A double-blinded comparison of in situ TUNEL and aniline blue versus flow cytometry acridine orange for the determination of sperm DNA fragmentation and nucleus decondensation state index

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Summary

The impact of sperm DNA fragmentation on assisted reproductive technology (ART) successes, in terms of outcome, is now established. High levels of DNA strand breaks severely affect the probability of pregnancy. The importance of sperm nucleus condensation in early embryogenesis and, subsequently, on the quality of the conceptus is now emerging. In this article we have compared in situ analyses with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) (for DNA fragmentation) with aniline blue (AB) (for nucleus decondensation), versus flow cytometry (FC) after acridine orange staining, in a double-blinded analysis. In our hands, TUNEL and acridine orange give perfectly comparable results. For decondensation the results are also comparable, but the double-stranded green fluorescence obtained with acridine orange seems to slightly underestimate the decondensation status obtained with AB.

Keywords: Acridine orange, Aniline blue, Decondensation, DNA fragmentation, Flow cytometry, Sperm, TUNEL

Introduction

According to the World Health Organization (WHO) (2004) estimation, infertility may now affect one couple of five in western countries. Male reproductive failure is thought to be the cause of at least one-half of the total number of failures. During the past half century and before the advent of large scale in vitro fertilization, routine semen analysis was oriented towards numeration, concentration, motility and morphology. Rapidly it has become obvious that a better appreciation of sperm chromatin is needed. A ‘transitory’ interest in nuclear shape and chromatin texture, i.e. the organization of chromatin condensation, measured by aniline blue (AB) staining (Auger et al., 1990) or by chromomycin A3 (Sakkas et al., 1996) has emerged. But overall, interest has been focused on sperm DNA fragmentation (Evenson et al., 1980), especially in relation to oxidative stress.

The large waste observed for embryos conceived by assisted reproductive technology (ART) has led to the common consensus that fertilization and embryo development constitute only a small proportion of sperm development capacity, as it is also true that the quality of the first embryo cleavage is under paternal effect (Eid et al., 1994, Ward et al., 2001). True sperm capacity is defined as the ability to sustain embryo development to term. Progress has been made in the global determination of DNA strand fractures (i.e. fragmentation; Evenson et al., 1980) and the identification of specific insults such...
as oxidation products and adducts (Gaspari et al., 2003, Badouard et al., 2008, Ji et al., 2013), essentially those originating from pollution. Interest towards the tertiary structure of the nucleus, i.e. its condensation, is re-emerging (Kim et al., 2013, Sellami et al., 2013). The importance of the structural interactions between DNA and the packaging proteins is no longer a matter for debate: failures in condensation induce delays in the first cell cycle with further detrimental consequences at some point for the developing embryo (Rousseaux et al., 2008; Ward, 2010). This includes recurrent abortion (Kazerouini et al., 2009). This highly compacted structure is not inert. Some gene families that are highly important for early embryo development are associated with histones (in human spermatozoa: Hammoud et al., 2009). If the oocyte has an important, yet finite, DNA repair capacity, it is largely efficient in relation to DNA strand breaks (Menezo et al., 2007, 2010). The oocyte capacity to improve tertiary structure is rather limited. Chromatin assembly factor 1 (CAF-1), and H2AFX (H2A histone family, member X) are correctly expressed in the oocyte; however it is not clear if these factors are active at the correct time, immediately at fertilization. This means that determination and correction (when possible; Menezo et al., 2014) of decondensation is of paramount importance in ART. There is no real consensus on the technique of evaluation of DNA fragmentation, but most of all for decondensation, which is usually totally neglected. In a first approach, we have demonstrated that chromomycin A and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) have overlapping characteristics: chromomycin A3 (CMA3) is a DNA dye, a guanine-cytosine-specific fluorochrome that competes with protamines for access to DNA. Aniline blue (AB) is a better marker of the sperm nucleus chromatin decondensation state (Belloc et al., 2009). In this study we have compared, on the same samples, the DNA fragmentation index (DFI) measured by TUNEL in situ and the DNA decondensation state index (SDI) measured by AB on slides versus flow cytometry (FC) after staining with acridine orange.

Material and methods

The analyses were performed in a double-blinded manner in 88 patients, who were under consultation for infertility after at least one ART failure. The sperm was collected from patients by masturbation. After liquefaction (0.5 h), the sperm was aliquoted in 2 × 0.5 ml samples. Samples were frozen in liquid nitrogen until analysis for dosage using TUNEL on slides and FC analyses. For AB testing, the samples were directly analysed after liquefaction according to the technique of Hamadeh et al. (1996) (see also Belloc et al. 2008). In total, 200 sperm cells were examined for each in situ determination.

DNA fragmentation with TUNEL

For DNA fragmentation with TUNEL, the classical terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick-end labelling test was performed. The fluorescein isothiocyanate–labelled deoxyuridine triphosphate (dUTP) kit (Roche Diagnostics Corporation, Mannheim, Germany) was used according to the instructions of the manufacturer for the in situ technique (see Cohen-Bacrie et al., 2009).

Acridine orange staining and flow cytometry

For acridine orange, flow cytometry (FC), if the sperm concentration was lower than 0.5 × 10⁶/ml, before acridine testing, the sample was washed/concentrated using a phosphate-buffered medium containing albumin at 10 g/l, in order to reach a final concentration of at least 1 × 10⁶/ml (Menezo, 2003; unpublished data). After thawing, the sperm were diluted in Earles medium + 1% albumin. For either concentration or dilution, the presence of proteins was necessary in order to avoid agglutination. After dilution (1/10) the sperm sample was lysed in a 0.1% Triton solution in 1 M HCl. After mixing, the staining solution (acridine orange in a phosphate citrate buffer) is added. The suspension was shaken gently for 30 s before introduction into the cytometer. The number of cells analysed was roughly 8000. Sperm samples were thawed and prepared one by one at the last minute. This step is necessary to maintain reproducibility.

The samples were analysed on a flow cytometer FC500 Beckman Coulter equipped with a BioSense™ measurement cell (150 μM × 450 μM). The laser used was an Argon device 488 nm, with seven detection units, that was focused on a elliptic measurement area 10 μM × 80 μM. The flow reproducibility and reliability was controlled for each measurement using flow check microspheres. The optical fluorescence stability detection was controlled using Flowser microspheres. Quantification was realized by gathering the signals of sperm cells obtained at 525 nm for SDI and at 620 nm for the lesions of single DNA strands (DFI).

Statistical analysis

One of the comparison of the data obtained was performed using the Welch two-sample t-test; or an alternative two-sided test. The other was performed using the Wilcoxon test.
Figure 1 In situ TUNEL versus acridine orange flow cytometry. Individual variations: blue, TUNEL; red, acridine orange.

Figure 2 In situ aniline blue versus acridine orange flow cytometry. Individual variations: dark blue: aniline blue; light blue: acridine orange green fluorescence.
Acridine orange vs aniline blue and TUNEL for sperm DNA analysis

Results

DNA fragmentation (DFI)

DNA fragmentation (DFI): means observed TUNEL: 18.07, acridine orange 17.47.

- Welch two-sample t-test; alternative: two-sided T-test: array, confidence interval 95% $[-1.9772; 3.1748]$. P-value 0.65: no difference between the two techniques.
- Wilcoxon test: Z-score: –2.57. Asymptotic significance: 0.01. The tests cannot be considered as similar. Acridine orange gives a lower value for estimation of chromatin decondensation.

Individual variations obtained between the two techniques are shown in Fig. 1, in situ image for the test is presented in Fig. 3.

Chromatin decondensation (SDI)

Chromatin decondensation (SDI): means observed AB: 9.94, acridine orange: 9.01.

- Welch two-sample t-test: confidence interval 95% $[-0.63; 2.47]$. P-value 0.24: no difference between the two tests.

Discussion

In situ analysis has two main disadvantages/two obstacles: the number of cells analysed and the operator dependence. It has also to be said that sperm samples can be frozen before FC acridine orange testing for SDI; this is not the case for AB. The samples cannot be frozen before AB testing otherwise the sperm cells are fully (blue) coloured. Automated FC avoids these difficulties, comparing in situ TUNEL versus FC acridine orange (red fluorescence) on the one hand and in situ AB versus FC acridine orange (green fluorescence) on the other hand gives roughly...
In situ determination of sperm decondensation index (SDI) using aniline blue. Top panel: light staining (SDI: 3%); bottom panel: heavy staining (SDI: 51%).

the same results. This is a little bit less valid for AB: the Wilcoxon test give a significant lower quantification with FC acridine orange green fluorescence (SDI), whilst the Welch test leads to a similar quantification.

For sperm DNA fragmentation the distribution in our general population, is, on a yearly basis, the following: using TUNEL DFI < 25%: 86%; DFI > 25%: 14% (1336 patients). But 53% of the patients have a DFI between 20–25%. An important percentage of the patients consulting for infertility is in this grey zone 20–25%. The pathological values limits are still controversial and may vary according to the techniques employed (Kazerooni et al., 2009, Sharma et al., 2010, Simon et al., 2011, Lewis et al., 2013). The threshold of 20–25% for sperm DNA fragmentation for decreased fertility is not far from the consensus: this could be considered as a grey zone where fertility problems start. The upper limit for having a healthy delivery can be considered at 35–40%. The success of ART also depends of the quality of the oocytes and especially the importance of the DNA repair possibilities, i.e. the mRNAs coding for this process (Menezo et al., 2007). This is especially true for fragmentation: the repair mechanisms are complete and iterating in the healthy (young) oocyte. This is less valid for chromatin condensation,
i.e. tertiary structure: the oocyte has a poor capacity to manage with an abnormal chromatin structure (Menezo et al., 2007). In our overall population, 17% of patients have a SDI strictly over 25% (AB, 528 patients). The determination of the ‘risky’ threshold is not just a matter of semantics. SDI is a neglected parameter, even if it is now clear that chromatin structure is an important parameter for the capacity to produce live births. The negative impact of a high decondensation may occur immediately at the time of fertilization, with the ‘no fertilization’ syndrome in intra-cytoplasmic sperm injection (ICSI) (Junca et al., 2012) and early developmental arrests (Dattilo et al., 2014), up to miscarriages (Kazerooni et al., 2009). Considering DFI, numerous observations have focused on the risk for the outcome and the next generation (Hemminki et al. 1999, Belloc et al., 2008, Fernández-Gonzalez et al., 2008). This includes an increased risk of cancer for the next generation. In a recent analysis of babies born after ICSI, Davies et al. (2012) demonstrated an increased risk of birth defects. The DFI increases with age and it is obvious that the patients seeking ART are older compared with the general population. Only one-third of our patients consulting for hypofertility seems totally ‘clean’ of an impairing DNA fragmentation. Moreover, in a recent paper we were able to demonstrate that both DFI and SDI are stable with time in the absence of any treatment (Menezo et al., 2014). In conclusion, FC acridine orange allows us to check simultaneously both sperm DNA fragmentation and decondensation. The determination of these parameters seems increasingly necessary: it has to be clearly said that ICSI does not cure all male fertility problems and may even be hazardous for the next generation, as oxidative stress and imprinting are problems and may even be hazardous for the next generation. In offspring. Biol. Reprod. 78, 761–72.

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