Diagnosis of male infertility: A need of functional and chromatin evaluation

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Sperm DNA fragmentation; Terminal deoxynucleotidyl transferase dUTP nick end labeling; Nitroblue tetrazolium; Sperm function tests

Abstract
Objectives: To evaluate the incidence of functional and chromatin alterations on spermatozoids in patients grouped according to normality of standard semen parameters based on the 5th edition of the World Health Organization (WHO) guidelines. To identify and correlate the most frequently altered characteristics in the normal standard semen parameters sub-population.

Materials and methods: A prospective study was performed. It evaluated standard semen parameters (volume, sperm concentration, motility and morphology, round cells, peroxidase-positive cells) according to WHO guidelines, as well as functional tests (24 h survival, hypoosmotic swelling test, modified stress test), and additional assays (aniline blue, nitroblue-tetrazolium, TUNEL) in 110 semen samples from patients and 6 from fertile donors (control). Based on standard semen parameters values, patients were divided into two groups (A: all standard parameters normal; B: one altered standard parameter at least).

Results: At least one of the variables analyzed was altered in 96.61% of the samples. Groups A and B showed statistically significant differences in all the complementary tests. At least one of the complementary tests were altered in 93.68% of the samples in group A, and the most frequently affected variable was sperm DNA fragmentation (16.95%).

Conclusions: Performing a more in-depth seminal study within the routine functional and chromatin assays provides a more precise diagnosis of male infertility. The WHO standards should be considered as a primary approach.

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Introduction

It is estimated that infertility affects approximately 15% of couples of reproductive age,\(^1\) and that in about 50% of the cases, the male factor is involved.\(^2\)

Semen analysis is now a routine procedure for the determination of the contribution of the male factor in the study of the infertile couple. It is thus an essential tool for clinicians when deciding between algorithms to continue with the diagnosis, or directly between therapeutic alternatives.

In search of large-scale standardization of semen analysis, the World Health Organization (WHO) published in 1980 the first edition of its ‘Manual for the examination of human semen and sperm–cervical mucus interaction’.\(^3\) Following the progress in this area, the WHO reissued revised and updated versions of the manual in 1987,\(^4\) 1992,\(^5\) 1999\(^6\) and 2010.\(^7\) These publications generated controversy on the true value of the recommended characteristics and their reference values.\(^8\)\(^\text{13}\)

The sperm must exhibit a set of unique biological properties to achieve fertilization and normal embryonic development. Given this complexity, it is difficult to diagnose changes in its physiology using the standard semen analysis, this is, the study of the variables recommended in the WHO manuals.\(^14\)

Other tests, additional to those recommended in the manuals, have been developed in search of a more comprehensive evaluation. Studying both functional and chromatin features of the sperm, the results have been positively correlated with sperm quality and reproductive success, constituting potential diagnostic and prognostic tools\(^15\)\(^–\)\(^19\).

The aim of this work was to study the incidence of functional and chromatin alterations in the sperm of a population of patients with marital infertility subdivided according to the normality of the standard semen parameters, according to the latest edition of the WHO manual.\(^7\) In turn, we intended to identify and correlate the altered characteristics with greater frequency in the normal subpopulation for standard parameters.

Materials and methods

Patients and samples

This study included 110 patients with marital infertility, whose average age was 38 ± 7 years, selected between March and July 2011. A control group was made up of 6 volunteer fertile donors, aged 25 ± 1 years. After standard sexual abstinence, and following the WHO guidelines,\(^7\) the semen samples were collected by masturbation, in sterile containers tested and supplied by the laboratory personnel. All the subjects included in this study signed informed consent forms.

The standard semen parameters analyzed were: sample volume, sperm concentration, progressive sperm motility, percentage of normal forms, percentage of immature forms, round cell concentration and concentration of peroxidase-positive cells. We also carried out the following functional tests: sperm survival at 24 hours, hypo-osmotic swelling test (HOST), modified stress test (MOST), and the following additional tests: nitroblue tetrazolium test (NBT), aniline blue
test (AB), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Standard parameters were studied according to the latest WHO manual and they were classified as normal or abnormal taking as a reference the ranges published in the manual. According to the above, the total set of patients was divided into two groups: group A, which included the patients whose samples were classified as normal for all standard parameters; and group B, with the patients whose samples showed alterations in at least one of these parameters.

Analysis of standard semen

After the liquefaction and determination of the volume of the samples, the sperm concentration and motility were analyzed, as well as the round cell concentration, by phase-contrast microscopy (Olympus BX51) using a Makler counting chamber type (Makler counting chamber, Sefi-Medical Instruments, Haifa, Israel). In order to show the peroxidase-positive cells, we used the benzidine—cianosine—H2O2 technique, and the reading of it was carried out by light-field microscopy (Olympus BX51). The sperm morphology was determined by analyzing stained smears according to the Papanicolaou technique described in the last WHO manual. Immature sperm forms were counted in the same preparations.

Functional tests

24-h survival
The survival rate was determined by the ratio between the percentages of motile sperm counted after and before 24 h of incubation at room temperature, of an aliquot of washed semen (half Earle's Balanced Salt Solution [EBSS] + SAH 10%).

Hypo-osmotic test
This test was carried out by incubating 100 μl of semen for 30 min at 37 °C, in 1 ml of hypo-osmotic solution (0-fructose 0.075 M, sodium citrate 0.025 M). These conditions cause a passive flow of water through intact and functional membranes into cells, which in turn causes them to swell. This swelling is evidenced by the winding of the sperm tails. Dead sperm or with altered membranes do not swell, and their tails are straight. The result of the test is expressed as the percentage of swollen sperm.

Modified stress test
The rate described as a result of this test was calculated as the ratio between the percentages of motile sperm counted before and after 4 h of incubation, at 40 °C of an aliquot of washed sperm with EBSS + SAH 10%.

Additional tests

Aniline blue test
For this study, smears with fresh semen were performed that were fixed in glutaraldehyde 3% in 0.2 M phosphate buffer, and stained with aniline blue solution at 5% in the same buffer. The test result was expressed as the percentage of stained sperm.

Nitroblue tetracentium test
It was diluted 1:1, an aliquot of fresh semen with an NBT solution at 0.1% (Sigma–Aldrich, St. Louis, USA) in phosphate buffered saline (PBS – Dako North America Inc., CA, USA), and incubated for 30 min at 37 °C. After washing the suspension, smears were performed which were stained with dye according to Wright. Under immersion and 1000× in a light-field microscope, the sperm were classified as NBT-positive as formazan granules were detected therein, and the result of the trial was expressed as the percentage of these sperm.

TUNEL trial
For this technique, we used the In Situ Cell Death Detection Kit-Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's specifications. Briefly, aliquots of washed semen were transferred onto multicol slides and fixed in 4% paraformaldehyde in PBS for 30 min. After washing in PBS, the preparations were permeabilized in a solution of cold Triton X-100 at 0.1% in sodium citrate at 0.1%. Labeling was subsequently performed in a moist chamber at 37 °C with the TUNEL reaction solution. Each experiment included a negative control (omitting the enzyme in the reaction solution) and a positive control (treated with 1 mg/ml DNase). As counterstaining, we used Hoechst 33342 (Molecular Probes, Invitrogen Detection Technologies). The preparations were analyzed under fluorescence microscope (Olympus BX40) and the DNA of the sperm was classified as intact or fragmented (nuclear green fluorescence). The result of the test was expressed as the percentage of sperm with fragmented DNA. The sperm of the negative controls showed no fluorescence, while 100% of the positive controls had their DNA fragmented.

Statistical analysis

In order to determine significant differences between groups, we used the one-way ANOVA test. In the cases that showed correlations, we used the Spearman Rank Order Correlation test. Statistical significance was assumed with p values lower than 0.05. The statistic was carried out on the SigmaPlot 11.0 platform (Systat Software Inc.).

Results

For the full set of patient samples, the average for volume, sperm concentration, progressive motility, and percentage of normal forms was within the reference ranges, while the percentage of immature forms and the round cell concentration was slightly altered. The results of the additional tests were within the reference ranges as well (Table 1). Interestingly, only in 3.39% of the samples, all the variables tested were classified as normal. This means that 96.61% of the samples showed at least one of the analyzed parameters altered.

The division of the total set of samples according to the normality of the standard semen parameters resulted in the composition of group A with 53.64% of the samples and group B with 46.36%. Along with this, and taking into account
the previous data, it was obtained that for group A, 93.68% of the samples showed altered at least one of the complementary tests.

Considering only the altered parameters not included in the recommendations of the last WHO manual,7 or the complementary tests, and analyzing them one by one for each group, we obtained the results presented in Table 2. Surprisingly, in group A we found a significant percentage of samples classified as abnormal for some of these parameters. As it might be suspected, these percentages were higher in group B. In the control group, we obtained values included in the reference ranges for all variables.

Statistically significant differences were found between groups A and B in all complementary tests and in some standard analysis parameters (volume, concentration, progressive motility, and normal forms). None of the variables showed significant difference when we compared group A to the control group, while the latter differed with statistical significance from group B in the concentration, progressive motility, normal forms, and HOST, AB, NBT and TUNEL tests (Table 3).

Table 2 Parameters altered depending on the group of patients analyzed. The table shows the parameters not considered by the 2010 WHO manual.7 The values correspond to the percentage of samples of the total in each group.

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature forms ≥ 2%</td>
<td>11.86%</td>
<td>27.45%</td>
</tr>
<tr>
<td>Round cells ≥ 1 ml/ml</td>
<td>13.25%</td>
<td>29.41%</td>
</tr>
<tr>
<td>24 h survival ≤ 20%</td>
<td>13.56%</td>
<td>41.18%</td>
</tr>
<tr>
<td>HOST ≤ 60%</td>
<td>8.47%</td>
<td>43.14%</td>
</tr>
<tr>
<td>MOST ≤ 0.50</td>
<td>0%</td>
<td>3.92%</td>
</tr>
<tr>
<td>TUNEL ≥ 20%</td>
<td>16.95%</td>
<td>47.06%</td>
</tr>
<tr>
<td>Aniline blue ≥ 25%</td>
<td>15.25%</td>
<td>59.90%</td>
</tr>
<tr>
<td>NBT ≥ 23%</td>
<td>5.08%</td>
<td>19.61%</td>
</tr>
</tbody>
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From the correlation analysis between the additional tests, we obtained direct and significant correlations between NBT and AB (r = 0.271; p < 0.01); NBT and TUNEL (r = 0.279; p < 0.005); and AB and TUNEL (r = 0.258; p < 0.01).

Discussion

Our results show that by analyzing only the parameters recommended by the WHO in the latest edition of its manual,7 we get a partial view of the actual situation of the studied sample. Even finding these parameters within the reference ranges, and therefore classifying the samples as normal, when performing the other tests, not all these samples were really normal.

Several considerations arise from the above observation. First, careful handling of the term ‘normality’ is required, especially taking into account the effect it may have on this particular type of patients consulting for infertility, given the emotional, social, etc. burden to which they are usually subjected. Then, an important role of the manuals and reference ranges is to provide a prognosis to help determine if medical interventions are necessary, if they are, to establish which is the best suited to the patient’s conditions and what chances of success exist, or if other actions such as the use of donors or adoption are to be considered.13 Clearly, this becomes difficult if we take into account only the parameters of the standard semen analysis with their reference ranges recommended in the fifth edition of the WHO manual.7

Furthermore, the standard seminal study provides no information on the pathogenesis, that is, the mechanism underlying the observed alteration in a certain parameter.13 Introducing functional tests and the additional tests evaluated in this paper in the seminal routine evaluation means providing valuable information for the diagnosis, prognosis, and etiology of infertility.

The fact that a sample may have alterations in the functional tests or in the other sperm parameters evaluated in this paper is documented, even taking standard parameters within the reference ranges.10,11 In 2002, Saleh et al. described significant levels of sperm DNA damage in normal patients for the standard sperm parameters.14 In line with this, an interesting finding is that published in the papers by Avedaño et al. The authors demonstrated that infertile patients may present fragmentation in the DNA of sperm classified as morphologically normal, and that this condition has a negative impact on the quality of the embryos generated by ICSI.22,23

Our results are consistent with the previous observations, and they show that the vast majority of the patients whose sperm samples would be classified as normal, according to the WHO criteria for standard semen parameters,7 has at least one of the other tests performed in this paper altered. It is striking that the feature that is more frequently affected in this group of patients is the sperm DNA integrity. If to this is added that its correlation with the sperm fertilizing capacity has been documented for a long time both in vivo and in vitro,24,25 it is clear that its evaluation should be included in the diagnostic algorithms of male infertility almost routinely. The exception is given by those cases in which from
the study of standard semen parameters, a clear diagnosis can be deduced, such as azoospermia.

It is now accepted that there are three fundamental mechanisms that give rise to sperm DNA damage: (a) oxidative stress generated by an imbalance between the production of reactive oxygen species by sperm and leukocytes and the seminal plasma antioxidant activity; (b) alterations in the chromatin packaging provided by altered activity of the topoisomerase II enzyme during the replacement of histones with protamines in the sperm maturation process; and (c) "abortive apoptosis", that is, apoptotic events that normally occur during the spermatogenesis go haywire for reasons still unknown, throwing in the ejaculate sperm that have entered the apoptotic pathway.26 While it would be wrong to think that these mechanisms act independently, when what is most likely to take place is a combination of the three of them,27 it is possible to investigate them, especially in cases of patients with high levels of sperm DNA damage.

In this paper, we examine two of the mechanisms: The sperm production of reactive oxygen species by the NBT test, and erroneous replacement of histones with protamines through the AB test. Through these trials, we obtain indirect vision of the contribution in the sperm DNA damage of the action of reactive oxygen species and of the errors in the chromatin packaging during sperm maturation. We consider this approach effective, technically simple and inexpensive that can be included in the routine of any laboratory. Moreover, the correlations found between these tests and the percentage of sperm with damaged DNA reinforces the idea that the mechanisms studied indirectly by these two trials are detrimental to the sperm DNA integrity. On the other hand, and because of the correlation we find between both tests, we adhere to the idea that those mechanisms would act in parallel and not individually.

Further studies are needed, increasing the number of cases and adding the apoptotic pathway analysis as a third source mechanism of sperm DNA damage, to continue to contribute to the understanding of the etiology of male infertility, and concomitantly to the development of efficient tools for its correct diagnosis.

Going beyond the parameters recommended by the WHO,7 deepening in the seminal study by introducing trials evaluating the sperm functionality, as well as its chromatin integrity, results in a more refined diagnosis and a better strategy.

Conflict of interest

The authors declare that they have no conflict of interest.

References

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