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Molecular Evidence of Apoptotic Pathway Activation in Semen Samples with High DNA Fragmentation

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Abstract. *Background/Aim: Male infertility is diagnosed by semen parameters, such as concentration, motility and morphology; however, these are not sufficient for the prediction of male fertility capacity. In the clinical routine, several other sperm functions have been introduced, including the sperm DNA fragmentation test. The objective of the present study was to evaluate sperm chromatin integrity in semen samples. Materials and Methods: Sperm chromatin dispersion test (SCD) was used in ejaculates from men divided into five groups: normozoospermic, oligozoospermic, asthenozoospermic, oligoasthenozoospermic and cryptozoospermic. Results: The data obtained showed that the SCD percentage appeared to be significantly associated with oligozoospermia diagnosis. We also evaluated total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and inhibin B serum hormonal levels in all samples examined, in order to assess whether DNA fragmentation increase could correlate with abnormal hormonal values. Finally we selected certain samples with an increasing DNA fragmentation and analyzed the molecular activated apoptotic pathways. Conclusion: A significant relationship was found between caspase-3 activation and increased DNA fragmentation.*

The golden standard in male infertility diagnosis is traditionally represented by the assessment of the semen parameters, such as concentration, motility and morphology. Although these parameters are essential to provide the fundamental information on sperm quality, however, they are not sufficient for the prediction of male fertility capacity. Indeed, they do not provide information regarding chromatin defects that represent an important component of the reproductive outcome (1). The various aetiologies like genital infections, endocrine disturbances and immunological factors have been studied extensively, as the most common causes of male subfertility. However, today, more often genetic/molecular causes are identified as contributing factors (2).

In the clinical routine, several other sperm functions have been introduced, including vital staining, antisperm antibody test, hypoosmotic swelling test, sperm penetration assay, reactive oxygen species (ROS) tests, biochemical analysis of semen and sperm DNA fragmentation test (3, 4). In particular, the assessment of DNA damage in the male germ line and its impact on reproductive outcome has received significant attention since an increasing amount of data demonstrate an association between sperm DNA damage and fertility (5, 6). Several studies have shown that high levels of sperm DNA fragmentation are positively correlated with lower fertilization rates in *in vitro* fertilization, impaired implantation rates and an increased incidence of abortion (7).

The sperm chromatin structure damage can occur at any step of the spermatogenesis and can be caused by intrinsic factors like abortive apoptosis, unrepaired DNA breaks during the spermatogenetic chromatin remodelling and packaging or oxidative stress (8) and extrinsic factors, such as storage temperatures, extenders, handling conditions, time after ejaculation, infections and reaction to medicines or post-testicular oxidative stress (9, 10).

The main objective of this study was to evaluate sperm chromatin integrity by sperm chromatin dispersion test (SCD) in ejaculates, analyzed using the World Health Organization

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criteria (WHO) (11), from men divided into five groups: normozoospermic, oligozoospermic, asthenozoospermic, oligoasthenozoospermic and cryptozoospermic.

Next, we divided the semen samples only into two groups: samples with percentage of DNA fragmentation >30% (DFI >30%) and samples with percentage of DNA fragmentation <30% (DFI <30%) independently from diagnosis because, this value, in the cut-off and, therefore, a sperm DNA fragmentation value higher than 30% is considered clinically significant. In the samples of both groups and in order to assess whether DNA fragmentation increase could correlate with abnormal hormonal values, we evaluated total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and inhibin B serum hormonal levels that play an important role in the spermatogenic process.

Finally, we analyzed the molecular activated apoptotic pathways in several samples with an increasing DFI percentage (from 5% to 56%).

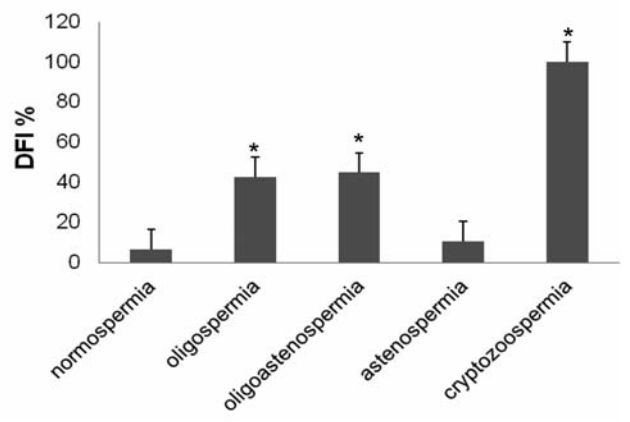

Figure 1. *Effect of % of DNA fragmentation index (DFI) on normozoospermic, oligozoospermic, oligoasthenozoospermic, asthenozoospermic and cryptozoospermic samples. *Significantly different compared with control ($p<0.05$).*

Materials and Methods

Semen samples. The samples were classified into five groups according to semen analyses: normozoospermia (157 cases), oligozoospermia (189 cases), oligoasthenozoospermia (132 cases), asthenozoospermia (98 cases) and cryptozoospermia (57 cases). The semen samples of the five groups were collected by masturbation after 2-5 days of sexual abstinence.

After complete liquefaction of the sample, semen analysis was performed according to the WHO guidelines (11). Sperm count was performed in a Neuberger counting chamber. After immobilizing the cells with distilled water, morphology was evaluated by the Diffquick staining technique (12). Motility was expressed as a percentage of rapid and/or progressive spermatozoa.

SCD test. The SCD test was performed according to the manufacturer's instructions (Halosperm kit; ABanalitica, Padova, Italy). Briefly, aliquots of 0.2 ml of fresh sample semen were diluted in medium to obtain sperm concentrations that ranged between 5 and 10 $\times 10^6$/ml. The suspensions were mixed with 1% low-melting-point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37˚C. Aliquots of 50 μl of the mixture were pipetted onto Coverslips and then carefully covered; the slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 7 min. The denaturation was then stopped and proteins were removed by a transfer of the slides to a tray with neutralizing and lysing solution (0.4 M Tris, 0.8 M DTT, 1% SDS, 2 M NaCl, 0.05 M Triplex) for 25 min at room temperature. Removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loop. Slides were thoroughly washed twice in water for 5 min, dehydrated in sequential 70%, 90% and 100% ethanol baths (2 min each) and air-dried. At the end, cells were stained with Wright and PBS (1:1) for 10 min. After air drying, the degree of DNA dispersion was assessed by bright field microscopy. A minimum of 500 spermatozoa were evaluated (13). The samples with sperm DNA fragmentation value higher than 30% were considered clinically significant.

Measurement of serum levels of total testosterone, FSH, LH and inhibin B. Serum total testosterone, FSH and LH concentrations were measured with an enzyme immunoassay (BioMerieux SA, Marcy l'Etoile, France). Instead, plasma inhibin B (Ansh Labs, RD-RadioDiagnostics, Frankfurt, Germany) were measured by a solid-phase sandwich ELISA.

Protein extraction and western blotting analysis. Aliquots of 1ml of fresh semen were centrifuged at 2,000 × *g* at 4˚C and the pellet was washed in PBS. Then, sperm cells were lysed in lysis buffer (50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride and 10 mg/ml leupeptin) for 30 min on ice. Fifty micrograms of protein from each cell lysate were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes and filters were stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. Membranes were probed with primary antibodies against cleaved caspase-3 and procaspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Hsp70 antibody (heat shock protein; Santa Cruz Biotechnology) was used to estimate equal protein loading. Primary antibodies were incubated following suggestions of the manufacturers. Membranes were then incubated in 1:5,000 peroxidase-conjugated anti-mouse immunoglobulin for 1 h at 22˚C. They were extensively washed and finally analyzed using the ECL system (Amersham, Milan, Italy).

Statistical analysis. The results were analyzed by performing ANOVA tests, with $p<0.05$ considered as statistically significant. The mean and standard deviation (SD) was also calculated for each value.

Results

Sperm chromatin dispersion test (SCD) into normozoospermia, oligozoospermia, oligoasthenozoospermia, asthenozoospermia and cryptozoospermia semen. A spermiogram was performed

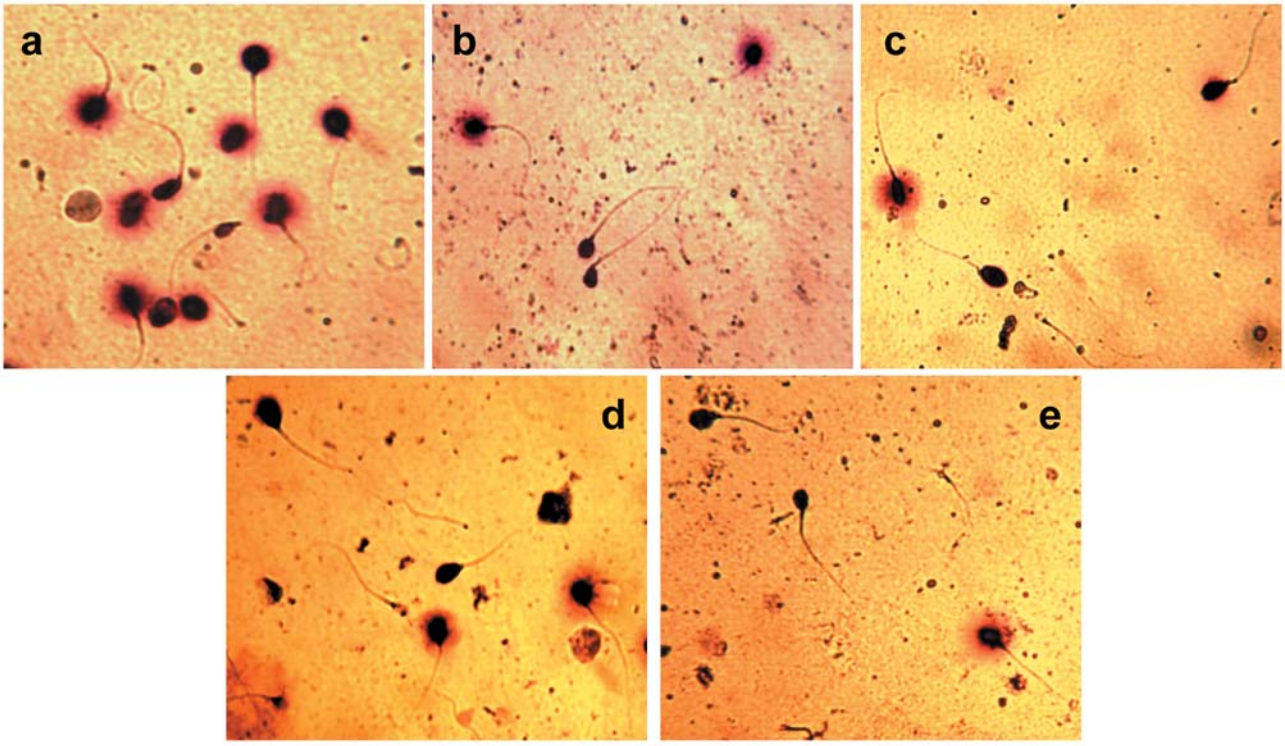

Figure 2. *Representative sperm chromatin dispersion (SCD) test in semen samples. Normozoospermia (a), oligozoospermia(b), oligoastenozoospermia (c), astenozoospermia (d) and cryptozoospermia (e).*

according to the standardized methods recommended by WHO and the semen parameters analyzed were volume, sperm concentration, sperm count, percentage of motile spermatozoa and percentage of normal spermatozoa. The samples were classified into five groups according to semen analyses: normozoospermia, oligozoospermia, oligoasthenozoospermia, asthenozoospermia and cryptozoospermia. The diagnosis of oligozoospermia was defined as the sperm cell count less than 15×10^6 cells/ml in seminal liquid, whereas the diagnosis of asthenozoospermia was defined as the motility of total (progressive and *in situ* mobility) sperm cell less than 40%.

In particular, we showed that the percentage of DNA fragmentation index (DFI), calculated with the SCD test, in oligozoospermia, oligoasthenozoospermia and cryptozoospermia increased significantly compared to the control and asthenozoospermia groups (Figures 1 and 2).

Specifically, in oligozoospermia, oligoasthenozoospermia and cryptozoospermia groups the percentage of DFI increased about 36-fold, 38-fold and 94-fold compared to the control groups, whereas in the asthenozoospermia groups the increase of DFI was of 4-fold compared to the control group.

Correlation of serum hormonal levels with DNA fragmentation. Next, we investigated if the increase of the DFI percentage was correlated to a variation in serum total testosterone, FSH, LH and inhibin B levels. For this purpose, we divided the semen samples into two groups: samples with DFI >30% and samples with DFI <30%, independently from diagnosis because, this value is the cut off and, therefore, sperm DNA fragmentation values higher than 30% were considered clinically significant.

We did not observe any statistically significant differences in serum hormonal levels between the two groups (Figure 3).

Caspase-3 activation in semen samples with high DNA fragmentation. Finally, we selected several samples with an increasing percentage of DNA fragmentation (from 5% to 56%) and evaluated the relationship between the activation of the apoptotic pathway and the increase of the DFI percentage using apoptotic markers. We performed immunoblot analysis using procaspase-3 and caspase-3 antibodies able to recognize both the inactive and active form of caspase-3 in order to measure the expression of apoptotic proteins. For this, we loaded samples with a DFI of 5, 17, 36 and 56%, respectively. As DFI percentage increases, there is a marked reduction of procaspase-3 and an activation of caspase-3 (Figure 4).

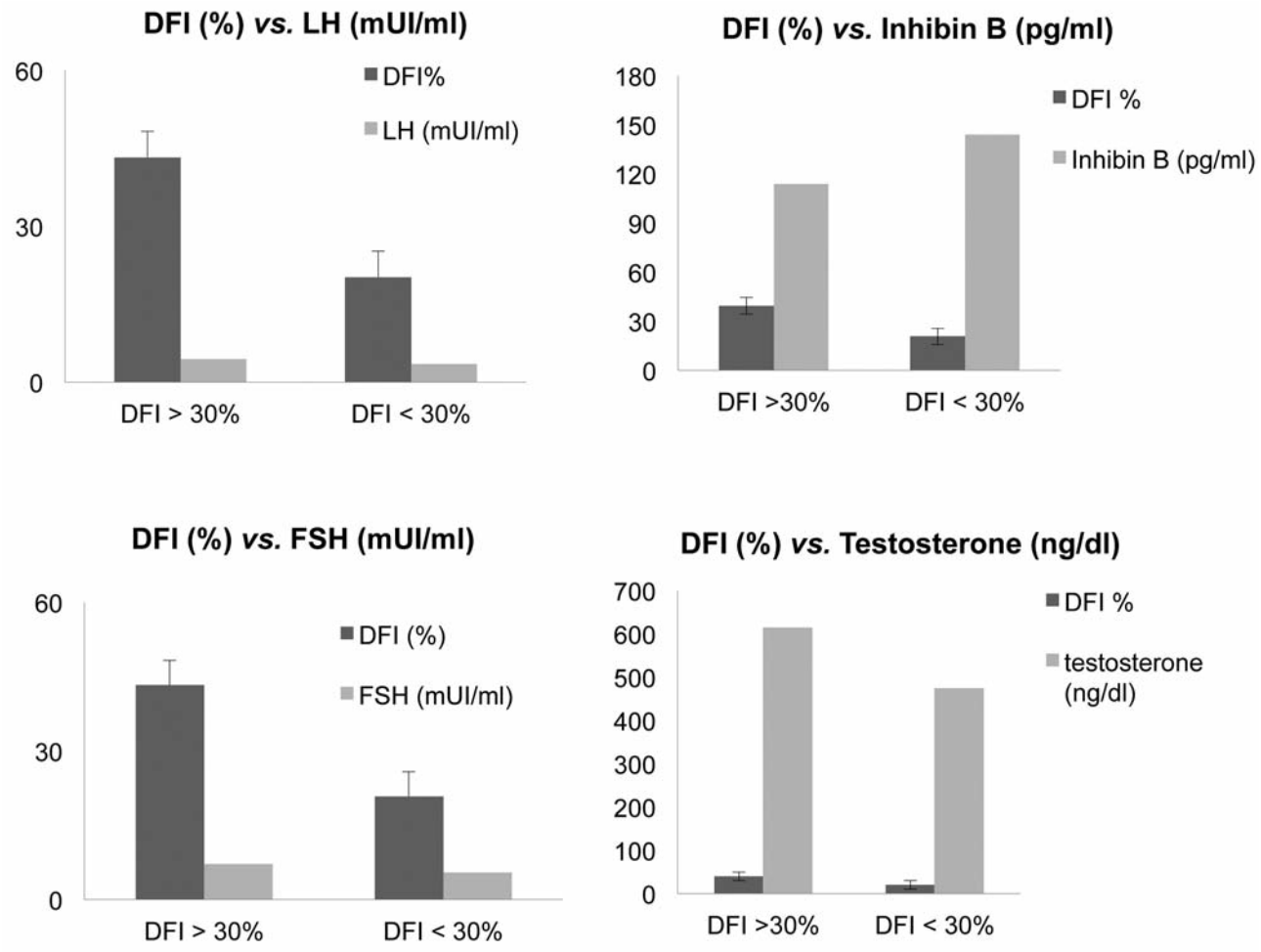

Figure 3. *Comparison of serum hormonal levels between the two groups (DFI >30% and DFI <30%). DFI, DNA fragmentation index; *Significantly different compared to control (p<0.05).*

Discussion

The number of infertile couples opting for assisted reproduction techniques (ART) is continuously increasing and evidence suggests that higher rates of sperm DNA fragmentation in *in vitro* fertilization cycles are associated not only with lower successful fertilization rates and pregnancy rates per cycle but also with a greater risk for miscarriages, even if embryo transfer is successful (14). In fact, some evidence shows that abnormalities of sperm DNA structure appear to be correlated to higher post-implantation spontaneous abortion rates (15). On the other hand sperm DNA fragmentation seems to have a negative impact on the sperm oocyte penetration (16).

Sperm morphology, motility and concentration are the 3 most important factors for male reproduction potential (17). However, these parameters are not sufficient to assess every aspect of sperm function and quality. Sperm DFI is an important parameter used to assess sperm quality.

It has already been demonstrated that abnormal sperm morphology has been correlated with high sperm DNA fragmentation and DNA instability (15).

In our study, we correlated the concentration and motility semen parameters with the sperm DNA fragmentation and showed that a low concentration (less than15×10^6 cells/ml) is more probably associated with a higher sperm DNA fragmentation (DFI>30%), as apoptotic events are also likely to influence the mechanisms of maturation of germ cells. According to literature data, sperm DNA fragmentation may originate in the testis or occur as a consequence of different insults after spermiation and during transit in the male genital tract (18).

Certain studies suggest that chronic stress, including infection, malnutrition, anxiety and depression, can lead to

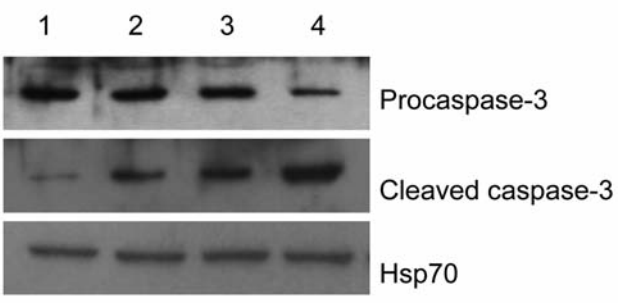

Figure 4. *Western blot showing anti-procaspase-3 and cleaved caspase-3 expression in semen samples with a DNA fragmentation index (DFI) of 5 (lane 1), 17 (lane 2), 36 (lane 3) and 56% (lane 4), respectively. Hsp70 antibody was used to ensure equal protein loading.*

reproductive dysfunction through a rise in glucocorticoids that suppress reproductive functions along the hypothalamic–pituitary–gonadal (HPG) axis and represses gonadotropin-releasing hormone (GnRH) secretion (19).

The hypothalamus directs many of its actions through secretion of GnRH, which acts on the pituitary gland to stimulate the synthesis and release of LH and FSH. In the testis, LH binds its receptor on Leydig cells to stimulate testosterone production. Within the seminiferous tubule, FSH binds its receptor in the Sertoli cell to stimulate spermatogenesis and influence the number of LH receptors through paracrine regulation (20).

In addition to the rapid effects of glucocorticoids on testosterone production, glucocorticoids have also been reported to induce Leydig cell apoptosis, reducing the number of Leydig cells per testis; they also promote apoptosis of spermatogonia within the seminiferous tubules (21, 22). For this reason, we evaluated total testosterone, FSH, LH and inhibin B serum hormonal levels to assess whether a DFI increase could correlate with abnormal hormonal values. We did not find any significant differences in the serum hormonal levels in the various samples analyzed. This suggests that probably the factors inducing higher sperm DNA fragmentation are intrinsic to the testicular cells. Another hypothesis could be that the hormonal assays should be performed before the DNA fragmentation test implying that further studies are tequired to clarify these mechanisms.

Finally, we analyzed if caspase-3 activation occurs in the samples with a DFI >30%. DNA fragmentation of differentiating germ cells could occur in the testis as part of the apoptotic process (which is known as the abortive apoptosis theory) (23), or during chromatin compaction (the defective maturation theory) (24). The theory is based on studies demonstrating high expression of Fas receptors (25), as well as the presence of ultrastructural apoptosis-like features, such as cytoplasmic vacuoles, in ejaculated sperm (26).

In our approach, the experimental sample was tested against increasing DFIs. Western blot analysis, to detect procaspase-3 and active caspase-3, revealed reduction of procaspase-3 and increase in cleaved caspase-3 in samples with increasing DFI. Caspase activation is a well-defined point of no return for apoptotic progression in somatic cells (27). Caspase-3 is considered to be a major player in apoptosis initiation as it generally participates in all major apoptotic signal transduction pathways, both in the receptor-mediated pathway and the mitochondrial pathway. Also, caspase-3 activity has been previously detected in the midpiece of ejaculated human sperm and shown to be significantly associated with low sperm motility (28), or with decreased normal sperm concentration, motility and morphology (29). Our present results confirm that, in the samples with progressively higher DFI, there is an increase of caspase-3 activation.

In conclusion, these data also show that the DFI percentage appear significantly associated with oligozoospermia diagnosis and not with asthenozoospermia diagnosis, which is in agreement with the hypothesis that apoptotic events may originate in the testis and, consequently, influence the sperm concentration. Also, no relationship was found between total testosterone, FSH, LH and inhibin B serum hormonal levels and DNA fragmentation. Finally, a significant relationship was found between caspase-3 activation and increased DNA fragmentation.

Conflicts of Interest

The Authors declare that they have no conflicts of interests.

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