Relationship Between Sperm DNA Fragmentation and Nuclear Vacuoles

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ABSTRACT

Objective: The aim of the present study is to assess the correlation between the presence, quantity and size of nuclear vacuoles and DNA damage and chromatin status in sperm samples of men who underwent to assisted reproduction technology.

Methods: Forty six males who underwent to assisted reproductive technology (ART) were considered. According to their latest semen analysis (<3 months), were grouped into: (A) strict morphology index ≤4% (26) and (B) strict morphology index ≥14% (20). Motile sperm were selected by density gradient, and MSOME study was conducted to assess the number and size of nuclear vacuoles. DNA fragmentation (TUNEL) and DNA strand status (acridine orange) were assessed over the selected spermatozoa accordingly to their vacuole pattern.

Results: In group A, sperm without vacuoles (1^o) have similar levels of DNA fragmentation (TUNEL) in compare to the rest of observed patterns (2° - 6°). Regarding to AO, spermatozoa with large or several vacuoles that cover more than 30-50% of the nuclear surface are AO+, but not necessarily TUNEL positive. The first three patterns of vacuoles patterns had lower levels of AO in compare to grades 4° and 6°. In group B, those sperm with one or more vacuoles greater than 30%-50% (4° and 6°), had a significant increase in TUNEL values, in relation to group 1°- 3°. Considering AO, it was found that the 4° and 6° pattern had a significantly elevated level of this marker, as same of group A (*P* <0.05).

Conclusions: There is no relationship between the greater number and size of sperm vacuoles with high levels of DNA fragmentation in patients with severe teratozoospermia (Kruger <4%). Conversely, this relationship is evident in normal semen samples (normal morphology. Sperm selection by IMSI technique, to select non-fragmented sperm in patients with Kruger <4%, is not necessarily secured when non-vacuolated sperm is selected.

Keywords: DNA fragmentation, Vacuoles, Sperm Morphology.

INTRODUCTION

Paternal genome integrity is vital to start and maintain a pregnancy. The presence of DNA strand breaks and/or changes in DNA nucleotides (resulting from the paternal genome) in the embryo's genome, is not compatible with normal embryonic and fetal development. Recently it has been shown that sperm DNA fragmentation is a potential cause of unexplained infertility. Likewise, a significant increase in DNA fragmentation levels would be associated with low rates of fertilization, embryo implantation and high rates of miscarriage (Carrell *et al*., 2003; Seli *et al*., 2004; Virro *et al*., 2004; Lewis & Aitken, 2005; Meseguer *et al*., 2008; Aitken *et al*., 2009; Sakkas & Alvarez, 2010).

Physical and metabolic factors can induce sperm DNA damage. This can occur in spermatogenesis or during the transit through the epididymis (Aitken *et al*., 2005, Aitken *et al*., 2006; Aitken & De Iuliis, 2007; Banks *et al*., 2005). Furthermore, many risk factors associated to increased

DNA fragmentation have been identified, e.g. varicocele, cryptorchidism, advanced age, severe teratozoospermia, episodes of high fever, radio and chemo therapy (Angelopoulou *et al*., 2007; Delbes *et al*., 2007; Smith *et al*., 2007; Smit *et al*., 2010; Vagnini *et al*., 2007; Wu *et al*., 2009; Moskovtsev *et al*., 2009; Mehdi *et al*., 2009).

DNA damage could be single and/or double-stranded, and has been assessed by several methods: SCSA (Sperm chromatin structure assay), SCD (Sperm chromatin dispersion test), TUNEL (deoxynucleotidyl-transferase-mediated-dUTP -nick-end-labeling) (Evenson *et al*., 1980; Gorczyca *et al*., 1993; Fernández *et al*., 2005). In our experience, and in the literature, there is vast evidence that the TUNEL technique is presented as a valuable tool for clinical use in infertility (Sakkas & Alvarez, 2010; Sergerie *et al*., 2005; Greco *et al*., 2005; Avendaño *et al*., 2009).

It has been reported that DNA fragmentation events may be associated with sperm apoptosis (Wu *et al*., 2009; Sakkas *et al*., 2002, 2003; Oehninger *et al*., 2003; Singh *et al*., 2003). Regarding to this, apoptotic markers have been detected in human spermatozoa, as: externalization of phosphatidylserine, active caspase 3, etc. (Almeida *et al*., 2009; Kotwicka *et al*., 2008; Paasch *et al*., 2004; Shen *et al*., 2002). On the other hand, some authors have stated that there is a positive correlation between DNA fragmentation and the presence of nuclear vacuoles, especially those that occupy more than 50% of the nuclear surface (Franco Jr *et al*., 2008; Nadalini *et al*., 2009; Oliveira *et al*., 2010). We have previously reported that in a population of high levels of sperm DNA fragmentation, there is no difference between the proportions of vacuolated spermatozoa (Alvarez Sedó *et al*., 2011). On the other hand, other reports state that the presence of vacuoles could be associated to the state and/or abnormalities of the acrosome and, thus, not necessarily with DNA fragmentation (Perdrix *et al*., 2011; Kacem *et al*., 2010).

Several sperm selection techniques have been proposed with the idea of removing sperm with damaged DNA and/or presence of nuclear vacuoles, in that sense and in our experience, both, Annexin V columns and IMSI (intracytoplasmic morphologically selected sperm injection) respectively, would be the techniques proposed for this purpose. However, to be possible (according to the authors mentioned above) that both events (DNA fragmentation and vacuoles) represent "the same" or could be related, there may not be a clear indication for both techniques. It is for this reason that this study aims to assess the presence, quantity and size of nuclear vacuoles and their association with DNA damage and chromatin status in sperm samples of men who underwent to assisted reproduction treatments.

MATERIAL AND METHODS Population

For the present study, a total population of 46 males who underwent to assisted reproductive technology (ART) was considered. According to their latest semen analysis (no longer than three months), were grouped into: (A) 26 patients who had strict morphology index ≤4% and (B) 20

Figure 1. Left: IMSI-MSOME inverted microscope. Right: Patterns assessed by MSOME.

Figure 2. Incubation and detection system in multiwell glass slide for AO and TUNEL assessment.

Figure 3. TUNEL assay for DNA fragmentation assessment. Fluorescence 488nm, positive TUNEL (green) bright field.

Figure 4. Acridine Orange test. Green sperm (normal), Yellow and Red (affected spermatozoa). **Table 1.** Acridine Orange and TUNEL levels in group A

patients with strict morphology index ≥14%. In all cases, sperm concentration was $> 10 \times 10^6$ /mL and progressive motility >32%.

Sample preparation

The samples were evaluated and subjected to discontinuous density gradient separation (PureCeption, SAGE) according to the World Health Organization (WHO, 2010) guidelines.

Sperm vacuoles assessment and selection (MSOME)

The sperm vacuole assessment was performed using an inverted microscope (Nikon - Eclipse TE 200U. - Differential phase contrast (DIC) and a 100X objective immersion. Therefore a secondary magnification system was used to get a final magnification of 7600X. Figure 1 depicts the patterns considered to assess the number and size of nuclear vacuoles. After identifying each pattern, we proceeded to select (by micro-manipulation) different sperm populations. Each group of sperm was subsequently subjected to the evaluation of DNA fragmentation and acridine orange stain.

Sperm DNA fragmentation (TUNEL)

Some spermatozoa, from each group, were loaded into a multi-well glass slide (Figure 2), and previously embedded in Poly-L-Lysine for 5 minutes. After that, 20 µL of 2% of formaldehyde in PBS was added in order to fix the cells for a period of 1 hour. The samples were washed with PBS and permeabilized with cold methanol for 3 minutes. It was then incubated with TUNEL solution (Roche) for 1 hour at 37°C, then washed and mounted with Vectashield H-1000 (Vector Laboratories). The observed patterns are represented in Figure 3.

Acridine Orange stain (AO)

The AO assay measures the susceptibility of sperm nuclear DNA to acid-induced denaturation in situ by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). The fluorochrome AO intercalates into double-stranded DNA as a monomer and binds to single-stranded DNA as an aggregate.

The monomeric AO bound to native DNA fluorescence green, whereas the aggregated AO on denatured DNA fluoresces red (Figure 4). For the procedure, spermatozoa were loaded into the multi well glass slide and let them dry. Starting from a stock solution of AO 1%, a working solution was prepared with 0.1M of citric acid, 0.3M of Na2HPO47H2O at pH 2.5. Samples were fixed with Carnoy´s solution for 1-2hs. Sample were carefully washed with distilled water, then stained with AO solution for 5

patients.

Markers at Group A

Table 2. Acridine Orange and TUNEL levels in group B patients.

TUNEL: Group A vs. Group B

minutes and finally rinsed with distilled water. Immediately the samples were mounted and observed under Fluorescence Microscopy.

Statistics

The Student's t-test was used for between-group comparisons, and the Mann-Whitney U-test was used to assess homogeneity. Differences were considered significant when *P*<0.05. MedCalc 12.4 software (Belgium) was used for the statistical analysis.

RESULTS

Table 1 shows the averages of Acridine Orange and TUNEL in group A (558 sperm evaluated). All spermatozoa were grouped according to stablished vacuole patterns. In this group of patients can be observed that sperm without vacuoles (1º) have similar levels of DNA fragmentation by TUNEL in compare to the rest of observed patterns (2°- 6°), although it is true, the patterns 4, 5 and 6 show a slightly increase of TUNEL, but these are not statistically significant. Regarding to AO, it's clearly seen that spermatozoa with large or several vacuoles that cover more than 30-50% of the nuclear surface are AO+ (*P*<0.05), but not necessarily TUNEL positive. The first three patterns of vacuoles patterns had lower levels of AO in compare to grades 4° and 6° (*P*<0.05).

Moreover, in Table 2, corresponding to the group B (380 sperm evaluated). Unlike the previous group, those sperm with one or more vacuoles greater than 30%-50% (4° and 6°), had a significant increase in TUNEL values, in relation to group 1°-3° (*P*<0.05). Regarding the AO, it was found that the pattern 4° and 6° had a significantly elevated level of this marker, as same of group A (*P*<0.05).

Table 3 shows the comparison of TUNEL values between

the groups A and B, demonstrating in all vacuoles patterns, even 1, an increase is observed (*P*<0.05) in group A. In both groups pattern 4° had the higher level of DNA damage (*P*<0.05).

DISCUSSION

It has been described, in recent reports, the possible positive correlation between higher rates of sperm DNA fragmentation and the presence of larger nuclear vacuoles (4th pattern) (Franco Jr *et al*., 2008; Nadalini *et al*., 2009; Oliveira *et al*., 2010). In these cited papers, there is no specification of what type of patients was used for the study, i.e., not previously identified populations. By contrast, in our experience, the experimental design was created to identify the population that could benefit from the techniques of sperm selection here proposed. In our results, in a population with an altered morphology (Kruger <4%) and elevated levels of DNA fragmentation, an indistinct presence of sperms with size-number of vacuoles was observed, normal spermatozoa had the same proportion of DNA damage that those sperm with different vacuole patterns. These results are in accordance with those previously described by our group, where in a population enriched with apoptotic sperm and morphology <4%, no differences in sperm counts with or without vacuoles were observed, regarding the non-apoptotic population of the same sample (Alvarez Sedó *et al*., 2011). On the other hand, it is important to mention that this work confirms that patients with morphology index $<$ 4% present a higher proportion of DNA fragmentation levels compared to patients with morphology index >14%; in previous reports from our group this increase was further defined as/when age is greater 40-45 years (Uriondo *et al*., 2011).

By contrast, in a population of spermatozoa with normal morphology (Kruger ≥14%), morphologically normal sperm or with small vacuoles compromising less than 20% of its nucleus surface, exhibit a significantly lower proportion of DNA damage compared to sperm with 4th or 6th patterns, ie, one or more vacuoles representing 30-50% of the nuclear surface, which would coincide with that reported by previously mentioned authors.

In this sense, we can say that IMSI technique for selecting non-fragmented sperm in a group of patients with lower than 4% of morphology index and high levels of fragmentation, taking into account the vacuole pattern, would not be ensuring such purpose. Moreover, even considering the possibility of finding normal spermatozoa in these samples is low; still, they can be equally fragmented. Nonetheless, in patients with normal morphology, selection of morphologically normal sperm or those with small vacuoles (representing less than 20% of the nuclear surface) could help to select sperm with non-fragmented DNA.

We are aware that further work is needed (e.g, what happens in patients with Kruger 5-13%), however, we believe so far that our results reveal that in patients with severe alterations in sperm morphology, there would be no predominance of sperm larger-number vacuoles with high spermatic apoptosis. Probably these patients would not benefit "at all" with the election of spermatozoa selected through higher magnification with the purpose of choosing non-fragmented sperm.

CONCLUSION

There is no relationship between the greater number and size of sperm vacuoles with high levels of DNA fragmentation in patients with severe teratozoospermia (Kruger less 4%). Conversely, this relationship is evident in normal semen samples (normal morphology. Sperm selection by IMSI technique, to select non-fragmented sperm in patients with Kruger <4%, is not necessarily secured when non-vacuolated sperm is selected.

CONFLICT OF INTERESTS

No conflict of interest have been declared.

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