress, as well as genetic mutations and chromosomal abnormalities may interfere with the mechanisms of spermatogenesis. These changes may lead to abnormal chromatin structure that is incompatible with fertility [Figure 1]. The defects of genomic material found in mature sperm may impair packaging (defective histone and protamine replacement) and maturation of the nucleus, leading to DNA fragmentation (i.e. single-or double-strand breaks) and defects of DNA integrity or chromosomal sperm aneuploidy.[1]

In the case of atypical and immature sperm, the DNA can fragment and lose its functional integrity, and thus lead to functional defects of the sperm. In fact, DNA fragmentation is particularly common in men subfertile sperm.[1]

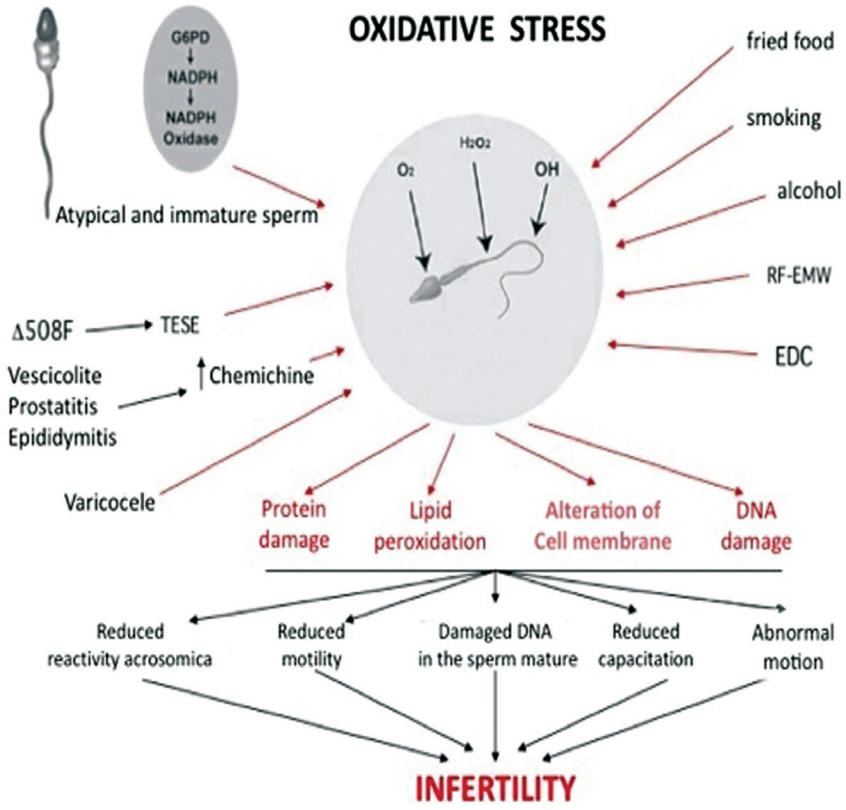

Figure 1: Causes of sperm oxidative stress

The p53 molecule, so called because of its molecular mass[5] is a sequence-specific transcription factor that responds to a wide range of stress signals and cellular functions, acting as a coordinator of cell destiny.[6]

It is closely involved in the cellular response to DNA damage, by controlling cell cycle arrest, apoptosis and the transcription of genes induced by DNA damage.[7]

Under normal conditions, the level of p53 protein is low in most cells of the body. However, when normal cells are deprived of oxygen or exposed to DNA damaging treatments, such as UV light or gamma rays, there is an increase in the concentration of p53 via a reduction of the rapid degradation process of the molecule.[8]

It is not surprising that p53 the "guardian of the genome"[6-8] seems to play a role in spermatogenesis.[9]

It has been suggested, in fact, that the role of the p53 ancestral gene was to ensure the integrity of the genomic germline and the fidelity of the development processes.[10]

p53 performs several functions in the meiotic and premeiotic spermatogenesis phases.[11] It is possible that p53 plays different roles in DNA repair, depending on the type of damage,[12] on the phase in which the cell has been damaged and on the possible recovery ways available.[11]

In brief, p53 helps the sperm to deal safely with DNA damage.[7]

The aim of this study was to analyze and compare the p53 values (DNA damage marker) measured by ELISA assay and the sDFI (sperm DNA Fragmentation Index) values obtained by the Acridine Orange (AO) test, in human sperm samples of seminal fluid from men with normal and pathological parameters.

The ultimate goal of this work was to verify the usefulness of the p53 measurement as an objective and repetitive method in the evaluation of sperm DNA compared to the current laboratory techniques. The ELISA method could, within the medically assisted procreation, identify subjects with low fertility and be predictive on the outcome of *in vitro* fertilization.

MATERIALS AND METHODS

Study population

A total of 103 samples of human semen were examined in the period from January 2011 to June 2012. Subjects were

aged between 27-35 years, and the ejaculate volume varied from 0.7 to 5.4 ml.

The samples were divided according to their diagnostic evaluations in: Normospermia, 44/103 (42.7%); mild oligospermia, 10/103 (9.7%); medium oligospermia, 23/103 (22.3%); severe oligospermia, 26/103 (25.2%).

The subjects examined did not have chronic diseases, they had not used medicines or drugs in the last 3 months prior to the collection of semen, their work did not involve exposure to environmental stresses[13,14] and the preliminary Doppler results did not indicate any symptoms caused by pathological varicocele.[15,16]

The subjects signed the informed consent form for the processing of personal and sensitive data, as well as of genetic and biological samples collection in compliance with the applicable laws.[17,18] The signed informed consent was obtained from the human participants of this study. The procedures followed were in accordance with the ethical standards of experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2000.

Seminal fluids according criteria, the WHO manual fifth edition 2010, were examined.

A Makler chamber (Makler Counting Chamber, Sefi-Medical Instruments ltd, Haifa, Israel) was used for the evaluation of sperm concentration, the total number of spermatozoa and for the study of the cellular component of non-sperm cells (leukocytes, erythrocytes, and germ line cells).[6-19]

The processing steps of seminal fluid samples were performed 30 minutes after ejaculation.

The samples were then aliquoted into two parts one of which was immediately analyzed and the other frozen at –20°C for future investigations.

AO test

The Acridine Orange Test allows the calculation of the percentage of mature spermatozoa containing the normal and double-stranded native DNA, using the acridine orange dye and by observation with fluorescence microscopy.

It is based on the principle that DNA possesses a different susceptibility to partial denaturation induced by heat shock or by contact with an acidic solution. The test utilizes the metachromatic properties of Acridine Orange, a specific fluorochrome for nucleic acids that, when binding to the DNA double helix (the native form) emits green fluorescence, whilst when binding to single-stranded DNA (denatured form) emits red fluorescence. The staining protocol was applied to each sample after 30 min from the liquefaction of semen at 37°C.

The coloration of Acridine Orange was performed according to the Tejada and coll. Method.[20] The sample was analyzed within 1 h of staining.

The slides were blindly read by two independent observers. The coloration has given results within a 10% of discrepancy. The nuclei of at least 300 spermatozoa from each individual were examined and classified as fluorescent green, red, orange, or yellow. Spermatozoa with green/yellow fluorescence were considered as having intact DNA, while those showing red/orange fluorescence were considered as having damaged DNA, like those showing green and red simultaneously [Figure 2]. The threshold for green fluorescence was set at 75%, and <25% values were considered positive in this test.[21]

Separation of sperm from seminal fluid

To perform the isolation of sperm from semen, the Differex System™ — for use with the Differex Magnet™, and the DNA IQ™ System-Small Sample Casework Protocol kits were used (Promega Corporation, Madison WI, USA). In 1985 Gill and coll.[22] developed a method to separate spermatozoa from epithelial cells in a sample of human semen. The separation protocol reported below has been developed for this project. It requires about 150 min to obtain the complete separation and purification of the sperm DNA.

The number of sample and reagents quoted has been calculated for a single sample and a single experiment in the following protocol.

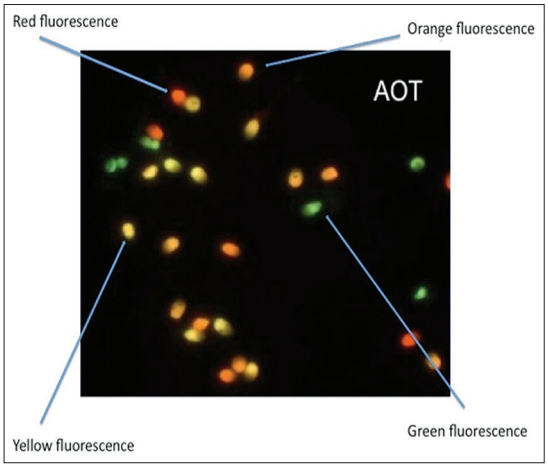

Figure 2: Photography by fluorescence microscopy of sperm stained with Acridine Orange. Fluorescence was reported as sDFI, which expresses the percentage of sperm with fragmented DNA (single-strand red fluorescence) over the total sperm counted (with single and double strand DNA, red fluorescence and green)

One hundred microliters of sample were placed in a 1.5 ml tube with 400 µl of Digestion Solution containing 6 microliters of diluted Proteinase K and 364 microliters of Digestion Buffer. The tube was vortexed for 30 s at 14,000 rpm, incubated for 90 min at 56°C and then centrifuged for 10 min at 14,000 rpm in a microcentrifuge at room temperature, not before having marked the position where the pellet would form.

Then 3.5 µl of DNA IQ™ Resin were added in the opposite position of the pellet and the tube was placed on Differex Magnet™ so that the resin, attracted by the magnet, would coat the pellets. The yellow liquid layer, containing epithelial cells, was then removed.

The sample was then washed three times with 500 µl of nuclease free water and the last washing volume was not removed.

The tube was centrifuged again at 14,000 rpm for 10 min and 3.5 µl of DNA IQ™ Resin were added in a position opposite the pellet and positioned in Differex Magnet™ so that the resin would coat the pellets. After three washes, a further 500 µl of nuclease free water together with 100µl of Separation Solution were added, so that the resin would coat the pellets. The washing and separation solutions were then removed and the pellet was resuspended by adding 400 microliters of 0.9% sodium chloride solution. Three hundred microliters of the solution thus obtained were aliquoted ("no lysis sample ").

To extract the sperm DNA, 250 microliters of Lysis Solution (containing 2.5 microliters of DTT and 252.5 µl of Lysis Buffer were added to the tube, which was then vortexed for 3 sec at high speed and incubated for 5 min at room temperature. After having vortexed again for 3 s, the tube was positioned in the Differex Magnet ™ so that the separation would occurs instantaneously and the supernatant was then removed and stored in another tube ("lysed sample").

Then 100 µl of Lysis Solution were added and the tube was removed from the Differex Magnet™. After vortexing for 2 s, the tube was put back in Differex Magnet™ and the entire Lysis Solution eliminated.

To perform the washing 100 µl of Wash Buffer were added after removing the tube from the Differex Magnet™ and the sample was vortexed for 2 s at high speed.

Once the tube was repositioned in Differex Magnet™, the entire wash solution (containing 500µl of Wash Buffer, 250 µl of Isopropyl Alcohol and 250 µl of ethanol) was eliminated.

The washing procedure was repeated three times.

The resin was allowed to air dry for 5 min, leaving the tube with the cap open.

Subsequently, 100 µl of Elution Buffer were added (10 mM Tris pH 8.0 and 0.1 mM EDTA), and the tube was vortexed for 2 s. incubated for 5 min at 65°C vortexed again and placed immediately on Differex Magnet™. The solution containing the DNA was carefully transferred in a new tube ("lysed sample and refined DNA").

Quantitative p53 dosage with ELISA assay

A direct and quantitative ELISA assay was used to measure p53 (DuoSet IC, Human Total p53 R and D Systems Inc. Minneapolis, MN USA).

Briefly, 100 µl of the capture antibody, appropriately diluted, were pipetted into each well of a 96 well microplate, which was then sealed and incubated overnight at room temperature. The next day the plate was washed three times with 400 µl Wash Buffer (0.05% Tween 20 in PBS, pH 7.2-7.4, filtered at 0.2 µl).

Each well was blocked with the addition of 300 µl of stop solution (Sample Diluent Concentrate: 5X PBS, 5 mM EDTA, and 2.5% Triton X-100). The plate was then incubated at room temperature for 2 hours. Extraction and washing steps were repeated. The standards were prepared by diluting in IC Diluent # 4 (1 mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2-7.4.) and using IC Diluent # 4 like standard zero.

Then 100 microliters of sample or standard were added ("no lysis," "lysed," and "lysed and purified DNA" cell preparations), the plate was sealed and incubated for 2 h at room temperature. After the incubation, extraction and washing were repeated and 100 µl of detection antibody (Total p53 Detection Antibody), appropriately diluted, were pipetted in each well.

The plate was sealed and incubated for 2 h at room temperature. After the incubation, extraction and washing were repeated.

One hundred microliters of Streptavidin-HRP were then added and the plate was incubated for 20 min at room temperature. After the incubation, extraction and washing steps were repeated and 100 µl of Substrate Solution (1:1 mixture of Reagent A and Reagent B) were added to each well and the plate was incubated for 20 min at room temperature.

Finally 50 µl of Stop Solution were added to each well.

The optical density was analysed using a microplate reader set at 450 nm with a software that automatically calculated the concentrations expressed in pg/100 microliters.

Statistical analysis

Pearson correlation coefficient "*r*" (a dimensionless index ranging between –1,0 and + 1,0, which reflects the extent of a linear relationship between two data sets) was used for the statistical analysis of the groups. Fisher transformation "x" was used to perform a hypothesis test on the correlation coefficient. Finally, for the development of probability and data significance, the student's "t" test was used. These statistical calculations were performed using Microsoft Excel 2008 (Microsoft Corporation, Redmond WA, USA).

RESULTS

The sDFI values obtained in the AO tests were "reinterpreted," as the sDFI is a parameter which alone has a limited prognostic value for *in vivo* procreation, except when correlated to sperm counts.[23] In fact, a high sDFI value (pathological) and a "high sperm count" do not indicate that the subject is potentially infertile. Likewise, a low sDFI (normal) and a low sperm count do not indicate potentially fertile subjects. This is why it was considered appropriate to evaluate the sDFI/tsc value for each sample instead of the simple sDFI, were tsc (total sperm count) is the result of the ejaculate volume multiplied by the sperm count/ml.

$$\text{sDFI/tsc} = \frac{\text{sDFI}}{\text{Ejaculate volume} \times \text{number of spermatozoa/ml}}$$

The p53 values were also corrected ("corrected p53"), because the dosage of p53 was performed on 100 microliters of sample. The p53 values were therefore related to 1/10 of the value of sperm counts and the corrected p53 is a reliable estimate calculated for 1 ml of semen.

$$\text{Corrected p53} = \frac{\text{Value of p53 ng / 100 µl}}{\text{1 / 10 of the spermatic count / ml}}$$

The value of p53 was measured in three different cell preparations ("no lysis," "lysed," and "lysed and purified DNA") for each sample.

Given the physiological localization of p53 on the DNA, there is a considerable difference of concentration in the 3 cell preparations. To make data interpretation more visible, the sDFI/tsc were plotted in ascending order, while the p53 values were correlated to the sample location associated with the sDFI/tsc value.

For all semen samples, we correlated the corrected p53 values and sDFI/tsc in the three different cell preparations ("no lysis", "lysed" and "lysed and purified DNA"). This showed that the cell preparations "lysed and purified DNA" has the best correlation among the parameters (r = 0.964, P < 0.025) [Figure 3].

DISCUSSION

In the case of atypical sperm, whether immature or subject to external stress, the DNA can fragment and lose its functional integrity. DNA fragmentation is often the cause of reproductive failure. In addition, several studies have shown that the p53 molecule is involved in maintaining the integrity of the DNA sperm.

Today, the AO test is one of the most popular methods used to verify the integrity of sperm DNA. However, it is not an objective methodology as it is linked to the individual operator's experience. In order to achieve greater objectivity, it is advisable to use an alternative method.

In this study we analyzed 103 samples of seminal fluid with both the AO test and the ELISA methodology for the quantitative p53 assay. The results were expressed in sDFI/tsc and corrected p53, respectively.

The evaluation of the data shows a correlation between sDFI/tsc and corrected p53.

We therefore suggest that the p53 ELISA method for assessing sperm DNA damage may be a viable alternative to the AO test and all interpretation tests of DNA sperm integrity currently used by laboratories. In fact, this method looks promising for routine use in the laboratory, as it enables to analyse multiple samples simultaneously, ensuring greater precision, repeatability and accuracy [Figure 4].

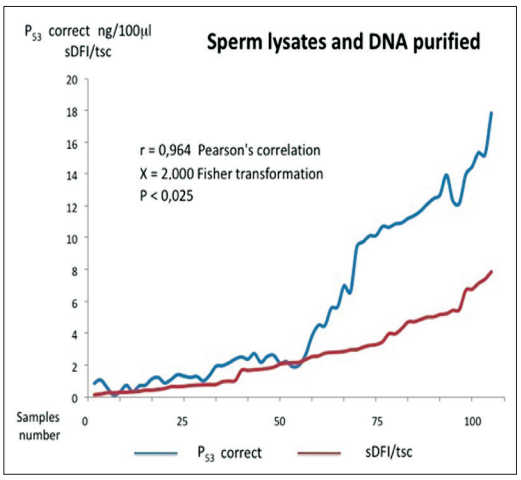

Figure 3: Graphical and statistical analysis of the results obtained

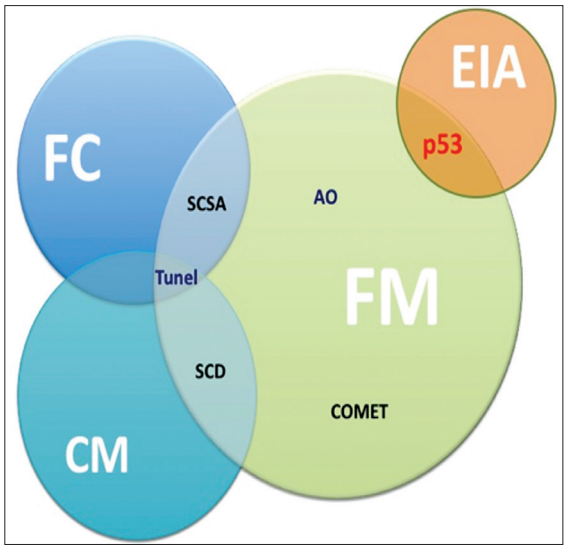

Figure 4: p53 ELISA assay correlates with the methods currently used to assess DNA sperm fragmentation

CONCLUSIONS

Although our findings need to be confirmed by further studies, the corrected p53 value could be really useful in the assessment of DNA sperm damage. The test could represent a powerful tool in order to prove the efficacy of treatment protocols, in view of a medically assisted procreation and as a predictive test for procreation *in vivo*.

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