

The study of spermatic DNA fragmentation and sperm motility in infertile subjects

Giuseppina Peluso¹, Alessandro Palmieri², Pietro Paolo Cozza¹, Giancarlo Morrone¹, Paolo Verze², Nicola Longo², Vincenzo Mirone²

¹U.O.S. of Andrology and Physiopathology of Reproduction-A.O. of Cosenza, Italy;

²Urological Clinic, University Federico II of Naples, Italy.

Summary

Introduction: Although the pathophysiology of the testicular damage associated with varicocele remains unclear, sperm DNA damage has been identified as a potential explanation for this cause of male infertility. The current study was designed to determine the extent of sperm nuclear DNA damage in patients with varicocele, and to examine its relationship with parameters of seminal motility.

Materials and method: Semen samples from 60 patients with clinical varicocele and 90 infertile men without varicocele were examined. Varicocele sperm samples were classified as normal or pathological according to the 1999 World Health Organization guidelines. Sperm DNA damage was evaluated using the Halosperm kit, an improved Sperm Chromatin Dispersion (SCD) test.

Results: The DNA fragmentation index (DFI: percentage of sperm with denatured nuclei) values was significantly higher in patients with varicocele, either with normal or abnormal (DFI 25.8 ± 3.2 vs 17.4 ± 2.8 - $P < 0,01$) semen profiles. In addition, an inverse correlation was found between spermatic motility and the degree of spermatic DNA fragmentation in patients with clinical varicocele.

Conclusions: Varicocele is associated with high levels of DNA-damage in spermatozoa. In addition, in subjects with varicocele, abnormal spermatozoa motility is associated with higher levels of sperm DNA fragmentation. DNA fragmentation may therefore be an essential additional diagnostic test that should be recommended for patients with clinical varicocele.

KEY WORDS: Spermatic DNA fragmentation; Oxidative stress; Varicocele; Male infertility.

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INTRODUCTION

Varicocele is one of the major causes of infertility in men, present in between 15-20% of the general male population (1, 2). It's predominant in adolescents but found in 41% infertile men and 80% of those with secondary infertility (2). This anatomical abnormality due to the dilation of the venous plexus which covers the testicles (pampiniform plexus) is probably one of the most common causes of oligoasthenozoospermia (1, 3). The pathogenetic mechanism through which varicocele causes testicular dysfunction and subsequent alterations in spermatogenesis, is, however, not completely understood. Although various factors may be involved (venous stasis which leads to testicular hypoxia) the increasing of internal testicular tem-

perature is probably the most likely link between varicocele and infertility. In fact, elevated scrotal temperature caused by vascular defects can cause altered production of spermatogenic cells by germinal epithelium (1, 3). In fact, induced varicocele in laboratory animals leads to elevated intratesticular temperature and sperm dysfunction with decreased sperm motility (1, 3).

Another link between varicocele and infertility may arise from impairment of the hypothalamic-gonadal axis or oxidative stress (OS) (4, 5). In fact, studies evaluating the role of oxidative stress in male infertility have recently shown that OS could be considered as an important cause of sperm dysfunction in varicocele infertile men (6-8),

with negative impact on sperm plasma membranes which contain higher amounts of polyunsaturated fatty acids (PUFA) which easily experience lipid peroxidation by ROS (9, 10). It is a result of cascade of events including lipid peroxidation (LPO) of sperm plasma membrane that ultimately affect an axonemal protein phosphorylation and sperm immobilization (9-11). These spermatozoa would therefore not only be particularly susceptible to reactive oxygen species (ROS) (12), with compromised membrane fluidity and integrity and greatly reduced motility (11-13). Metanalysis studies support this hypothesis, demonstrating that infertile patients with varicocele have higher levels of ROS when compared to other typologies of infertility and controls (14, 15). In addition, the seminal plasma of varicocele patients exhibited reduced total antioxidant capacity (TAC) (7, 15-18). Recently, various studies have also demonstrated that an elevated presence of DNA fragmentation is present in the spermatozoon nuclei of infertile patients with clinical varicocele (19, 18). Interestingly, DNA damage are higher when compared to spermatozoon of patients suffering from other types of infertility; moreover the spermatozoon of infertile varicocele patients exhibit higher levels of ROS, suggesting correlation between oxidative damage and DNA fragmentation. Studies using rat models have confirmed this, demonstrating that nitric oxide (NO) released by endothelial cells of dilated spermatic veins and peroxynitrites generated from reaction with superoxide radicals cause intracellular oxidative damage (20-22), particularly regarding membrane lipid peroxidation, thus altering the integrity of chromatin in spermatozoon nuclei which may be a direct expression of such oxidative damage (17). In addition, sperms of varicocele patients exhibit elevated levels of 8-hydroxy-2 deoxyguanosine which are associated with a deficiency in the pro-oxidant defense system which would cause oxidative damage to the DNA by modifying the base, DNA strand breaks and chromatin cross linking, since spermatozoa have limited defense mechanisms against oxidative attack on their DNA mainly due to its exclusive structural composition for the complex packaging arrangement of DNA (18, 23, 24).

Other indications of increased ROS in patients with varicocele are elevated quantities of cytoplasmic droplets in the young spermatozoa. The droplets are indicative of immaturity and functionally defective spermatozoon (25-27) and, containing high concentrations of cytoplasmic enzymes such as G6PDH and SOD, are additional sources of ROS (21, 26). Together, these studies indicate a positive correlation between spermatozoon immaturity, elevated levels of ROS and increase of concentration of mature spermatozoa with damaged DNA in ejaculates of these patients (21, 25-27).

Therefore, in this study, DNA fragmentation in spermatozoa of infertile individuals diagnosed with clinical varicocele was quantified with respect to infertile men and controls. In addition, the amount of DNA fragmentation was correlated to sperm motility.

MATERIALS AND METHODS

One hundred and fifty subjects analyzed in this study ranged in age between 20-50 with the median age of 35

and attending to Andrology Service for infertility diagnosis and other andrological problems. Once consensus had been granted to be involved in this study, patients were given a thorough andrological analysis according to the guidelines provided by WHO 2001 which included complete anamnesis, scrotal scan, scrotal Doppler ultrasound, hormonal profile based on serum/blood levels basal gonadotrophines (FSH, LH and total blood Testosterone), urethral sample for common microbes, two spermograms at a week's interval with a minimum of three days since sexual activity. Considering the results of these analysis, it was possible to identify 80 patients with varying degrees of clinical varicocele but predominantly unilateral. All patients with urinary tract infections, leucocytospermia, hypogonadism (testicular volume < 15 ml), a history of excess of cigarette, alcohol or drug use were excluded from the study. The control group consisted of 100 healthy males with normal genitalia and normal seminal parameters, as defined by the WHO 2001 guidelines.

For each patient included in the study, the spermatozoon population was determined. In addition, sperm DNA fragmentation was quantified using the sperm chromatin dispersion (SCD) test (*Halosperm kit-Indas Laboratories, Madrid Spain*) which allows to express the spermatic DNA fragmentation as a percentage index (*DFI: DNA Fragmentation Index*). On the basis of the usual seminologic criteria, 34 patients with varicocele showed isolated asthenospermia, whereas spermatic concentration and morphology have normal values.

Patients with severe dispermia in conjunction with abnormal standard semen parameters such as oligoasthenozoospermia (OAT) where 3%: in such case high levels of damaged sperm DNA are usually observed, consequently they were excluded from this study. Patients considered in this phase of the study were therefore characterized by asthenozoospermia with various degrees of motility, ranging from 5-45% of the progressive linear a+b motility, according to the cut-off of normal sperm motility established by WHO, where a+b is greater than or equal to 50% values.

The degree of DNA damage in spermatozoa of these patients with only altered motility parameter was then correlated with the a+b motility seminal parameter.

ANALYSIS OF SPERM DNA FRAGMENTATION

Spermatozoon DNA fragmentation was quantified using *Halosperm (Diasint-CGA, Florence-Italy)* which has been used in the well-developed sperm chromatin dispersion (SCD) test (16, 17). The basis of the technology lies in the differential response offered by the nuclei of spermatozoa with fragmented DNA compared to those with their DNA intact.

The controlled denaturation of the DNA followed by the extraction of the nuclear proteins, gives rise to partially deproteinized nucleoids in which the DNA loops expand, forming halos of chromatin dispersion.

The nucleoid, which corresponds to the massively deproteinized nucleus of the spermatozoon, is composed of two parts: spermatozoon nucleus silhouette, called the "core", positioned centrally, and a peripheral halo of chro-

matin/DNA dispersion. Likewise, when DNA fragmentation is present, the nucleoid do not exhibit a dispersion hallow or, if present, is negligible.

The tail of the spermatozoon is visible and serves as an important morphological parameter to distinguish the nuclei of nemaspermic cells from others.

An aliquot of each sperm sample was diluted in phosphate buffer solution (PBS) to a concentration of 5 million/ml; 25 µl of each sample was mixed and resuspended in fused agarose microgel, as provided by the kit. 20 µl of semen-agarose mix was pipetted onto an agarose pre-coated slide, provided in the kit, and covered with a 22-x 22-mm coverslip. The slide was placed on a cold plate in the refrigerator (4°C) for 5 minutes to allow the agarose to produce a microgel with the sperm cells trapped. The coverslip was gently removed and the slide immediately immersed horizontally in a denatured solution, previously prepared by mixing 80 µl of HCL from an Eppendorf tube in the kit, with 10 ml of distilled water, and incubated for 7 minutes at room temperature before transfer to 10 ml of lysing solution and left to incubate for 25 minutes. After washing 5 minutes in a tray with abundant distilled water, the slides were dehydrated in increasing ethanol bath (70%-90%-100%) for 2 minutes each and air dried at room temperature. For the latter, the slides were horizontally covered with a mix of Wright's solution (*Merck, Darmstadt, Germany*) and phosphate buffer solution (*Merck*) (1:1) for 5 to 10 minutes, with continuous airflow.

Then the slides were briefly washed in tap water and allowed to dry. Strong staining is preferred to easily visualize the periphery of the dispersed DNA loop halos. A minimum of 500 spermatozoa for sample were scored under the 100x objective of the microscope.

SCORING CRITERIA

The categorization of the different halo sizes is performed using the minor diameter of the core from the own nucleoid as a reference to which the halo width is compared.

Five SCD patterns were established (28):

- a) Sperm cells with large halos: those whose halo width is similar or higher than the smallest diameter of the core.
- b) Sperm cells with medium-size halos: the halo size is between those with high and with very small halo.
- c) Sperm cells with very small-size halo: the halo width is similar or smaller than one third of the minor diameter of the core.
- d) Sperm cells without a halo.
- e) Sperm cells without a halo-degraded: similar to point d), but weakly or irregularly stained.

Sperm cells with very small halos, without halos, and without halo-degraded contain fragmented DNA. Nucleoids that do not correspond to sperm cells were separately scored.

STATISTICAL ANALYSIS

All data were calculated as average + standard deviation on experiments which were repeated and analyzed statistically using the statistical program SPSS. The statistical tests used were Student's t for continuous values and c2 for parametric values. In addition, statistical correlation was used to test non-linear regression with evaluation of the correlation coefficient.

RESULTS

The concentration of nemasperms in varicocele patients included in the study, was significantly lower than that of controls (19.8 + 6.5 e 39.7 + 7.1 respectively: P < 0.01. Motility (type a+b) considered was also significantly lower than that of controls (30.4 + 9.7 and 49.9 + 8.5 respectively: P < 0.01). In addition, the frequency of DNA damage in nemasperms of infertile patients with varicocele (% DFI) was statistically higher compared with infertile patients without varicocele (25.8 + 3.2 and 17.4 + 2.8 respectively: P < 0.01: figure 1). Besides the DFI of patients with varicocele was higher compared to values in patients with varicocele and infertile patients for other causes. As a whole the values measured is reported in Figure 1.

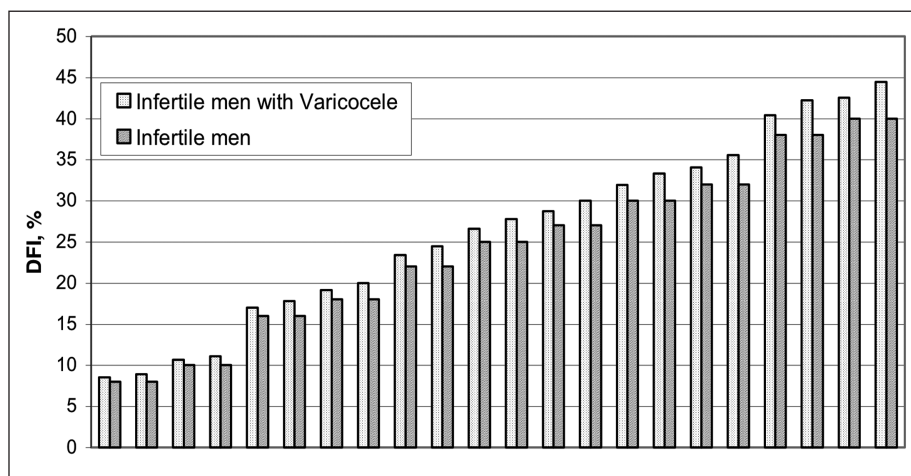
Subsequently the values were grouped together, for everyone of the two categories of patients, referred to threshold values, established equal to 15, 20, 25, 30, 35, and 45. These values referred to conditions in a range from normality to extreme pathological condition.

Therefore the average values of groups under each threshold value were calculated, and the comparison is showed in the Figure 2.

The average of these groups were evaluated for differences

Figure 1.

Comparison of DNA Fragmentation Index (DFI) experimentally evaluated for infertile men affected from varicocele (dots) or other causes (lines).



such as varicocele-DFI infertile-DFI. Such differences were correlated to the DFI values of the two categories and revealed that such differences were always positive and higher for patients with varicocele (Figure 3).

Therefore, the most significant data of our study came from the analysis of correlation between the DFI values calculated and progressive a+b motility values expressed in % and calculated in patients with varicocele associated with the condition of isolated asthenozoospermia.

Statistical analysis of the data found a semi-empirical correlation of 0.9982 between the index of percentage fragmentation (DFI) and percentage sperm motility according to the:

Eq.1: $DFI = 49,48 * EXP (-0,022 * motility)$, as seen in Figure 4.

DISCUSSION

While varicocele is one of the most common andrological pathologies in the general population, it is particularly common in infertile men (2). That varicocele negatively influences spermatid function is well documented (29, 30), although the exact underlying mechanisms are still not understood. In fact, infertility may be associated to a variety of spermatidogenic conditions ranging from normozoospermia to moderate oligoasthenoteratozoospermia (OAT), to azoospermia (4, 5).

Recently, several authors have suggested that human patients with varicocele have a significantly higher DNA fragmentation index (27). Studies show that varicocele samples contain a higher proportion of spermatozoa with abnormal DNA and immature chromatin than those from fertile men as well as infertile men without varicocele (31). A cause of this phenomena may be the increased production of ROS in varicocele patients which is significantly higher in patients diagnosed with 2° and 3° degree varicocele in whom altered sperm motility is common in these patients (8, 32).

An enhancement in OS, both due to an increase in ROS production and a decrease in the antioxidant capacity, has

Figure 2.

Comparison of DNA Fragmentation Index (DFI) experimental evaluated from different groups of infertile men. For each group, the threshold value has been selected on the basis of different relevance of the pathology.

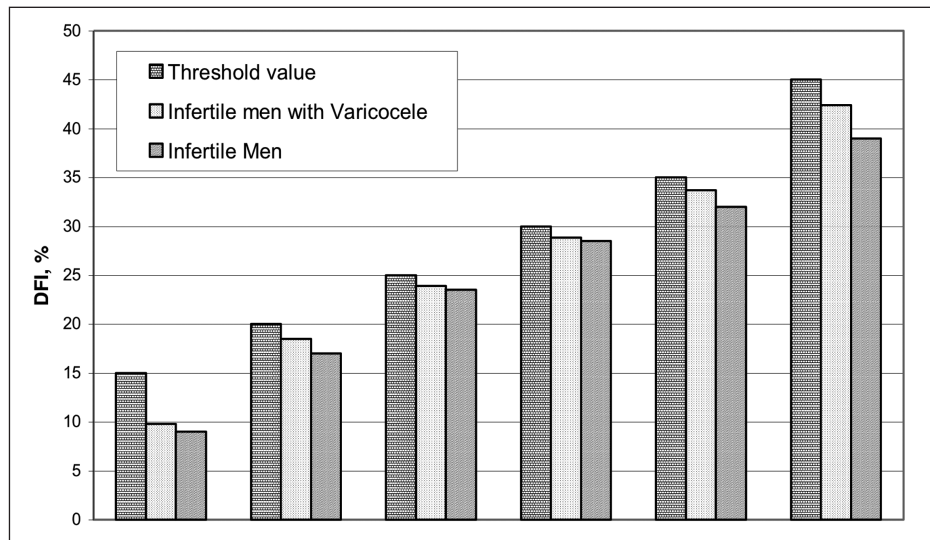
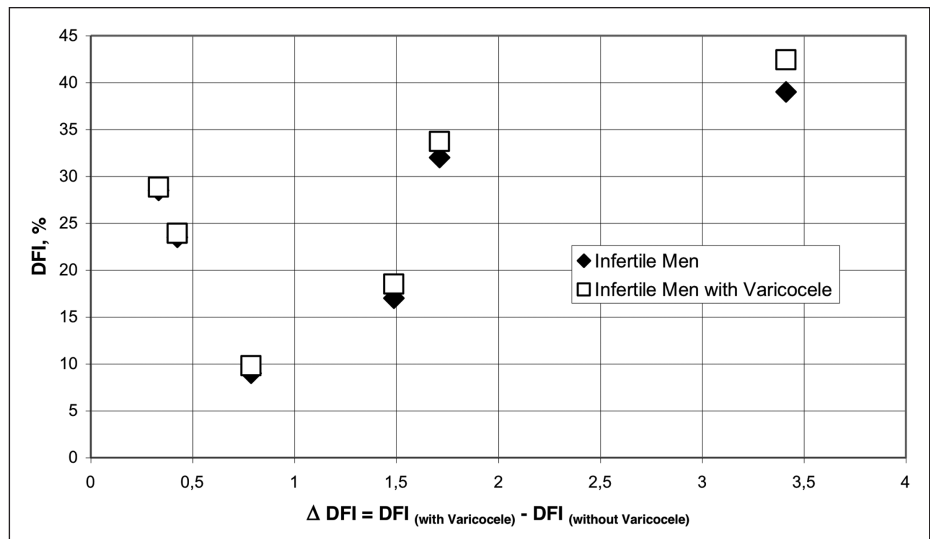


Figure 3.

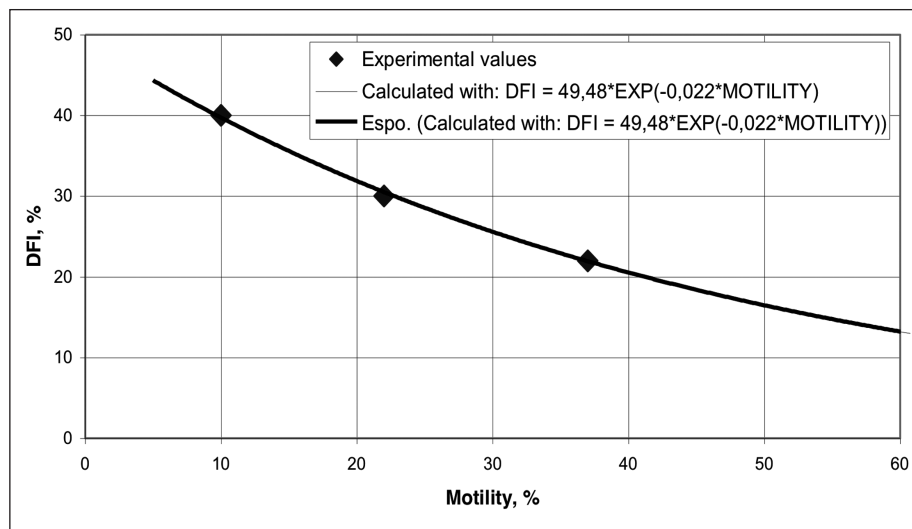
Detailed comparison of the relevance of the varicocele on the DNA Fragmentation Index (DFI) for infertile men. It's evident that DFI% is higher for varicocele infertile men.



been reported in men with varicocele (16, 17, 33, 34). NO and peroxynitrite, a potent oxidant ROS, have been demonstrated to be produced in high concentrations in the dilated spermatid veins, so they could be main contributors to the high OS level in varicocele (20, 22, 35). In addition NO can improve sperm DNA fragmentation that is associated with infertility in men with varicocele (36). Besides the dilated veins, ROS may be released in the seminiferous tubules by the cytoplasmic droplets retained in immature spermatozoa, which seem to be frequent in the sperm samples from infertile men with

Figure 4.

Relationship estimated between DNA Fragmentation Index (DFI) versus linear progressive motility (a+b) for varicocele infertile patients; (Correlation = 0,9982).



varicocele (37). Immaturity is a consequence of defective spermiogenesis that could also lead to differences in disulfide crosslinking and in susceptibility toward DNA fragmentation (38, 39).

Because all the varicocele samples in this study showed abnormal standard semen parameters, we compared them with those from idiopathic infertility, either normozoospermic patients and from patients with abnormal semen parameters, all attending the infertility clinic.

Significant differences were found between the 3 groups in the frequency of sperm cells with fragmented DNA using the Halosperm kit, particularly between the varicocele group and the infertile group, except those samples with more intense and combined abnormalities that could have more frequency of sperm cells with fragmented DNA.

Results of our study, on the higher frequency of DNA fragmentation presented in the sperm cells of infertile patients with varicocele compared to patients suffering from other typologies of infertility and the fertile controls, supporting the hypothesis which has been proposed by other authors.

Besides, a higher proportion was evidenced in our varicocele samples in relation to the fertile controls.

In addition, we also found a high inverse correlation between low sperm motility and index of DNA fragmentation in sperms of varicocele patients and patients manifesting isolated asthenozoospermia.

These findings suggest that a common pathological mechanism underlies this pathological condition. This mechanism may be the presence of ROS or other types of agents which compromise the energetic metabolism of gametes (32, 40).

These can react negatively upon sperm membranes, probably by disrupting the equilibrium between antioxidants and prooxidant. In addition, these can also act upon genomic integrity and chromatin structure of the sperma-

tozoon, damaging sperm cell DNA (36, 41).

The fact that sperms of subjects with varicocele exhibit higher levels of DNA damage with respect to sperms from other typologies of infertility, even when normal seminal parameters are attained, clearly demonstrate the importance of studying sperm DNA fragmentation (29). This evaluation can be included as a routine test in the clinical management of patients with varicocele, followed by suggestions regarding the potential negative effects that the elevated presence of damaged DNA may have on eventual future fertility (30). Future research is needed to better understand the exact mechanism by which DNA is damaged in the spermatozoon of varicocele patients so that

treatment to repair varicocele may be successful in the treatment of infertility and the reduction of such damage.

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Correspondence

Giuseppina Peluso, MD (Corresponding Author)
Via G. Verdi 82/d - 87036 Rende (CS), Italy
pina.peluso@libero.it

Pietro Paolo Cozza, MD
Giancarlo Morrone, MD
U.O.S. of Andrology and Physiopathology of Reproduction
A.O. of Cosenza, Italy

Alessandro Palmieri, MD
Paolo Verze, MD
Nicola Longo, MD
Vincenzo Mirone, MD
Urological Clinic, University Federico II of Naples, Italy