DNA DAMAGE MEASUREMENT USING A MODIFIED PROTOCOL OF SPERM CHROMATIN DISPERSION TEST

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Summary

Introduction: Infertility affects approximately 15% of all couples trying to conceive, male factor is the sole or contributing factor in roughly half of cases infertility. Men with normal semen analysis may also have a high degree of DNA fragmentation, which can be a major cause of undiagnosed/unexplained infertility. In this work, we estimate the level of damage DNA, using a modified protocol of the Sperm Chromatin Dispersion (SCD) test. **Materials and Methods**: We performed a DNA controlled denaturation to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. Later, nuclear proteins were removed to form nucleoids with a central core and a peripheral halo of dispersed DNA loops. Such DNA halos are absent or extremely small in nucleoids with nonfragmented DNA. To demonstrate the sensitivity of the SCD test, aliquots of sperm samples, from two different subjects were exposed to increasing concentrations of the nitric oxide donor sodium nitroprusside (SNP), for 1 hour at room temperature, to induce DNA damage. To estimate interobserver variability in sperm cells scoring, three technicians analyzed 500 sperm cells from each sample, three times a day. Means of DNA fragmentation level obtained by the observers of each sample were compared. **Results and Conclusions:** The modified protocol of SCD test was sensible to detect DNA fragmentation from 60 μM of SNP. Analysed samples showed a dose–response effect by SNP. There wasn't significant differences (Pearson Test χ^2, $P>0.05$) in the mean of sperm cells with fragmented DNA observed, among the observers. SCD is a simple, fast, cheap and reproducible technique does not require complex or expensive equipments. Sperm DNA damage can be determined with accuracy using this assay, in laboratories with a basic instrumentation. The analysis can be carried out with conventional bright-field microscopes.

Key words: Biomarkers, DNA oxidative stress, sperm chromatin dispersion test

Introduction

Data accumulated over the past few decades indicate that male factor infertility plays a rolein approximately 50% of infertile couples(1). Normal sperm genetic material is required for fertilization, embryo and fetal development and postnatal child well being. Abnormal DNA can lead to derangements in any of these processes. Defects in the genomic material may take the form of condensation or nuclear maturity defects, DNA breaks or DNA integrity defects and sperm chromosomal aneuploidy. Recently, sperm DNA has been recognized as an independent measure of sperm quality. The quality of sperm DNA has been shown to affect fertility both in vivo and in vitro (2).

In some cases, standard investigations of the infertile couple reveal no detectable abnormalities, and therefore the couple is diagnosed with unexplained or idiopathic infertility. It is clear that abnormalities in the male genome characterized by damaged DNA may be indicative of male subfertility regardless of the semen analysis. The evaluation of sperm DNA integrity, in addition to routine sperm parameters, could add further information on the quality of spermatozoa. This could help in the correct identification of male infertility and in advising couples on the management of infertility.

Multiple techniques are reported to measure sperm DNA defects in human spermatozoa (3,4). Some methods like terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphatenick end labeling (TUNEL) (5), comet assay (6),, and sperm chromatin structure assay (SCSA(7-12)) can evaluate the integrity of sperm DNA. Recently, a new method, the sperm chromatin dispersion test (SCD) (13), was introduced for evaluating sperm DNA fragmentation. The objective of this study was estimate the level of DNA fragmentation, using a modified procedure of the SCD test.

Methods

Semen samples voluntarily donated were incubated for 30 minutes at 37^{o}C and diluted to 10^{7} per mL in PBS, to obtain semen suspensions. Such suspensions were mixed with LGT agarose 1 % and extended in a previously cover with 0.65% regular agarose microscope slide. We performed a DNA controlled denaturation to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. Then, slides were treated with HCl (0.08 N) 7 minutes at room temperature (RT) and placed in lysing solution (NaCl 2.5 M, EDTA 100 mM, Tris10 mM, Triton X-100 1ml y DTT 25 mM) for 15 minutes at RT, to remove nuclear proteins and form nucleoids, with a central core and a peripheral halo of dispersed DNA loops. Such DNA halos are absent or extremely small in nucleoids with nonfragmented DNA. After, we wash the slides during 5 minutes in a coplin with abundant distillated water. Silver staining was performed using a stain solution (0.47 M Na_2CO_3, 5 mM $AgNO_3$, 10 mM NH_4NO_3, 0.86 M tungstofosforic acid, 15 mM formaldehyde). Acetic acid 1% was used to stop staining. Then, slides were washed 3 times with distillated water and dried at RT. Using 40 X objective of a Bright Field microscope, 500 cells per sample were counted. Evaluation criteria used was that cells in levels 1 and 2 were considered nonfragmented. Level 1, big halo comprehends those cells with a halo width similar or higher than the minor diameter of the core. Level 2, medium halo were those cells with a halo size between big and with very small halo. Fragmented classification includes cells in levels 3, 4 and 5. Level 3, small halo, corresponds to cells with a halo width similar or smaller than one-third of the minor diameter of the core. Level 4, no halo, were cells without a halo. Level 5, degraded, includes level 4 like-cells, weakly or irregularly stained. Other nucleoids usually without tails and big, corresponding to spermatids, epithelial cells, leukocytes and other nongerm cell, were ignore in scoring (13).

Intraobserver variability in scoring was determined by scoring DNA fragmentation gel three times a day, per sample, per technician. To estimate interobserver variability in sperm cells scoring, three technicians analyzed 500 sperm cells from each sample. Means of DNA fragmentation level obtained by the observers of each sample were compared using Pearson's χ^2 test . In order to demonstrate the sensitivity of the SCD test, aliquots of sperm samples, from two different subjects were exposed to increasing concentrations of the nitric oxide donor, Sodium Nitroprusside (SNP), for 1 hour at room temperature, to induce DNA fragmentation.

Results

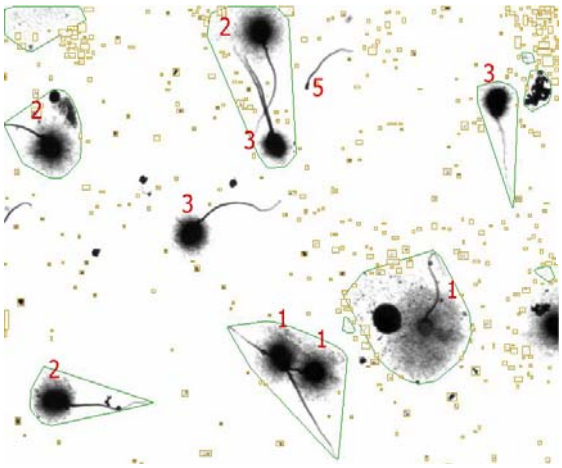

Figure 1: General view of a field, containing fragmented and nonfragmented DNA spermatozoa.

Table1. Evaluation of DNA fragmentation using SCD assay.

Observer	Big halo %	Médium halo %	Small halo %	No halo %	degraded %	fragmented %
1	7.6	16.4	59	12.2	4.8	**76**
	6.2	18.8	60	10	5	**75**
	6	25.4	57	12	5.6	**74.6**
2	5.6	18.4	55.2	16.8	4	**76**
	3.4	25.2	48.8	16	6.6	**72**
	5.2	21	53.6	16.2	4	**73.8**
3	1.6	23.4	46.6	22.4	6	**77**
	2.2	23.2	50.8	19.4	4.4	**75**
	2.4	24	47.5	21.1	5	**73.6**

Table 2. Evaluation of DNA fragmentation of spermatozoa treated with Sodium Nitroprusside, using SCD assay.

Subject	**SNP**	**Big halo**	**Médium halo**	**Small halo**	**No halo**	**degraded**	**fragmented**
	(μM)	%	%	%	%	%	%
1	0	51.4	23.6	11.2	10.8	3	**25**
	60	12	44	22.8	16.8	3.4	**42.8**
	250	5.6	18.4	55.2	16.8	4	**76**
2	0	49.6	22.7	4	1.2	1	**6.2**
	60	42.8	46	5.4	4.4	1.4	**11.2**
	250	7	76.6	14	2.4	0	**16.4**

Discussion

Infertility affects approximately 15% of all couples trying to conceive, male factor is the sole or contributing factor in roughly half of cases infertility. Though, sperm analysis is the most used test to predict male infertility, standard parameters, such as: spermatozoa concentration, mobility and normal morphology do not reveal defects of the genetic material. Sperm DNA fragmentation is being increasingly recognized as an important cause of infertility. Thus, men with normal semen analysis may also have a high degree of DNA fragmentation, which can be a major cause of undiagnosed / unexplained infertility.

A number of tests are currently available for the sperm DNA fragmentation measurement (3,4), the TUNEL assay (5), the Comet assay (6), the Chromomycin A3 test (14), DNA Breakage Detection-Fluorescence In Situ Hybridization (DBD-FISH) test (15-17) , and the SCSA test (7-12) and Sperm Chromatin Dispersion (SCD) test (13). Sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed in sperm with nonfragmented DNA, following acid denaturation and removal of nuclear proteins, this is the base of SCD assay. In the present work, we estimate the level of DNA fragmentation, using a modified procedure of the SCD test.

Our work team introduced some changes to the original SCD procedure. Ditiotreitol concentration was reduced from 0.8 M to 25 mM**.** Wright staining was substituted for silver staining, commonly used in our laboratory. Introduced modifications did not affect chromatin integrity or density, which allows a proper staining and discrimination among fragmentation levels. Spermatozoa tails remains attached to the head during the process, it make easier to identify germinal cell among other that could be present in the analysed fluid (Figure 1). Besides, one sample analysis using commercial kits cost about 32 USD dollars, our version reduce the cost 28 folds.

The standardized version was sensible to detect interindividual variation in sperm DNA fragmentation as can be see it in Table 1. Intra and Interindividual variability in DNA fragmentation scoring, using the modified SCD procedure, was quite low, reflecting the easy and reliability of the test endpoints. Although visual scoring is clearly precise, we do not discard the usefulness of imaging analysis software integrated to microscope coupled in the slides processing.

Nitric oxide (NO) is an important regulator of several physiological processes. In sperm NO, may be produced during capacitation and acrosome reaction and might react with superoxide radical yielding peroxinitrite, a potent DNA fragmentation inductor. Thus, in order to demonstrate the sensitivity of SCD test in the induced DNA fragmentation detection; aliquots of sperm samples from two subjects were exposed to increasing concentration of SNP. A dose response in the fragmentation frequencies was evident, starting from a concentration of 60 μM SNP (Table 2)

Conclusions

- SCD is a simple, fast, cheap and reproducible technique does not require complex or expensive equipments. The modified procedure of SCD test was sensible to detect DNA fragmentation from 60 μM of SNP.
- Analyzed samples showed a dose–response effect by SNP.
- There wasn't significant differences (Pearson Test χ^2, P>05) in the mean of sperm cells with fragmented DNA observed, among the observers.
- Sperm DNA damage can be determined with accuracy using this assay, in laboratories with a basic instrumentation. The analysis can be carried out with conventional bright-field microscopes.

We recommended the use of the modified procedure of SCD, as a routine test in the Cuban Genetic Network laboratories, for the studies of idiopathic male infertility and individuals above 40 year old suffering varicocele grade I y II and in couples with a history of repeated abortion.

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