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Research Article

Sperm DNA Fragmentation is Significantly Increased in Those Men with Morphologically Abnormal Spermatozoa

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Abstract

Purpose: To evaluate the levels of DNA fragmentation regarding to spermatozoa morphology in male infertility patients attending Andrology Laboratory at fertility center.

Methods: Semen samples of 196 patients were analyzed using computer-assisted semen analysis (CASA). Sperm DNA fragmentation was measured by the sperm chromatin dispersion test. According to the values of sperm morphology, the groups were conformed by teratozoospermic (105 patients) and normozoospermic (91 patients) men.

Results: Subjects' ages ranged from 21 to 68 years. Teratozoospermic men were older that the normozoospermic men (39.72 ± 7.86 vs. 36.57 ± 6.29 years; $P<0.05$). Values of pH were similar in both evaluated groups (P:NS). Parameters of volume, concentration, motility and vitality in teratozoospermic men were significantly lower compared to those normozoospermic patients ($P<0.05$). High levels of DNA fragmentation were observed in those patients with abnormal sperm morphology compared to normozoospermic men (44.31 ± 7.52 vs. 34.92 ± 5.89; $P<0.05$).

Conclusions: Men with abnormal spermatozoa morphology showed high levels of DNA fragmentation. These significantly high percentages of sperm DNA damage will be an additional factor that drastically reduces the possibility of success in these infertile men.

Keywords: Teratozoospermia; Sperm DNA fragmentation; SCD test; Infertility; Semen parameters

Introduction

Semen quality is frequently used as an indirect measure of male infertility. Ejaculate volume, sperm concentration, motility and morphology determined according to the World Health Organisation (WHO) are the most important parameters evaluated in infertility centers as part of routine semen analysis. Spermatozoa with abnormal morphology or Teratozoospermia have been associated with infertility and the Intracytoplasmic Sperm Injection (ICSI) technique is frequently used as the treatment of choice. However, several concerns about safety and impact of ICSI on the offspring have been raised due to the forced injection of putative abnormal spermatozoa [1]. As a result, sperm with morphology and normal genetic material is required for successful fertilization, as well as for further embryo and fetal development that will result in healthy offspring [2-6].

Teratozoospermia is usually defined as ≤ 4% normal sperm morphology at semen analysis with normal sperm count and normal progressive motility [7]. Many studies have shown that semen samples with teratozoospermia produce lower fertilization rates when conventional IVF was used [8-10].

Currently, the integrity of sperm DNA is being recognized as a new parameter of semen quality and a marker of male infertility [11,12]. Sperm DNA fragmentation can be caused by apoptosis in the seminiferous tubule epithelium, defects in chromatin remodeling during the process of spermiogenesis, oxygen radical-induced DNA damage during sperm migration from the seminiferous tubules to the epididymis, the activation of sperm caspases and endonucleases, damage induced by chemotherapy and radiotherapy, and the effect of environmental toxicants [13]. In humans, high levels of sperm nuclear DNA damage have been related to low fertility potential, failure to obtain blastocysts, blockage in embryo development after embryo implantation, increased risk of recurrent miscarriages, reduced chances of successful implantation, and negative effect on the health of the offspring [13-17].

During the past two decades, a number of tests have been introduced for the analysis of sperm DNA fragmentation. These tests include TUNEL assay [18], Comet assay [19], CMA3 [20], *in situ* nick translation [21], DNA breakage detection fluorescence in situ hybridization (DBD-FISH) [22], SCD [23], and the SCSA [24]. In the present study, the presence or absence of DNA fragmentation was determined by examining the halo size utilizing the SCD test; a simple, highly reproducible and less expensive technique, yielding results highly correlated with those from other procedures like the DBD-FISH and the SCSA [25]. Chohan et al. [26] shown that the percentage of sperm that failed to show the characteristic halo of dispersed DNA loops under SCD correlated well with SCSA %DFI values and the percent of TUNEL-positive cells.

The aim of the present study was to evaluate the levels of DNA fragmentation in a population of teratozoospermic patients and to compare to those levels observed in normozoospermic men.

Materials and Methods

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Patients

A total of 196 patients for infertility evaluation at Andrology Laboratory, FERTILAB Laboratory of Assisted Reproduction Lima, Peru were included in this study. From the total of patients, the study group consisted of one hundred and five teratozoospermic men (<4% normal morphology sperm; WHO) and ninety-one normozoospermic men were the control group. All the patients had a normal 46, XY karyotype, a testicular volume within the normal range, no history of radiotherapy, chemotherapy, chronic illness, medication or varicocele. This protocol was approved by the Institutional Review Board (IRB) and the corresponding Ethics Committee.

Semen samples

The semen samples were collected by masturbation in aseptic conditions into sterile cups after 3-5 days of sexual abstinence. Semen analysis was performed following semen liquefaction for 30 min at room temperature. Seminal volume, seminal pH, sperm motility, sperm morphology, and sperm concentration were assessed using CASA according to World Health Organization guidelines [7].

Sperm DNA fragmentation assessment

The sperm DNA damage was evaluated by Sperm Chromatin Dispersion (SCD) test [23] using the Halosperm® Kit (Halotech Dna, Spain). Sperm samples, which contained not <5 million and not >10 million spermatozoa per milliliter after dilution, were used. The kit contains aliquots of agarose gel in Eppendorf tubes. Each semen sample was processed after the agarose gelled (from immersion in a water bath at 90ºC for 5 min). When the Eppendorf tubes reached a temperature of 37ºC (5 min at 37ºC in a dry atmosphere), 25 µL of sperm were added and gently mixed. Twenty microliters of this mixture were placed on precoated slides and covered with 22x22 mm coverslide. The slides were maintained at 4ºC for 5 minutes to produce a microgel containing embedded spermatozoa. The coverslides were gently removed, and the slides were immersed in a previously prepared acid solution (80 µL of HCl added to 10 mL of distilled water) for 7 minutes. After removal from this solution, the slides were incubated for 25 minutes in 10 mL of lysing solution (provided in the Halosperm kit). After rinsing in distilled water, the slides were dehydrated for 2 minutes in three concentrations of alcohol (70%. 90% and 100% vol) for 2 minutes each and either were stored (storage was possible several months in optimal conditions) or were processed immediately with staining solution for 10 minutes with continuous airflow. Staining was performed with 1:1 (vol/vol) by using Wright's solution (Merck, Darmstadt, Germany) and phosphate-buffered saline solution (Merck). The slides were rinsed in tap water, allowed to dry at room temperature, processed for upright or inverted bright-field microscopy at 100X, and covered with 22x22 coverslide. Operators scored ≥ 500 spermatozoa for each patient according to the patterns established by Fernández *et al.* [23]. Strong staining is preferred to visualize the dispersed DNA loop halos. Removal of sperm nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. The sperm tails remain preserved. The acid treatment produces DNA unwinding that is restricted in those nuclei with high levels of DNA strand breakage. After the subsequent lysis, sperm nuclei with fragmented DNA produce very small or no halos of dispersed DNA. However, nuclei without DNA fragmentation release their DNA loops to form large halos.

Statistical Analysis

Statistical analysis was carried out using the statistic package Stata 10 (StataCorp, College Station, TX). Data are represented as Mean ± SD. Group comparisons were made using the χ^2 test and Student's t-test. It was considered a statistical significant difference when $P<0.05$.

	Teratozoospermic men	Normozoospermic men	P *value*
Patients	105	91	
Age (Mean ± SD)	39.72 ± 7.86*	36.57 ± 6.29	0.0012
Volume (mL)	2.91 ± 1.32*	3.28 ± 1.51	0.0241
pH	7.93 ± 0.36	7.91 ± 0.37	0.3491
Concentration (x10^6/mL)	219.76 ± 192.03*	290.68 ± 191.40	0.0004
Motility (%)	40.24 ± 17.38*	59.29 ± 15.40	0.0001
Vitality (%)	82.26 ± 8.94*	88.44 ± 6.47	0.0001
Round cells (x10^6/mL)	4.10 ± 3.14*	3.26 ± 1.89	0.0121
Leukocytes (x10^6/mL)	0.42 ± 0.19*	0.37 ± 0.10	0.0112

Table 1: Descriptive statistics and comparison between four evaluated age groups.

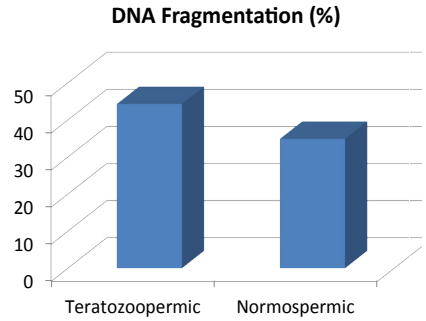

Figure 1: Percentages of sperm DNA fragmentation in both evaluated groups.

Results

A total of 196 seminal samples from infertile men were analyzed regarding the semen parameters and DNA fragmentation. The results of the basic semen parameters are shown in Table 1. Values of volume, concentration, motility and vitality were significantly lower in teratozoospermic men group compared to the normozoospermic men ($P<0.05$). The pH values were similar between both evaluated groups (P:NS).

On the other hand, we showed that the percentages of sperm DNA fragmentation increases with advancing age. These values are significantly high in men with teratozoospermia compared to those values from normozoospermic men ($P<0.05$) (Figure 1).

The regression analysis showed a significant association between DNA fragmentation and age (a; $r=0.208$; $p=0.012$) (Figure 2a) as well as morphology (b; $r=2.464$; $p=0.000$) (Figure 2b).

Discussion

The information obtained by the conventional sperm parameters reflects to a certain extent the quality of the spermatogenic process, which determines the functional competence of the spermatozoa and therefore the fertilizing potential of the ejaculate [27]. Sperm concentration, motility and morphology have been correlated with fertilization rates *in vivo* and *in vitro* [28]. Of these, several clinical studies have demonstrated a clear association between sperm head morphology and improvement in IVF success rate [29,30]. There are several methods for the evaluation of sperm morphology but the results are highly variable, causing difficulty in the interpretation of results. The CASA techniques facilitate a more detailed morphology analysis and makes detection of subtle variations in sperm head morphometry possible, which was used in the present study.

On the other hand, normal sperm genetic material is required

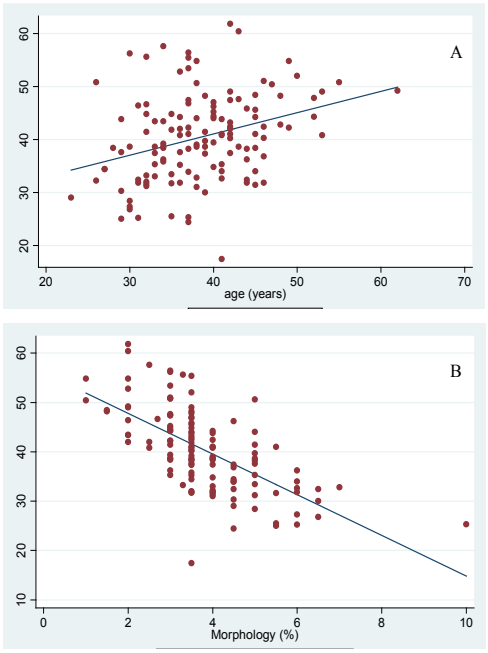

Figure 2: Scatter graph illustrating associations between DNA fragmentation and age (a; r=0.208; p=0.012), as well as morphology (b; r=2.464; p=0.000).

for successful fertilization, as well as for further embryo and fetal development that will result in healthy offspring. Sperm DNA contributes half of the offspring's genomic material and abnormal DNA can lead to derangements in the reproductive process. Sperm DNA damage has been attributed to a variety of intra and extratesticular factors [31]. The most important is the production of Reactive Oxygen Species (ROS), which is excited by excessive stress, competitive sports, alcohol and drug abuse or nicotine. If produced in abundance, ROS can enter the cell nucleus, bind to the DNA and cause its fragmentation [32-35]. Several studies have reported that redox balance is deregulated in the ejaculates from infertile males, with glutathione peroxidase 4 as one of the main enzymes involved in this issue [36]. Collateral effects of various pathological iatrogenic and environmental factors include cancer, antineoplastic drugs [37], varicocele [38], high fever [39], leukocytospermia [40] and also advanced male age [41,42]. However, DNA fragmentation is also a feature of physiological processes like apoptosis and necrosis [43,44].

Previous studies have demonstrated a positive correlation between teratozoospermia and DNA fragmentation rate [45,46]. Some head abnormalities are associated, with overall increase in head length with minor deviation in width, and the percentage coverage of acrosome in head is decreased, resulting in poorly packed chromatin and an increase in the incidence of chromosomal aneuploidy [47,48]. Therefore, spermatozoa with tapered heads were also reported to have a higher incidence of failed fertilization rates post ICSI due to chromatin abnormalities [49]. Similarly, Abdelrazik *et al.* [50] analyzed sperm morphology using computer assisted morphometry, and demonstrated that spermatozoa with several abnormal forms (in particular amorphous and micro heads) containing immature chromatin and higher DNA fragmentation rate compared with other forms of head abnormalities resulting in an increase in aneuploidy incidence and mutations in the germ line [44,51]. In the present study, it was demonstrated that DNA fragmentation was significantly higher in teratozoospermic men compared with normozoospermic men, similar to the results shown by several authors [44,48,50,52,53].

In assisted reproduction programs, several studies show that high levels of sperm DNA fragmentation are related to lower pregnancies rates either natural or using IUI, IVF or ICSI procedures [54-57] and higher aneuploidies rates in embryos [52,58,59]. Greco *et al.* [60] reported 29 ICSI cycles in which the percentage of DNA-fragmented spermatozoa, detected by TUNEL assay, was >15%; only two pregnancies and no births were obtained. Muriel *et al.* [61] and Benchaib *et al.* [16] showed that the DNA fragmentation level was inversely correlated with fertilization rate, embryo quality to achieve blastocyst stage, and embryo morphological quality. Additionally, high incidence of DNA fragmentation has been frequently observed among infertile couples with unexplained aetiologies and with recurrent pregnancy failures and high abortion rates [62,63].

On the other hand, several techniques have been proposed to select sperm with lower DNA fragmentation, like the use of Annexin-V, a protein that binds specifically to phosphatidylserine and enables the identification of apoptotic cells [45] and significantly reduce the percentage of spermatozoa with DNA fragmentation, and a sperm selection method based on sperm Hyaluronic Acid (HA) binding [64]. HA-bound spermatozoa show low chromosomal aneuploidies and DNA fragmentation, and good nuclear morphology [65].

Finally, our study demonstrated that men with abnormal spermatozoa morphology show high levels of DNA fragmentation. These significantly high percentages of sperm DNA damage will be additional factors that drastically reduce the possibility of success in these infertile men.

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