Evaluation of sperm functional parameters in normozoospermic infertile individuals

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Abstract
Objective: This study evaluated the integrity of the chromatin structure, reactive oxygen species (ROS) levels, mitochondrial membrane potential (MMP), DNA damage and lipid per-oxidation of semen samples from infertile men classified as unexplained infertility.

Methods: Between February 2010 and July 2011 semen parameters and functional tests were evaluated in 10 subjects with proven fertility, 10 that belong to general population and 8 with idiopathic infertility. In addition to the conventional semen analysis, the following unconventional seminal analysis were conducted: evaluation of ROS, MMP, sperm chromatin structure assay (SCSA) by flow cytometry, assessment of sperm membrane lipid peroxidation by spectrophotometry, and alkaline comet assay by electrophoresis.

Results: We observed a significant increase (p < .05) in the production of ROS and the fragmentation or sperm DNA damage in the population of infertile men. There were no statistically significant differences (p > .05) in the analysis of sperm membrane integrity between the groups. Moreover, we observed significant correlations (p < .05) between SCSA and comet assay (r = 0.86) and the production of intracellular ROS (r = −0.588).

Conclusion: The sperm from individuals with idiopathic infertility showed high levels of intracellular ROS and increased levels of DNA fragmentation in the sperm. These results suggest that these two parameters are related to unexplained infertility and therefore have clinical importance as a possible diagnostic and prognostic tool in the evaluation of idiopathic male infertility.

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PALABRAS CLAVE
Infertilidad; Especies reactivas del oxígeno; Espermatozoide; Semen; Calidad espermática

Evaluación de los parámetros funcionales espermáticos en individuos infértiles normozooespermicos

Resumen
Objetivo: El presente estudio piloto evaluó la integridad de la cromatina, los niveles de especies reactivas del oxígeno (ROS), el potencial de membrana mitocondrial (PMM), el daño en el ADN y la lipoperoxidación de la membrana espermática en muestras de hombres clasificados como infértils de causa desconocida.

Método: Entre febrero de 2010 y julio de 2011 se evaluaron los parámetros seminales y pruebas funcionales en 10 individuos con fertilidad probada, 10 donantes pertenecientes a la población general y 8 con infertilidad Idiopática. Adicional al espermograma convencional se realizaron los siguientes análisis seminales no convencionales: evaluación de ROS, PMM, ensayo de la cromatina espermática (SCSA) por citometría de flujo, evaluación de la lipoperoxidación de la membrana espermática por espectrofotometría y el ensayo cometa mediante electroforesis alcalina.

Resultados: Se observó un aumento significativo (p < 0.05) en la producción de ROS y en la fragmentación o daño del ADN espermático en la población de hombres infértiles. No se encontraron diferencias estadísticamente significativas (p > 0.05) en los análisis de integridad de membranas espermáticas entre los grupos. Adicionalmente, se observaron correlaciones significativas (p < 0.05) entre SCSA y el ensayo cometa (r = 0.86) y la producción de ROS intracelular (r = −0.588).

Conclusión: Los espermatozoides de los individuos diagnosticados con infertilidad idiopática mostraron altos niveles de ROS intracelular y un aumento en los niveles de fragmentación del ADN espermático. Estos resultados sugieren que estos parámetros se encuentran relacionados con la infertilidad de origen desconocido, y por tanto tienen potencial importancia clínica como una posible herramienta diagnóstica y pronóstica en la evaluación de la infertilidad idiopática masculina.

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Introduction

Infertility is a condition that occurs in 10–20% of the world population and it is defined as the inability to carry a pregnancy to term after a year of sexual intercourse without contraception. The diagnosis of infertility requires the assessment of the reproductive health status of the couple, by means of a thorough and comprehensive study to identify the possible factors that are interfering with the ability to fertilize.

At least 50% of the infertility cases are associated to the male factor, analyzed mainly from the seminal analysis results, and comparing them to the reference limits established by the World Health Organization. Despite this consensus for evaluation of human semen, the spermogram is a subjective analysis, unable to distinguish between fertile and infertile individuals and to describe the functional and biological properties of the sperm. Some male infertility cases are usually classified as idiopathic because the underlying pathophysiology of the sperm dysfunction is only partially known.

Several studies have reported a relation of the DNA damage to the sperm function and infertility and including recurrent miscarriage; however, the mechanism of this condition is still unknown. In recent years, some mechanisms that could be related to the decline in sperm quality and infertility have been suggested, among which are: oxidative stress, apoptosis, sperm DNA damage, and genetic disorders.

Therefore, the objective of this study was to assess some conventional and unconventional semen parameters such as DNA damage, the concentration of reactive oxygen species (ROS), the functional status of the mitochondria, the sperm membrane lipid peroxidation, and the sperm chromatin integrity in men with idiopathic infertility, individual donors from the general population, and a control group with proven fertility.

Materials and methods

Study population

Between June 2010 and December 2011, we conducted a pilot cross-sectional study involving 28 normozoospermic men with an age range of 20–40 years, divided into 3 groups: (a) 8 consulted for fertility problems to the Human Fertility Institute -InSer, Medellin, Colombia, and they were diagnosed with unexplained infertility by a group of specialists in the field; (b) 10 with proven fertility, whose partner had at least one child younger than 1 year, or whose partner was pregnant with a gestation period exceeding 3 months; and (c) 10 apparently healthy donors from the general population. The ethical endorsement was obtained from the respective Committee of the University Research Center (SIU, from the Spanish Sede de Investigación Universitaria) of the University of Antioquia.
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Conventional semen analysis

The semen samples were obtained by masturbation after a period of 3–7 days of sexual abstinence. Each ejaculate was evaluated for the routine parameters established by the World Health Organization.²

Evaluating levels of intracellular reactive oxygen species

The sperm intracellular ROS levels were measured by flow cytometry (H₂O₂,•OH: ONOO⁻) using 2', 7'-dichlorofluorescein diacetate (DCFH-DA), a signal molecule, permeable, non-fluorescent, highly sensitive to changes in intracellular oxidation/reduction (redox).¹¹

Lipid peroxidation

In order to determine the status of lipid peroxidation of the sperm cell membrane we used malondialdehyde as the indicator molecule. Some modifications to the protocol previously described by Laudat et al.¹¹ were made. The sperm lipid peroxidation was defined as the nmol of MDA formed by 10 × 10⁶ sperm.

Analysis of the integrity of the sperm chromatin structure

The analysis of the chromatin structure was carried out following the protocol described by Evenson et al.¹³ with some modifications of our laboratory.⁷,⁸ The DNA fragmentation index (DFI) was determined as: DFI = red fluorescence intensity/red + green fluorescence intensity × 100.⁷,⁸

Single cell gel electrophoresis (comet assay)

In order to detect sperm DNA damage, we carried out the alkaline comet assay based on the protocol described by Shamsi et al.¹⁴ The readings were performed under the fluorescence microscope (Leica DMLS) with 515–560 nm excitation filter and 590 nm barrier filter, lens 40×, by adding 40 μl ethidium bromide (20 μg/ml) on the agarose. 25–50 pictures were taken per plate and they were analyzed using the Comet Assay Software Project (CASP).

Assessment of mitochondrial membrane potential

In order to evaluate changes in the sperm mitochondrial membrane potential (MMP) we used as dye 3,3'-di-hexiloxacarbocyanine iodide (DiOC6), a cationic carbocyanine, permeable and lipophilic, capable of emitting high fluorescence by being incorporated by viable mitochondria; however, in proapoptotic cells, the MMP is altered and its signal is weak or non-existent due to loss of the mitochondrial electrochemical gradient.¹⁵

Statistical analysis

The data obtained from the experiments were tabulated, processed, plotted, and analyzed statistically using the program GraphPad prism° 5.0 (Windows version). The Excel program (Office 2007) was used to determine the nmol MDA/10 106 × 10⁶ sperm of each individual in the study, from the equation of the line established by the standards of the different concentrations of hydrolyzed TEP. We used the Kruskal–Wallis test to compare the groups and Spearman test to correlate the variables. The results of the flow cytometries were analyzed using the WinMDI 2.8 program (Trotter J., The Scripps Research Institute, San Diego, CA, U.S.A.).

Results

Table 1 summarizes the data from the seminal analysis of the samples analyzed in the study. No statistically significant differences were found when comparing the values obtained for each of the conventional semen analysis variables between groups.

By evaluating intracellular ROS generation of the samples of the individual participants, we observed higher levels of ROS in infertile men with regard to the samples of fertile men (p < 0.05, Fig. 1). The status of lipid peroxidation of the sperm cell membrane was not affected or altered between

![Figure 1](http://www.elsevier.es)
the samples of the male participants, where fertile individuals (0.18 ± 0.07) have a lower degree of oxidation with respect to the group of infertile individuals (0.26 ± 0.11) and donors (0.29 ± 0.23).

Regarding the assessment of the integrity of the sperm chromatin structure by flow cytometry, we evidenced a higher and statistically more significant fragmentation index in the infertile men group as compared to the group of fertile men (8.5 ± 3.7; p < 0.001) (Fig. 2). It is also noteworthy that by means of the alkaline comet assay, we also detected higher levels of sperm DNA damage in men with fertility problems compared to fertile individuals (p < 0.001) and donors belonging to the general population (p < 0.05) (Fig. 3).

**Fig. 4** shows the quantifications of the mitochondrial membrane potential for the 3 groups of individuals analyzed. Although there are no statistically significant differences, we observe that the individuals in the infertile population (84.0 ± 14.6) express a higher percentage of sperm with increased MMP compared to the fertile group (66.5 ± 10.3) and the donor one (64.5 ± 25.6).

Finally, correlations were made between the seminal parameters analyzed, in order to observe the behavior of the variables, and the following significant correlations were found: sperm chromatin assay (SCSA) and comet assay (r = 0.866; p < 0.001) and SCSA and intracellular ROS levels (r = −0.588, p < 0.05), Fig. 5.

**Discussion**

At present, the molecular basis of infertility is not fully understood: some studies have focused on identifying the
differences in the profiles of proteins, lipids, and sugars that allow the sperm of an individual to fertilize an oocyte; others have analyzed the possible spermatogenic character role in the fertilization process. However, the mechanism underlying this condition is still unknown.

In the past decade, we have related oxidative stress, apoptotic events, and sperm DNA integrity to male infertility, where factors such as age, smoking, and diet have proved to have an effect on this condition. However, the relation between the ROS, damage to DNA, mitochondrial membrane potential, membrane integrity, and structure of the sperm chromatin have not been studied together in idiopathic infertility.

In the present study, we found a significant difference between fertile and infertile men compared to DNA fragmentation, suggesting a susceptibility to fragmentation of the chromatin structure in the population of men diagnosed with infertility of unknown origin. These individuals had a high percentage (27.5%) in the DNA fragmentation index (DFI), approaching the prospective limit proposed by Evenson et al. (DFI ≥ 30%) in 1999, where the probability of in vivo fertilization or intrauterine insemination is reduced to a value close to 0 if the percentage of DFI is equal to or lower than 30%, this being a prognostic indicator of the probability of success of an assisted reproduction procedure, which is related to the fertilization rates.

The findings of this study report an increase in the sperm DNA damage in the infertile population group, indicating that these individuals have baseline damage higher than the fertile men group. The percentage of DNA damage is highly correlated with the percentage of DNA fragmentation (r = 0.86; p < 0.01) coinciding with previous reports, which in turn confirms that these tests are capable of detecting alterations in the quality of the final product of the spermatogenic process.

Regarding oxidative stress, the ROS are involved in the physiological functioning of male germ cells as molecules involved in the signaling and activation of cellular processes influencing their fertilizing capacity but due to their high reactivity, they can lead to negative effects by generating an imbalance in the sperm redox status. The origin of the ROS by the sperm is controversial, and some authors claim that their increase is due to the cytoplasmic retention of immature sperm, suggesting a less efficient regulation in spermiogenesis. In the present study we found an increase in the generation of the ROS in the samples of individuals diagnosed with idiopathic infertility, making it possible to propose these ROS as an important factor in this type of infertility. Some authors also report an increase in sperm ROS in individuals who showed abnormalities in at least one of the conventional parameters of the semen
analysis, which confirms the importance of the ROS in male fertility.\textsuperscript{19}

The excessive production of ROS could have serious implications in the structure and functionality of the sperm plasma membrane because this exhibits a special composition of phospholipids, sterols, and saturated and unsaturated fatty acids, the latter present in a greater proportion, making it susceptible to oxidation.\textsuperscript{29,30} Although this study did not find a difference between the populations studied in the MDA levels detected, there is a relation between the degree of oxidation of the plasma membrane and the amount of sperm ROS, confirming the mild effect of intracellular ROS on the membrane, indicating that the contribution by the NADPH oxidase plasma may be reduced, and there may be other sources of ROS generated in infertile individuals.

**Conclusion**

In conclusion, this set of sperm evaluation techniques would make it possible to make a solid contribution to the profile of the seminal analyses, and along with other parameters become an effective diagnostic and prognostic tool for evaluating male factor infertility of idiopathic origin and explore the events that generate such a condition, which must be taken into account in clinical practice for optimizing the classification of infertility.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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