ORIGINIAL ARTICLE

Sperm DNA status in infertile patients with clinical varicocele

Statut de l’ADN spermatique chez les patients infertiles atteints de varicocèle clinique

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KEYWORDS
DNA fragmentation index; Infertility; Varicocele; Semen parameters; Sperm DNA damage

Summary

Objective. — To evaluate if a potential relationship exists between sperm nuclear DNA damage and clinical varicocele and to determine the impact of clinical varicocele on standard semen parameters.

Materials and Methods. — A prospective study involving 30 infertile patients with clinical varicocele and 15 controls patients referred to our laboratory for routine spermiological exploration. Spermograms were performed and analyzed according to World Health Organisation (WHO) guidelines 2010. The DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay.

Results. — The DNA fragmentation index (DFI) was significantly higher in patients with clinical varicocele compared to controls ($13.3 \pm 3.4\%$ versus $6.1 \pm 2.5\%$, $P=0.0001$). In addition, the DFI was positively and significantly correlated with the degree of severity of varicocele thus the DFI was $15.24 \pm 1.9\%$ in patients with grade 3 versus $12.92 \pm 3.5\%$ in those with grade 2 ($P<0.0001$). However, an abnormality of at least one of the spermatic parameters was found in 90% of varicocele patients, and all semen characteristics such as sperm count, vitality, mobility and typical forms were decreased compared to the controls. Furthermore, statistically significant negative correlations were noted between sperm DNA fragmentation index and sperm concentration ($P=0.0001$), motility ($P=0.03$), and normal sperm morphology ($P=0.03$).

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MOTS CLÉS
Indice de fragmentation d’ADN ; Infertilité ; Varicocèle ; Paramètres spermatiques ; Dommage de l’ADN spermatique

Introduction
Varicocèle est un abattement du système de spermatic veins from the pampiniform plexus. It is the most frequently identified etiology in males undergoing infertility evaluation. Varicocèle affects approximatively 22% of general population, as it is a common condition in men having normal spermatogenesis but also in men with abnormal semen parameters. Thus, it reportedly affects 35% of men with primary infertility and 75 to 81% of men with secondary infertility [1]. It is claimed that Varicocele progressively reduces spermatogenesis via elevated intratesticular temperature and altered testicular blood flow; the decreased supply of oxygenated blood and nutrients to the sperm secretion sites reduces sperm quality and quantity, and consequently, their fertility capacity [2].

Conclusion. — Clinical varicocele generates a significant increase of sperm abnormalities and DNA damage, and these changes are positively correlated with varicocele grade. Sperm DNA damage independent of its cause, may affect the quality of the ejaculated sperm and may have implications on patient’s fertility potential.

Résumé
But. — Évaluer s’il existe une relation potentielle entre les dommages de l’ADN nucléaire du sperme et la varicocèle clinique et déterminer l’impact de la varicocèle clinique sur les paramètres spermatiques.
Méthodes. — Une étude prospective incluant 30 patients infertiles atteints de varicocèle clinique et 15 patients témoins adressés à notre laboratoire pour une exploration sperméiologique de routine. Tous les spermogrammes ont été réalisés et analysés conformément aux recommandations de l’Organisation mondiale de la santé (OMS) 2010. La fragmentation de l’ADN a été détectée par le test TUNEL (terminal deoxyuréidyltransférase médiée par la désoxyuridine triphosphate biotine nick-end).
Résultats. — L’indice de fragmentation de l’ADN (DFI) était significativement plus élevé chez les patients atteints de varicocèle clinique par rapport aux témoins (soit 13,3 ± 3,4 % versus 6,1 ± 2,5 %, p = 0,0001). De plus, le DFI était positivement et significativement corrélé avec le degré de gravité de la varicocèle, ainsi le DFI était de 15,24 ± 1,9 % chez les patients avec varicocèle grade 3 versus 12,92 ± 3,5 % chez ceux de grade 2 (p < 0,0001). Cependant, une anomalie d’au moins un des paramètres spermatiques a été trouvée chez 90 % des patients atteints de varicocèle, de même toutes les caractéristiques spermatiques telles que la concentration spermatique, la vitalité, la mobilité et les formes typiques sont diminuées par rapport aux témoins. De plus, des corrélations négatives et statistiquement significatives ont été notées entre l’indice de fragmentation de l’ADN du sperme et la concentration du sperme (p = 0,0001), la motilité (p = 0,03) ainsi que la morphologie normale spermatique (p = 0,03).
Niveau de preuve. — 3.
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Sperm DNA integrity represents one of the vital determinants of normal fertilization and embryo growth in natural and assisted conception [3,4]. Considering this, interest in sperm DNA is justified by the fact sperm DNA integrity affects fertilization and the early stages of embryonic development, implantation, as well as pregnancy rates [5]. Several studies have examined the relationship between sperm nuclear DNA fragmentation as measured by the sperm chromatin structure assay (SCSA) and the terminal deoxynucleobonucleotidyl transferase mediated deUTP nick-end labelling (TUNEL) assay with cleavage rates, embryo quality and pregnancy rates in assisted reproduction technique (ART) [6—10]. Overall, these studies suggested that high levels of DNA fragmentation can have a negative impact on embryo development and pregnancy rates.

The compact nature of human sperm DNA ensured by chromatin condensation protects against damage that can
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occur during sperm transport. Some degrees of DNA damage were usually repaired by the oocyte’s cytoplasm. Yet the oocyte’s repair capacity had not been sufficient when the damage was considerable. Several hypotheses have been put forth to explain the mechanisms by which varicocele led to spermatogenic failure and DNA fragmentation. Sperm DNA damage may occur due to one of three main mechanisms: defect in chromatin condensation, apoptosis and oxidative stress [11].

Not all men with varicocele were infertile, and patients with clinically evident varicocele may have either normal or altered spermatogenesis [12]. Although a cause-effect relationship is not established, multiple reviews concluded that there was indeed evident association between varicocele and increased DNA fragmentation [13].

The purposes of the present study were to assess the levels of sperm nuclear DNA damage in ejaculated spermatozoa of infertile patients clinically diagnosed with varicocele and to determine the association between clinical varicocele and several semen parameters.

Materials and Methods

Study group

The study included 45 men assessed by our laboratory of reproductive biology. A prospective study was designed involving one control group of men with unknown fertility and normal semen parameters (n = 10) and one group of patients with clinically diagnosed varicocele and infertility (n = 30). The medical history of all cases was checked, including occupation, smoking habits, alcohol intake and the use of medication. The study excluded men with azoospermia, and severe oligospermia or leukocytospermia. For each patient, sperm analysis (in particular vitality and motility) and preparation for subsequent DNA Fragmentation test were assessed within maximum 1 h after ejaculation according to WHO 2010 [14] recommendations. The local ethics committee approved of this protocol and all patients had previously given informed consent for the study.

Sperm collection and semen analysis

Semen samples were collected by masturbation after three to five days of sexual abstinence and were analyzed according to the WHO 2010 [14] recommendations. After complete semen liquefaction, pH and volume were measured and a standard seminal analysis was performed including motility, vitality and total sperm count. Sperm morphology was assessed using modified David’s criteria. Sperm was conserved at −20 °C until realization of the DNA TUNEL fragmentation test.

Semen samples Conservation

Briefly, after semen analysis, each sample was cryopreserved. Samples were washed twice with PBS diluted in equal volume (1:1) in order to eliminate exceeding seminal liquid; and then fixed using 3 volumes of ethanol and 1 volume of acetic acid under agitation. The mixture was centrifugated 1500 tours during 5 min. The pellet was recuperated and a second fixation was done using same proportions of ethanol and acetic acid. The pellet was finally maintained in a suspension of ethanol and acetic acid, and the sample was cryoconcentrated at −20 °C for storage. After cryostorage duration, the specimens were thawed at room temperature for 10 min, and then washed with PBS.

Measurements of DNA fragmentation by TUNEL assay

Sperm nuclear DNA integrity was evaluated by use of the TACS® TdT-DAB in situ apoptosis detection Kit (Trevigen®, Gaithersburg, United States) based on TUNEL assay conceived for fixed cells and cryoconcented tissues with the use of optical or electronic microscopy.

We followed the manufacturer’s instructions with few modifications. The kit included: the mixture dNTP, enzyme TdT, solution DAB, DAB Enhancer, Streptavidine-HRP, Counterstaining with methyl green, buffers (labelling buffer and labelling stop buffer), Tacs nuclease, ions Co2+, Mg2+, Mn2+. TUNEL assay was based on an enzymatic reaction of labelling free 3′-OH termini marked with biotin by incorporating a highly purified form of TdT enzyme. Marking with biotin was realized using the streptavidine rainfort peroxide and the staining substrate of diaminobenzidine (DAB) which gave brown color of sperm cells with fragmented DNA. Then, a counterstaining using methyl green would lead to a green color for normal sperm cells.

In day 1, Sperm sample was washed 3 times with PBS in order to eliminate ethanol and acetic acid. About 10³ sperm cells were disposed on a slide and left to incubate 20 min at 45 °C or 2 h at 37 °C. Afterwards, the slide was covered with 70% ethanol and left at room temperature a night long.

Then in day 2, the slide is dehydrated with PBS for 15 min. Then, 50 μl of proteinase K was added on the slide for 25 min and washed twice with PBS. Hydrogen peroxide was added for 5 min and then washed with PBS. After that, the labeling Buffer was disposed on the slides for 5 min. The slides were covered with the labelling reaction mix and left to incubate at 37 °C for 60 min in a humified atmosphere in the dark. After incubation, the slides were washed with Labelling Stop buffer for 5 min and washed twice with distilled water. The slide was covered with 50 μl of Strep HRP solution for 10 min at 37 °C, with DAB solution during 5 min and finally with methyl green for 5 s. The slides were finally washed gently with water and left to dry.

A total of 500 sperm cells of each semen sample were analyzed using optic microscope 100×, normal cells considered as having intact DNA were colored in green and sperm cells with fragmented DNA were colored in brown.

Statistical analysis

For statistical analysis, we used the SPSS software (version 20). Group comparisons were made using student’s test. We performed Pearson’s correlation to examine the relationship between the percentage of sperm with DNA fragmentation and standard semen parameters. To compare qualitative data, we used Chi² test. Odds ratio was calculated with a confidante interval of 95%. If the P-value was under 0.05, the
correlation coefficient was interpreted as being statistically significant.

Results

Our study involved 45 patients that were divided into two groups. The first group included 30 infertile patients with clinical varicocele (detected by physical examination and confirmed by Doppler ultrasound). The second group included 15 patients without clinical varicocele and with satisfying semen parameters. The average age (years) of the patients at the time of diagnosis was 26 ± 4 years (19–35) and 35 ± 7 years (27–47) for the control group. Most of our patients (60%) work as soldiers. 40% of patients of our series had primary infertility and 13.3% secondary infertility.

Ninety percent of our patients had unilateral varicocele, and ten percent bilateral location. A total of 83.3% had grade 2 while 16.7% had grade 3 varicocele.

Standard semen parameters analysis

Table 1 represented the main sperm parameters of controls and varicocele patients. 10% of the varicocele patients had normal sperm characteristics, while 40% had moderate asthenospermia, 13.3% had severe asthenospermia and 36.6% had severe oligoasthenoteratospermia.

Analysis of Sperm DNA fragmentation

According to WHO 2010 guidelines [14], sperm motility, concentration and normal morphology proved to be lower in varicocele group with a statistically significant difference compared to the control group (P < 0.05). As shown in Fig. 1, the sperm DNA fragmentation index (DFI) was 6.1 ± 2.5% in control group. Sperm DNA fragmentation was significantly higher in patients with clinical varicocele compared to control group 13.3 ± 3.4%, P = 0.0001. DFI tended to be higher in patients with grade 3 varicocele (15.24 ± 1.9%) compared to grade 2 (12.92 ± 3.5%) (P = 0.001), (Fig. 1).

Relationships between DNA fragmentation and semen parameters

As shown in Fig. 2, DNA fragmentation correlated negatively with sperm concentration (r = −0.5, P = 0.001), sperm motility (a + b) and normal sperm morphology (r = −0.3, P = 0.03). However, the percentage of DNA fragmentation and sperm volume, or paternal age did not show any evident correlation (P > 0.05).

Discussion

Varicocele is the leading cause of male infertility due to its impairment of spermatogenesis through several distinct pathophysiological mechanisms [15]. It may be associated with a variety of spermatogenetic conditions, ranging from normozoospermia to moderate oligoasthenoteratospermia or azoospermia. The pathology of varicocele is marked by the dilatation and tortuosity of the pampiniform plexus, leading to higher temperature of the testes and thereby testicular atrophy with infertility. Several reports suggested that several factors associated with varicocele may induce pathways that lead to DNA damage and apoptosis. Sperm DNA damage might occur through at least one of three fundamental mechanisms: defective chromatin condensation or abortive apoptosis during spermatogenesis or transportation of sperm through the male genital tract [16].

This study aimed to answer the following question: did patients with clinical varicocele have higher sperm DNA damage than normal men?

The results of our study indicated that there was clearly a negative influence of clinical varicocele on semen parameters. In fact, in our study only 10% of varicocele patients were normozoospermic. Most common abnormalities included abnormal morphology (53%), asthenozoospermia (33.4%), necrozoospermia and oligozoospermia (6.6%). The presence of more than one defect was the most frequent case and Oligo-astheno-teratospermia (OAT) was notified in 36.6%. Our data was in line with the report of several studies, which showed an OAT as the main spermatogenic profile of clinical varicocele patients [11].

According to the literature research, many studies demonstrated that clinical varicocele led to significant decrease of DNA polymerase activity, increases of oxidative stress due to the induction of high free radicals, and a significant decrease of anti-oxidant activities, that all had harmful effect on spermatogenesis [17].

The recent meta-analysis of Wang and al, concerning the effect of clinical varicocele on genetics of DNA lesions, highlighted the important DFI of patients with varicocele compared to fertile men without varicocele [18]. DFI provided additional information about the fertility potential and showed less biological variation when compared to conventional semen analysis [19]. It was rather interesting to determine DNA fragmentation status especially in cases of couples with repeated pregnancy loss after ICSI as sperm DNA fragmentation significantly affects embryo post-implantation development [20]. As known, sperm DNA fragmentation may compromise the embryo post-implantation development in ICSI cycles, resulting in spontaneous miscarriage or biochemical pregnancy.

In our study we demonstrated a higher average DFI when varicocele is grade 3 compared to grade 2: 15.24% versus 12.92% respectively. Our results matched with findings of the literature namely Wang and al that proved that infertile men with clinical varicocele grade have greater oxidative DNA damage than patients who are either fertile or infertile but without varicocele [18]; Clinical Varicocele grade was positively correlated with 8-hydroxy-é-desoxyguansine (8OHdG) in the seminal fraction that was a marker of oxidative DNA damage [18]. Several studies, have also demonstrated a strong correlation between semen parameters and reactive oxygen species concentrations [21]. However, some authors admitted that patients with a clinical varicocele might also have a diminished seminal antioxidant capacity and sperm DNA fragmentation increases [22].

When semen characteristics were correlated with sperm DNA fragmentation index, we noted statistically negative significant correlations with sperm concentration, motility, and normal sperm morphology. However, we did not register any evident correlation between the percentage of sperm DNA fragmentation and other semen parameters or...
paternal age. Regarding the results of the literature, correlations of DFI with sperm parameters had been extremely variable. DFI has been found to be negatively correlated with sperm concentration, progressive motility, vitality, and normal forms in many studies [17,23].

The fact that spermatozoon’s cytoplasmic membrane was very rich with polyunsaturated fat which are predicted target to free radicals, explained the previous result. This explained the decline of sperm parameters in response to lipoperoxidation. As consequence, the lipidic peroxysina
tion altered motility and sperm concentration. It could also alter cytoplasmic and acrosomic membranes, a spontaneous acrosomic reaction and cell apoptosis [24]. The significant negative correlation between DFI and sperm motility could also be explained as follows, the fact that motility was the most sensible oxidative stress indicator, since high level of reactive derives of oxygen blocked many phosphorylation enzyme or oxidative glycolysis, which limited ATP production necessary to sperm mobility [25].

Finally, some authors expressed their interest in evaluating the beneficial effect of varicocelectomy and elucidate predictors of improvement after repair, especially by controlling DFI after varicocelectomy repair. So, Abdelbaki et al. demonstrated a significant reduction in DFI and ROS levels in association with a significant improvement in various seminal variables [26].

Another prospective trial conducted by Alhathal et al. found that varicocelectomy was associated with a significant improvement in sperm chromatin compaction and DNA integrity, using three different assays [27]. In another similar study in 92 patients, Kadioglu et al. reported a significant large decrease in DFI from a preoperative mean of 42.6% to a postoperative mean of 20.5%(P < 0.001) and concluded that varicocelectomy can improve seminal variables and sperm DNA damage in infertile men with varicocele [28].

Therefore, using DFI as a prognostic test in infertile patients with varicocele to help decision-making as regards the necessity and the anticipated outcome of varicocelectomy in patients with infertility could be recommended.

Indeed, the results of our research are intermediate. Thus, this study will be ongoing for the next years in order to determine and evaluate the efficiency of varicocelectomy repair on sperm DNA integrity and live birth rates.

The present study possesses several limitations. This study did not include the follow-up on pregnancy. The TUNEL assay had certain limits; unfortunately, it did not allow differentiating between normal and pathological DNA ruptures [29]. According to Mitchell and al, TUNEL assay

<table>
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<th>Table 1</th>
<th>Means of age and conventional semen parameters of control group, and varicocele patients.</th>
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<tbody>
<tr>
<td></td>
<td>All subjects Mean ± SD</td>
</tr>
<tr>
<td>Age</td>
<td>30 ± 12</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>3.5 ± 1.7</td>
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<tr>
<td>Concentration (million/ml)</td>
<td>75 ± 80</td>
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<tr>
<td>Progressive motility (a + b) %</td>
<td>33 ± 10</td>
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<tr>
<td>Total motility (a + b + c) %</td>
<td>40 ± 10</td>
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<tr>
<td>TZI</td>
<td>1.31 ± 0.17</td>
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<td>Typical form %</td>
<td>16 ± 7</td>
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<tr>
<td>Vitality %</td>
<td>80 ± 11</td>
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Figure 1. DNA fragmentation index assessed by TUNEL assay in spermatozoa from control group, grade 2 varicocele, and grade 3 varicocele (*P-value < 0.05 compared to control group).
underestimated damages in sperm DNA and proposed a modified protocol using Dithiothréitol (DTT) known as Cleland reactive in order to help deoxyxynucleotidyl-transferase (DTt) reaching DNA cleavage sites [30]. The results should be considered objectively using flow cytometry as it allowed rapid analysis of a larger number of sperm and provided a highly sensitive means of defining sperm with fragmented DNA in a more objective and precise manner [29]. Further investigations are also needed to reveal the cause and effect relationship between the increase of DNA fragmentation levels and clinical varicocele pathology, as well as the underlying mechanisms.

In conclusion, our data demonstrated significant increase of sperm abnormalities and DNA damage in clinical varicocele patients than men without varicocele and that these changes positively correlated with varicocele grade. Independent of its cause, sperm DNA damage might affect the quality of the ejaculated sperm and may have implications on their fertility potential. Therefore, we recommended the evaluation of sperm DNA status and extent of damage in infertile patients with a high clinical varicocele grade in order to optimize sperm quality and pregnancies rates in this population.

Disclosure of interest
The authors declare that they have no competing interest.
References