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Evaluation of the human sperm nucleus: ambiguity and risk of confusion with chromomycin staining

Yves Ménézo

Laboratoire Clément, Paris, France

Commentary

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Author for correspondence:
Yves Ménézo. Laboratoire Clément, 75016 Paris, France. E-mail: yves.menezo@gmail.com

It is now common knowledge that classical sperm parameters: number, mobility and morphology give only partial information on their developmental capacity. With the advent of intracytoplasmic sperm injection (ICSI), the evaluation of nuclear integrity is now an unavoidable parameter in studying human infertility, irrespective of whether fertilization and blastocyst formation are obtained/observed after ART procedures. Increased DNA fragmentation (SDF/DFI, DNA fragmentation index) and an increase in the decondensation index (SDI), also called high DNA stainability (HDS), in the sperm chromatin structure assay, SCSA, are the two major negative parameters of sperm developmental capacity. These two markers do not affect fertilization but decrease the developmental capacity of the embryo obtained leading to a dramatic increase to the time to pregnancy (Buck Louis *et al.*, 2014).

The negative effect of sperm DNA fragmentation is obvious: as the SDF is more or less linked to oxidative damages; this leads to DNA strand breaks (Ménézo *et al.*, 2014a) or adduct products (Badouard *et al.* 2008), oxidized bases and abasic sites formation. All these insults have to be repaired at the time and immediately after fertilization and the problem is equally shared between the male and the female genomes (Lopes *et al.*, 1998), not specifically protected during the quiescent time before maturation. The DNA repair process is mandatory to avoid transmission of mutations to the next generation. The oocyte's capacity to repair decay is important, redundant, but finite and decreases with maternal age (Ménézo *et al.*, 2010); overwhelming this defence leads normally to embryo apoptosis. SDF affects IVF outcomes (Evenson *et al.*, 1999) and increases with age (Belloc *et al.*, 2009; Deenadayal Mettler *et al.*, 2019) in relation to a decreased capacity to combat oxidative stress.

Three main processes are important in nucleus condensation, i.e. nucleus tertiary structure, measured by SDI/HDS. The replacement of histones by protamines, their padlocking and the final biochemical modification: deacetylation, methylation etc., lead to a spatial and biochemical nucleus conformation required for rapid access to the paternal genome after nucleus swelling (Ward *et al.*, 2001; Ward, 2010). This is mandatory for quick activation of the S-phase and the major methylation/epigenetic modifications affecting the male genome, which will be rapidly demethylated and then quickly remethylated (Park *et al.*, 2007). This aspect has been well documented in the bovine, in which fertility of one bull can be estimated from several thousands of inseminations (Eid *et al.*, 1994; Rahman *et al.*, 2018; Kutchy *et al.*, 2019). HDS/SDI elevated values are related to reduced protamination or a failed padlocking of them, leading to nucleus structural loosening. Tertiary structure anomalies finally reduce the size of both pronuclei (Rahman *et al.*, 2018). Sperm decondensation is highly significantly directly related to defective protamination in low fertility bulls (Kutchy *et al.*, 2019). The oocyte is poorly equipped to repair and manage a defective tertiary structure (Ménézo *et al.*, 2007, 2014a, 2014b).

In human, poor sperm morphology is linked to high HDS/SDI (Zini *et al.*, 2009). Pronuclear formation can be impaired, leading to a syndrome called 'no fertilization post ICSI', an unfortunate concept (Junca *et al.*, 2012). It may induce poor embryo development and lead to recurrent miscarriages (Booze *et al.*, 2019; Jerre *et al.*, 2019). The tertiary structure is of major importance to allow the correct timing in preimplantation embryo development. A three-phase first step, involving peroxidation of lipids by NADPH oxidase, followed by a reaction with oxidized glutathione, followed by glutathione peroxidase activity, is mandatory for this protamine padlocking (Ménézo *et al.*, 2014a). This is probably why sperm nucleus condensation increases with age and so HDS/DFI decrease: HDS/SDI and SDF/DFI are independent parameters.

Different evaluations of SDF/DFI and the effect of age

Current analyses: SCSA® and flow cytometry–terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL), modified or not, are the two major ways to test sperm nuclei and are perfectly linked, with high coefficients of correlation; on this issue there is no ambiguity in the scientific literature. The Comet assay has also a certain value, but it is less easy to use and not easy to automate.

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Since 1956 and the Denham Harman free radical theory of ageing (*Aging: A Theory Based on Free Radical and Radiation Chemistry*) sustained by Fenton's discovery of free radicals in 1894, it is now of common knowledge that age decreases defences against oxidative stress and that this is independent of sex. This leads naturally to an increase in sperm DNA oxidation and fragmentation with age. Whatever the testing technique used, TUNEL, DFI/SDF by SCSA, or cytology (Belloc *et al.*, 2009; Deenadayal Mettler *et al.*, 2019; Paoli *et al.*, 2019) SDF is strongly correlated with 8-oxodeoxyguanosine (8-OHdG), the main oxidation product of DNA (De Iuliis *et al.*, 2009; Aitken *et al.*, 2010) with significance over 10^{-3}, to confirm that oxidative stress is a major effector of sperm fragmentation. However, the dosage of 8-OHdG is delicate, as spontaneous oxidation of DNA may occur during processing (Badouard *et al.*, 2008; Aitken *et al.*, 2010) and this may lead to uncertain conclusions.

HDS/SDI evaluation: the problem of chromomycin (CMA3)

HDS/SDI is determined in SCSA using the green fluorescence of acridine orange. It corresponds to increased access of the dye to DNA, due to loosening and weakening of compaction. The results obtained with SCSA (HDS) and aniline blue (Hamidi *et al.*, 2015) were not statistically different (Welch two-sample *t*-test confidence 95%, $P = 0.24$) or slightly lower (Wilcoxon Z-test).

HDS (determined by SCSA) shows a slight, but significant, decrease with age. When measured by aniline blue, the slight decrease observed was not found to be significant (Belloc *et al.*, 2009; Ménézo *et al.*, 2014b; Evenson *et al.*, 2020), coherent with a better padlocking of the brotamines. HDS and AB give coherent similar data.

Chromomycin

Chromomycin (CMA3) is presented here as a standard for nucleus decondensation testing and protamination quality. Chromomycin is a fluorescent dye specific for DNA G–C bonds, as is oligomycin. It is first of all a marker of DNA, but also binds to protamines. If co-incubation is performed with soluble salmon protamines, CMA3 binding decreases (Manicardi *et al.*, 1995). However, if CMA3 staining is performed prior to TUNEL, the sensitivity to nick translation is 'drastically reduced' (Manicardi *et al.*, 1995). Moreover, De Iuliis *et al.* (2009) found first a strong overlap/correlation ($r = 0.956$, $P < 0.001$) between TUNEL and CMA3. This confirmed the strong affinity between DNA and CMA3. Finally, a very high correlation was observed by De Iuliis *et al.* (2009) between 8-OHdG, the major product of DNA oxidation, and CMA3 ($r = 0.610$, $P < 0.001$) that was similar to the correlation observed, in the same laboratory, between DNA fragmentation (TUNEL) and 8-OHdG ($r = 0.671$, $P < 0.001$; Aitken *et al.* 2010) (Table 1).

Therefore, CMA3 did not allow a dichotomy between condensation and DNA fragmentation, due to oxidative stress insults. Confirming this fact, the paternal age effect measured by CMA3 showed an increase in decondensation with age (Belloc *et al.*, 2009), which could be coherent with a decrease in protamination, but did not fit with the increase in sperm DNA fragmentation related to paternal age. CMA3 cannot be considered as the standard test for sperm decondensation. Manicardi *et al.* (1995) showed that the real measurement of condensation with CMA3 was the difference in staining between CMA3 staining minus TUNEL staining, if reduced by two-thirds. The real evaluation of condensation included protamination, padlocking and the final biochemical modifications of nuclear structure (methylation, phosphorylation, acetylation and their combinations).

Table 1. Correlation between, CMA3, TUNEL and 8-OHdG (de Luliis *et al.*, 2009; Aitken *et al.*, 2010)

	r	*P*-value
CMA3 vs TUNEL	0.956	<0.001
CMA3 vs 8-OHdG	0.610	<0.001
TUNEL vs 8-OHdG	0.671	<0.001

Conclusion

CMA3 testing leads to some confusion when not handled with care: if used as a reference for decondensation, it may lead to wrong interpretations (Mohammadi *et al.*, 2020). Stating that chromomycin reflects the degree of protamination is incorrect and not justified. Data dispersion and the absence of correlation observed by authors between CMA3 and the 'blue dyes' and between HDS and CMA3 is the image of a normal situation. The same processes are not measured and cannot be strictly correlated, contrary to that observed between CMA3 and TUNEL, or 8-OHdG for example (De Iuliis *et al.* 2009; Aitken *et al.* 2010), which have a rather good correlation. The difference Δ = CMA3 − TUNEL from the same sample seems a much more rationale evaluation of tertiary structure and should be compared with HDS. An important part of the binding/fluorescence of CMA3 corresponds to its interaction with DNA. HDS does correlate with decondensation and quality of the tertiary structure. Recent interesting observations (Dattilo *et al.*, 2016; Gallo *et al.*, 2018; Jacquesson-Fournols *et al.*, 2019) have clearly highlighted the importance of final methylation rearrangements in condensation measured by HDS; folate intake *per os* decreases HDS in some infertile patients and restores fertility. Sperm DNA fragmentation and nucleus decondensation are two independent major effectors of fertility; it is important to share correctly the contributions of these two parameters, to define therapeutic protocols and to remember that the spermatozoon not only provides the paternal genome, it also is key to a rapid, harmonious embryonic development to term (Ward 2010).

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