Zygote

cambridge.org/zyg

Comparison of sperm preparation methods to improve the recovery of mature spermatozoa in sub-fertile males

Chiara Fasano, Giuseppe D'Andolfi, Loredana Di Matteo, Claudia Forte, Brian Dale and Elisabetta Tosti

Centro Fecondazione Assistita (CFA), Via Tasso 480, 80123 Naples, Italy

Research Article

Cite this article: Fasano C *et al.* (2022) Comparison of sperm preparation methods to improve the recovery of mature spermatozoa in sub-fertile males. *Zygote*. **30**: 664–673. doi: 10.1017/S0967199422000132

Received: 13 September 2021
Revised: 7 March 2022
Accepted: 14 March 2022
First published online: 8 July 2022

Keywords:
Chromatin condensation; Gradient; Sperm preparation; Spermatozoa; Swim-up

Author for correspondence:
Elisabetta Tosti. Centro Fecondazione Assistita (CFA), Via Tasso 480, 80123 Naples, Italy.
Tel: +39 081641689.
E-mail: elisabettatosti@yahoo.it

Summary

The integrity of chromatin in the spermatozoon is essential for reproductive outcome. The aim of this study was to evaluate the most effective and cost-effective method to reduce the percentage of spermatozoa with defects in chromatin decondensation for use in assisted reproductive technologies (ART) procedures. Sperm samples from 15 sub-fertile males were examined at CFA Naples to determine the sperm decondensation index (SDI), using the aniline blue test, before and after preparation, comparing density gradients with two different swim-up approaches. All three techniques led to a reduction in decondensed spermatozoa with no statistical difference ($P > 0.05$) between the control and the treated sperm. In contrast, we found a highly significant decrease in SDI ($P < 0.01$) after the two swim-up methods in all the samples, confirming the efficacy of these methods in lowering the percentage of chromatin compaction damage. There was no statistical difference between the two swim-up methods, however swim-up from the pellet led to improved count, motility and the percentage of normal condensed spermatozoa. We suggest that swim-up from the pellet be used in ART on sub-fertile males, both to reduce cell stress by multiple centrifugation and improve the recovery rate of mature spermatozoa.

Introduction

Gametogenesis is the biological process of gamete formation occurring in the gonads and underlined by meiosis, the unique process of cell division whose final target is the production of mature haploid gametes competent for fertilization. Spermatogenesis is a complex process of proliferation and differentiation of spermatogonia, the male germ cell precursors give rise to primary and secondary spermatocytes and round spermatids. During spermiogenesis, the last phase of spermatogenesis, the spermatid undergoes a dramatic structural remodelling that transforms it from a spherical shape to the typical one of a mature spermatozoon consisting of the head, the neck and the tail. A crucial event associated with spermiogenesis is sperm nuclear compaction due to the condensation state of chromatin. This packaging differs from the chromatin in somatic cells (Ward and Coffey, 1991) and occurs by the replacement of the DNA-linked histones with transition proteins in the spermatocyte that are later replaced by basic protamines (P1 and P2). This process creates a specific protamine ratio and leads ultimately to tightly packaged chromatin (Poccia, 1986; Curry and Watson, 1995; Balhorn *et al.*, 1999; Hao *et al.*, 2019), with a compact and hydrodynamic nuclear structure aimed both to protect the sperm genomic integrity and to improve sperm motility (Evenson *et al.*, 1978; Caron *et al.*, 2005). The mature chromatin contains ~85% protamines and 15% histones, whose retention is associated with epigenetic information regulating important gene expression involved in embryo development (Ward, 2010; Ihara *et al.*, 2014). In addition to the biological role of the histone-bound chromatin, a protamine anomaly associated with an increased level of histones may account for incorrect DNA chain folding.

Correct spermatogenesis and appropriate animal and human sperm chromatin condensation are associated with sperm maturity, functionality and fertilization potential. Evidence is provided that altered sperm chromatin condensation may affect the dynamics of DNA methylation reprogramming in the male pronucleus and that both male and female pronuclei show a tendency of decreased size leading in turn to a lower fertilization rate (Rahman *et al.*, 2018). Furthermore, it is well documented that different degrees of decondensed chromatin in a sperm population may exert detrimental effects on normal embryo development, ongoing and term pregnancy and live birth outcome (Sakkas *et al.*, 1998; Esterhuizen *et al.*, 2000; Evenson and Jost, 2000; Agarwal and Said, 2003; Virro *et al.*, 2004; Lin *et al.*, 2008; Kazerooni *et al.*, 2009; Ward, 2010; Talebi *et al.*, 2012; Booze *et al.*, 2019; Jerre *et al.*, 2019; Kutchy *et al.*, 2019).

In ~50% of couples infertility is due to male factors (Daumler *et al*., 2016). Clinical screening of sub-fertile men starts with the evaluation of sperm parameters according to WHO guidelines (World Health Organization, 2010). The conventional spermiogram investigates sperm concentration, motility and morphology. However, over the latter decades there has been increasing evidence that these parameters have poor prognostic value for fertilization success and therefore it is recommended that a sperm functionality test is associated with traditional semen analysis (Lefièvre *et al*., 2007; Shamsi *et al*., 2011).

In particular, sperm genomic integrity has been shown to affect sperm functionality together with fertilization and developmental success and pregnancy outcome (Lewis and Aitken, 2005; Lewis *et al*., 2008). Systematic reviews by Zini (Zini *et al*., 2008; Zini, 2011) have demonstrated that sperm genomic damage is associated with reduced natural and intrauterine insemination (IUI), lower *in vitro* fertilization (IVF) and pregnancy outcome, and higher risk of recurrent pregnancy loss (Hammadeh *et al*., 1998; Esterhuizen *et al*., 2002; Sreenivasa *et al*., 2012; Coughlan *et al*., 2015).

In assisted reproductive technologies (ART), IUI is the first therapeutic treatment in couples with unexplained infertility and/or in cases of normal male factors combined with mild female factors (Tomlinson *et al*., 1996; Starosta *et al*., 2020). However, it is common practice that, after three IUI attempts, IVF and/or intracytoplasmic sperm injection (ICSI) is suggested. Currently ICSI is the most common technique used to overcome severe male infertility, giving rise to the birth of millions of babies worldwide since the 1990s (Palermo *et al*., 2017; Haddad *et al*., 2021).

All techniques used in ART, from IUI through to IVF to ICSI, require pre-preparation of the spermatozoa based on the removal of seminal plasma and *in vitro* capacitation. Swim-up (SU) and density gradient centrifugation (DGC) are the most widely used techniques aimed to recover an enriched high-quality sperm population (Henkel and Schill, 2003). In this study, using the acidic aniline blue staining test on human semen samples, we compared three different sperm preparation methods to evaluate the best protocol by which to enrich the population of mature spermatozoa for use in ART.

Materials and methods

Patients and sperm preparation

In total, 15 seminal fluids from patients attending the Centre of Assisted Fertilization (CFA, Naples, Italy) for primary infertility and with written informed consent, were randomly selected on the basis of the following parameters: normal pH, volume ≥ 4 ml, sperm number $\geq 5 \times 10^6$/ml, normal morphology $\geq 2\%$ and progressive motility > 10 %. Samples were collected by masturbation after 2–5 days of sexual abstinence. Each sample was processed after liquefaction at room temperature for at least 30 min and then classified according to the reference values suggested by the WHO (World Health Organization, 2010). Microscopic examination of sperm number and velocity was quantified using a Makler counting chamber (Sefi Medical Instruments). Sperm morphology was evaluated using Papanicolaou stain-coated slides (TestSimplets, Waldeck Gmbh, Germany) and scored according to the Krüger strict criteria (Krüger *et al*., 1987). To avoid intravariability, sperm concentration, motility and morphology were evaluated always by the same experienced observer.

Each sample of seminal fluid was split into four aliquots as follows: (i) raw ejaculate as control; (ii) ejaculate subjected to discontinuous DGC; (iii) ejaculate subjected to swim-up from the pellet after centrifugation (SU-P); (iv) direct swim-up from the ejaculate (SU-E) without centrifugation (Fig. 1).

All the preparations and centrifugations were performed in conical tubes and dilutions and pellet resuspensions were made in Sperm Preparation Medium (SPM, ORIGIO, Måløv, Denmark).

DGC were performed by stratifying 1 ml of 40% gradient (Origio, Måløv, Denmark) over 1 ml of 80% gradient. Next, 1 ml of ejaculate was then gently layered on the 40% gradient and centrifuged at 200 *g* for 20 min at room temperature. The pellet was then removed and resuspended in 0.4 ml of SPM.

SU-P were performed by mixing 1 ml of ejaculate with 2 ml of SPM and centrifuged at 300 *g* for 3 min. Then the supernatant was removed and 0.5 ml of SPM was gently stratified over the compact pellet and incubated for 1 h at 37°C. Then 0.4 ml of the supernatant containing spermatozoa was gently collected without disturbing the interface between the pellet and the medium.

SU-E was performed by gently stratifying 0.5 ml of SPM on 1 ml of ejaculate and incubating for 1 h at 37°C. Next, 0.4 ml of the upper layer containing spermatozoa was gently collected without disturbing the interface between the ejaculate and the medium.

To obtain reproducible data and a consistency between the control and the treated samples, after the treatments, each sample was observed for evaluating sperm concentration and motility to detect the count and motility of spermatozoa to be submitted for the decondensation test.

Nuclear chromatin decondensation test

This test allows the identification and discrimination of the ratio of histones and protamines in sperm nuclei. Briefly, 10 µl of each aliquot was spread and smeared on a glass slide previously washed in 70% ethanol and allowed to dry at room temperature. Smears were then fixed in 4% (v/v) buffered glutaraldehyde for 30 min and then rinsed in phosphate-buffered saline (PBS; Sigma, Italy) and in distilled water for 20 s each. Slides were left to air dry at room temperature and then stained with 5% aqueous aniline blue (Sigma, Italy) 5% mixed with 4% acetic acid for 15 min. Slides were then rinsed twice in distilled water to remove the excess of aniline and air dried. At least 200 spermatozoa were counted per slide using a phase-contrast microscope (Nikon, ×1000 magnification). Three categories of head staining intensities were observed as unstained, partially stained and fully stained.

Three different operators blindly performed microscopic evaluations of either sperm count, motility and SDI and the mean values in each group were compared.

Statistical analysis

Statistical analysis was carried out using Systat 11.0 release. Before the analyses, percentage values were transformed in arcsin and homogeneity of variances and their normal distribution were tested. Hypothesis testing was performed by parametric tests, which included linear regression analysis (LRA) and analysis of variance (ANOVA), Coefficients of correlation (R) were recorded for each LRA model. A probability (P) value of ≤ 0.05 was selected as a criterion for a statistically significant difference; a P-value of ≤ 0.001 was selected for high significant difference.

Results

The average age of the patients involved in this study was 37.9 ± 2.0 years (range from 23 to 57 years). Seminal fluid selection was based

https://doi.org/10.1017/S0967199422000132 Published online by Cambridge University Press

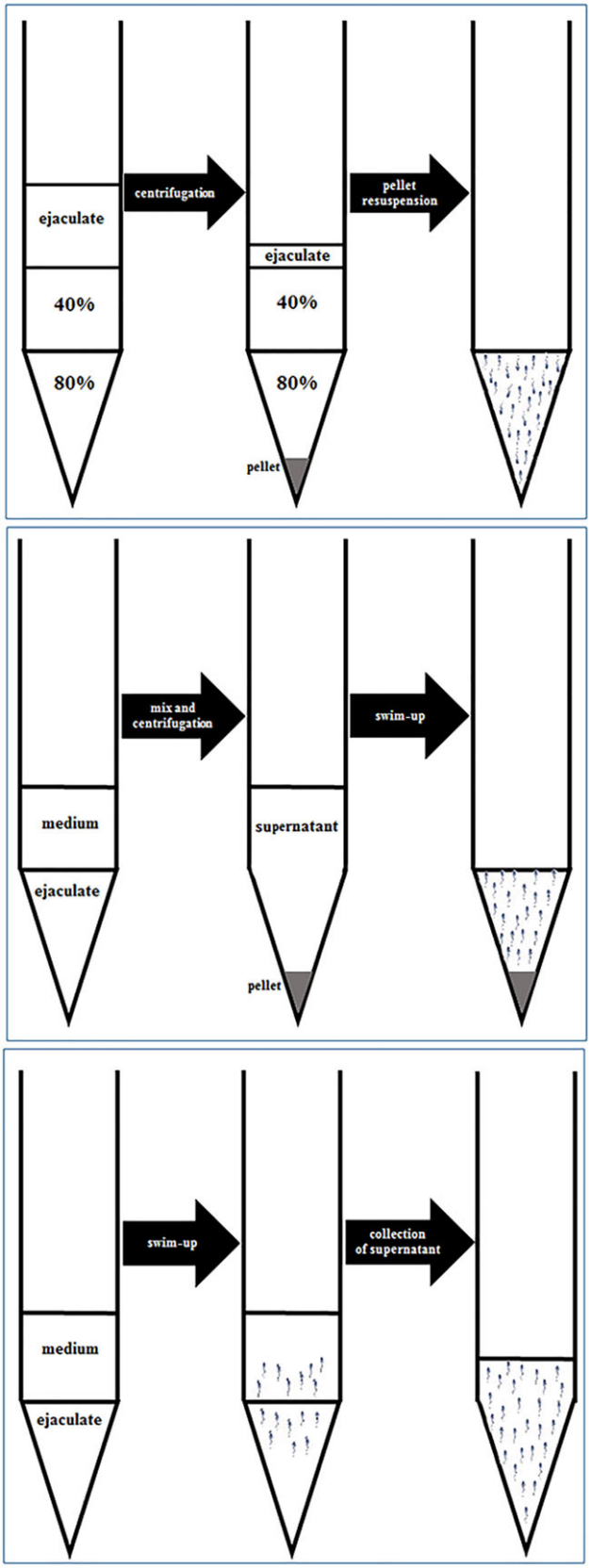

Figure 1. Three sperm preparation techniques. Top panel: density gradient centrifugation (DGC); middle panel: ejaculate subjected to swim-up from the pellet after centrifugation (SU-P); and bottom panel: direct swim-up from the ejaculate (SU-E) without centrifugation.

on sperm concentration and forward motility. Only samples exhibiting at least 5×10^6/ml and 10% forward motility were used in this study. Concerning sperm morphology, from the 15 seminal fluids 13 had normal morphology $\geq 3\%$, whereas two had normal forms $\geq 2\%$.

Using the aniline blue decondensation test, three types of sperm head staining were evaluated as follows: (i) unstained pale blue for normally condensed chromatin (NCC), (ii) partial pale and blue stained for partially condensed chromatin (PDC), and (iii) intense blue stained for highly decondensed chromatin (FDC) (Fig. 2). We considered PDC as decondensed spermatozoa as this pattern may reflect the amount of residue histones that however is a mandatory epigenetic marking for embryo development.

The percentage of sperm exhibiting chromatin decondensation was expressed as the sperm decondensation index (SDI) and evaluated as the percentage of decondensed cells over the total number of sperm examined.

Sperm number, progressive motility and SDI in control and after the three preparation methods are summarized in Table 1.

The mean sperm concentration in the control decreased after all three treatments, however, was not significantly different with respect to the DGC (59.9 ± 12.8 vs 44.9 ± 15.4) and significantly different to the other two treatments (59.9 ± 12.8 vs 14.5 ± 5.6 in SU-E and vs 17.3 ± 4.2 in SU-P, respectively; $P \leq 0.001$). No significant differences were reported between the two SU treatments. A different trend was observed for sperm progressive motility that significantly increased between control and the three preparations and between DGC and the two swim-up (41.3 ± 3.3 vs 61.0 ± 5.3 in DGC, vs 80.0 ± 3.1 in SU-E and vs 84.0 ± 2.4 in SU-P; $P \leq 0.001$). Similarly, for the sperm number no significant differences were observed between SU-E and SU-P.

Nuclear chromatin decondensation rate (SDI, threshold value > 30%) was evaluated in the freshly produced ejaculate and after three different preparation methods. From 15 semen control samples, 87% showed normal SDI in a range between 5 and 24 % whereas 13% showed abnormal SDI with percentage values of 35–36%. No significant correlation was observed between control and DGC treatment (17.3 ± 2.6 vs 12.8 ± 2.3; $P > 0.05$), nor between DGC and SU-E (12.8 ± 2.3 vs 8.1 ± 1.4; $P > 0.05$). In contrast, a high significant difference was observed between control and both the two swim-up techniques (17.3 ± 2.6 vs 8.1 ± 1.4 in SU-E and vs 6.4 ± 1.5in SU-P; $P \leq 0.001$).

Comparing the SDI decrease between DGC and the two swim-up, these were significant only vs SU-P ($P < 0.05$) whereas no correlation existed between DGC and SU-E (Fig. 3).

Discussion

It is well established that semen characteristics play a fundamental role in the outcome of infertility treatments (Zhao *et al.*, 2004). In this study, we showed a significant effect of different sperm preparation methods with respect to sperm nuclear tertiary structure.

In particular, we highlighted the finding that the two different SU methods represented the best techniques able to lower the SDI in seminal fluids, showing variations in semen parameters as number, motility, morphology and SDI. According to Sellami and colleagues (Sellami *et al.*, 2013), who suggested that chromatin decondensation is independent of basic parameters, our samples allowed us to evaluate the objective effect of the preparation methods on the SDI by excluding the interference of other conventional sperm parameters. The role of the spermatozoon was to activate

https://doi.org/10.1017/S0967199422000132 Published online by Cambridge University Press

Table 1. Mean and standard error of sperm concentration, motility and decondensation (SDI) observed in the three study groups

	Number × 10^6/ml, X ± SE ($n = 15$)	% progressive motility, X ± SE ($n = 15$)	SDI X, ± SE ($n = 15$)
Control	59.9 ± 12.8	41.3 ± 3.3	17.3 ± 2.6
DGC	44.9 ± 15.4	61.0 ± 5.3	12.8 ± 2.3
SU-E	14.5 ± 5.6	80.0 ± 3.1	8.1 ± 1.4
SU-P	17.3 ± 4.2	84.0 ± 2.4	6.4 ± 1.5

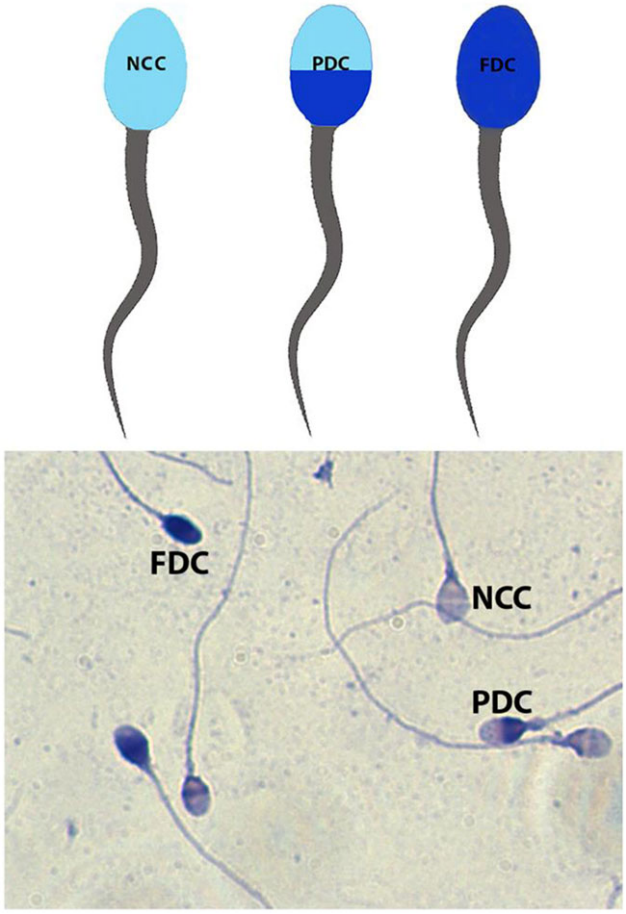

Figure 2. Top panel: three categories of sperm head chromatin condensation: normally condensed sperm head (NCC); partially decondensed sperm head (PDC) and fully decondensed sperm head (FDC). Bottom panel: phase-contrast photomicrograph of a sperm population including the three categories of condensed/decondensed sperm heads.

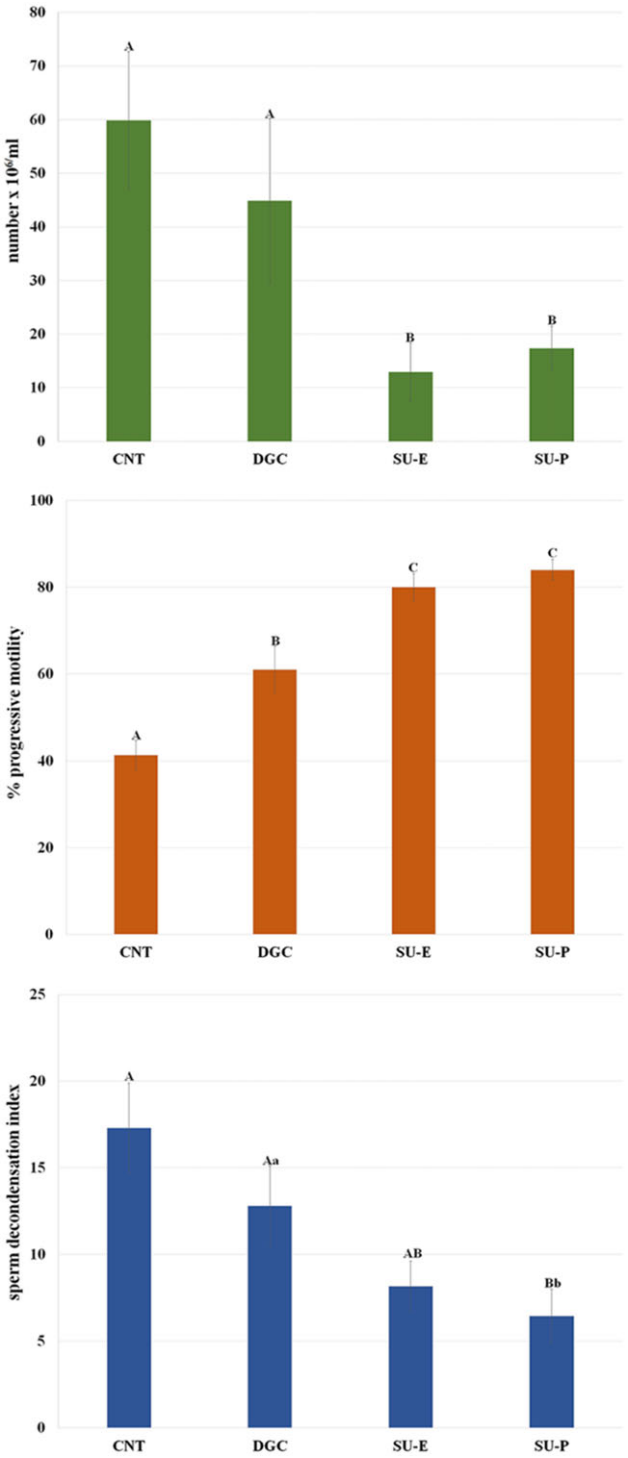

Figure 3. Sperm number (top panel), sperm forward motility (middle panel), and sperm decondensation index (SDI) (bottom panel) for: fresh ejaculate control (CNT) and after preparation by density gradient centrifugation (DGC), direct swim-up from the ejaculate (SU-E) and ejaculate subjected to swim-up from the pellet after centrifugation (SU-P). A vs B, *P*-value ≤ 0.001 (top and bottom panels). A vs B vs C, *P*-value ≤ 0.001 (middle panel); A vs B, *P*-value ≤ 0.001; a vs b, *P*-value ≤ 0.05 (bottom panel).

the oocyte, probably via a soluble sperm factor, provide the centrioles and deliver a haploid genome into the oocyte (Wilding and Dale, 1997; Dale *et al.*, 2010; Tosti and Ménézo, 2016). To become a mature spermatozoon, the haploid spermatid undergoes modifications leading to a striking compaction and remodelling of the chromatin. Several techniques set up to detect the rate of chromatin compaction are based on either staining or binding. The physiological method is based on the capacity to bind hyaluronic acid via hyaladherins, which are expressed on the sperm surface upon sperm maturation. Several studies have demonstrated that the capacity to bind hyaluronic acid was correlated with low levels of chromatin maturity and condensation (Torabi *et al.*, 2017). Nonetheless other methods have been tried such as erythrocyte–sperm separation; staining with aniline blue was found to be the most reliable, rapid and low cost method to identify condensation due to correct spermiogenesis (Soygur *et al.*, 2018).

https://doi.org/10.1017/S0967199422000132 Published online by Cambridge University Press

There is evidence that a mature chromatin is essential for fertility in humans and other mammals (Rathke *et al.*, 2014), in fact fertilization and ongoing pregnancy failure after ART have been shown to positively correlate with abnormally condensed sperm chromatin in the partner seminal fluid (Mohamed and Mohamed, 2012; Irez *et al.*, 2015, 2018). However, contrasting data have been presented by other groups. In a prospective clinical study, in fact, Gandini *et al.* (2004) obtained pregnancies and normal delivery even in the presence of high levels of SDI in either conventional IVF or ICSI. Similarly, results from 154 ICSI cycles showed that abnormal chromatin condensation did not correlate with fertilization rate, embryo score and/or pregnancy rate (Karydis *et al.*, 2005). In humans, seminal plasma is a collection of spermatozoa with different characteristics of maturity, vitality, morphology and genomic integrity. In the ART clinical practice sperm, preparation methods have been developed to mimic the natural selection process occurring in the female reproductive tract by also using a medium that resembles the tubal environment. The further aims of these methods were to select spermatozoa with ideal concentrations, motility and morphology to be associated with functional fertilization competence (Vaughan and Sakkas, 2019). However, both processing and incubation conditions were shown to negatively affect the DNA integrity of ejaculated human spermatozoa (Zini *et al.*, 2000; Matsuura *et al.*, 2010).

To obtain a subpopulation of numerous and highly motile spermatozoa, the classical SU for sperm preparation based on a two-step washing procedure was described by Bob Edwards in the late 1960s (Edwards *et al.*, 1969, 1980). Later on, density gradients were developed to process oligoasthenoteratozoospermic patients to enhance sperm recovery and motility and to separate the so-called 'good spermatozoa' from immotile, senescent and dead spermatozoa, in addition from germinal line cells, leukocytes, epithelial cells and cytoplasmic bodies (Gorus and Pipeleers, 1981; Gellert-Mortimer *et al.*, 1988; Henkel and Schill, 2003). Nonetheless, sperm processing, a mandatory step for ART procedures, can itself exert iatrogenic sperm damage compromising fertility potential (Mortimer, 1991; Muratori *et al.*, 2003). In fact, both these techniques rely on centrifugation steps that are aimed at removing the components of seminal plasma that may interfere with the sperm capacitation process (Erel *et al.*, 2000).

It is well established that sperm centrifugation may generate oxidative stress due to the removal of antioxidants from seminal plasma (Sabeti *et al.*, 2016). In contrast, although centrifugation also removes leukocytes that are the main source of reactive oxygen species (ROS) it may, in turn, promote new ROS production due to the centrifugation time and forces (Ghaleno *et al.*, 2014).

ROS in elevated levels and the absence of antioxidant defences may generate loss of sperm motility of membrane fluidity and a mitochondrial potential decline, together with a generalized impairment of fertilization success (Amaral *et al.*, 2013; Zorn *et al.*, 2003). Among the main mechanisms by which ROS impairs the sperm functionality, lipid peroxidation and DNA integrity damage are included. Nonetheless, sperm vulnerability to ROS has been shown since the 1940s; it is also well established that low levels of ROS are necessary and beneficial for sperm capacitation. Due to this clinical relevance, a balance between 'good' and 'bad' ROS seems to be achieved by identifying pro- and antioxidant management strategies in either *in vivo* or *in vitro* fertilization (Aitken *et al.*, 2012; Aitken, 2017). For sperm preparation, procedures involving high intracellular ROS generation, among a vast variety of antioxidants, have highlighted the key role of albumin due to its ability to prevent DNA damage by neutralizing lipid peroxide-mediated impairment to either sperm plasma membrane and chromatin (Twigg *et al.*, 1998a).

Contrasting data have been reported for ROS production after centrifugation-based sperm processing (Aitken and Clarkson, 1988; Aitken *et al.*, 1992; Zalata *et al.*, 1995; Twigg *et al.*, 1998b; Li *et al.*, 2012) further highlighting the need to identify new procedures aimed at minimizing collateral DNA integrity damage. In particular, Iwasaki and Gagnon (1992) showed that repetitive washing and centrifugation increased ROS species formation by 20-fold to 50-fold; however, more recent studies have reported no enhancement of oxidative stress following sperm preparation (Takeshima *et al.*, 2017).

A recent 2-year survey on more than 500 IVF cycles compared single and double centrifugation gradient sperm preparation methods, demonstrating good clinical outcomes in term of fertilization, embryo and blastocyst formation, pregnancy and live birth rates. However, an increased sperm DNA fragmentation index was observed, along with reduced sperm concentration, progressive motility and even normal morphology for the double centrifugation, suggesting that the latter subjected spermatozoa to excessive mechanical stress with potential impairment of reproductive processes (Dai *et al.*, 2020). In contrast, other similar clinical surveys on high numbers of couples enrolled in IVF programmes showed that sperm DNA damage levels were negatively correlated with IVF pregnancy outcomes, corroborating the fact that sperm DNA damage is a useful parameter for predicting the clinical pregnancy rate. Due to the frequent evidence that sperm with a high level of DNA damage negatively correlate with the IVF outcomes, the contrasting results may account for a possible DNA damage threshold that should be reached for generating adverse fertilization, development and pregnancy rates (Zhang *et al.*, 2016, 2018).

Although the choice of the more suitable preparation method should remain at the discretion of the operator and should be based on the quality '*in toto*' of the semen, in clinical practice DGC has almost totally replaced the SU, being less time consuming and permitting a higher concentration of sperm collected. In this study, we demonstrated that the decrease in sperm SDI after DGC was not significantly lower than the control, however high variability was observed. In fact, in two of 15 samples SDI did not change and in one sample SDI even increased. In light of these results DGC should be avoided for sperm preparation due both to the lack of improvement of the SDI ratio and because of potential damage induced by the double centrifugation.

In contrast with the two SU methods tested, we obtained a consistent and highly significant decrease in chromatin decondensation in the recruited sperm population with respect to the control. In the latter, only one of the 15 SDI values remained equal to the control, whereas in the SU-E one in 15 SDI values remained equal and one was greater than the control. From these data it is worth noting that DGC is less capable of selecting normally condensed spermatozoa compared with the other two SU techniques.

Previous studies have demonstrated the efficiency of preparation techniques in eliminating decondensed spermatozoa. In 1994, it was shown that glass-wool filtration was a superior method for separating normally condensed spermatozoa compared with the SU procedures; however, several disadvantages were reported such as a decreased sperm motility and damage due to the sample contamination by glass-wool fragments (Henkel *et al.*, 1994; Sánchez *et al.*, 1994; Henkel and Schill, 2003).

The effectiveness of the SU method in oligospermic samples was observed, although a worse sperm number recovery was

https://doi.org/10.1017/S0967199422000132 Published online by Cambridge University Press

achieved (Adiga and Kumar, 2001), In contrast, Sakkas *et al.* (2000) showed a decrease in poorly condensed chromatin evaluated by chromomycin (CMA3) staining after PureSperm and Percoll gradients techniques. In this study, we used aniline blue staining as CMA3 use is rather controversial, in fact nonetheless some authors have used CMA3 for evaluating sperm chromatin decondensation. A recent study highlighted that CMA3 and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) test for detection of sperm DNA fragmentation have the same target, therefore giving rise to misleading results (Manicardi *et al.*, 1995; Ménézo, 2021).

To our knowledge this is the first study that compared the effects of two different SU techniques on chromatin decondensation evaluated using an aniline blue test. In all the comparisons no significant differences were detected between SU-E and SU-P, however a trend of higher number and motility and SDI decrease favoured the SU-P, which was also corroborated by a significant SDI difference between DGC and SU-P. This may be attributed to the fact that SU-P relies on the stratification of the medium on a very compact pellet and the supernatant recovered is free from the components of the ejaculate. In contrast, SU-E, with no net interface between ejaculate and medium, may be more susceptible to disturbing vortices exerted by the pipette when collecting the supernatant. Therefore, when used for IUI and IVF, it should need a further centrifugation step to eliminate possible harmful residues in ejaculate components. This manipulative problem is strictly related to the ability of the operator and the fluidity of the seminal plasma. Consistently with other studies that suggested SU-P to be the best option for minimizing sperm DNA fragmentation (Volpes *et al.*, 2016), here SU-P appears to be the best selection method to decrease decondensation, even in samples showing SDI values within the threshold of 30%.

At present there are no recognized standard values for chromatin decondensation suggested by the WHO (World Health Organization, 2010). In ART, it was suggested that a normal semen sample generally should contain less than 25% stained spermatozoa. Subsequently, other studies from clinical observations identified a threshold value over 30%, as no pregnancy was achieved following *in vitro* fertilization and ICSI with semen samples exceeding this percentage (Evenson *et al.*, 1999, 2002; Ménézo *et al.*, 2007; Giwercman *et al.*, 2010).

Furthermore, it was speculated that even the apparently normally condensed spermatozoa in the remaining 70% may be susceptible to alterations and multiple defects.

Large numbers of studies over the last decade have addressed the problem of sperm genomic integrity and its repercussions for fertilization potential and outcome in fertile and infertile couples. Most of these studies, however, have taken in consideration DNA fragmentation and its effect on fertility and embryo development and pregnancy outcome (Evenson and Wixon, 2006; Rex *et al.*, 2017; Simon *et al.*, 2019). Fragmentation is the 'other face' of genome disorders and consists of a break in the filament of DNA, mostly induced by apoptotic process and oxidative stress (Muratori *et al.*, 2015).

During spermiogenesis, DNA fragmentation is a physiological process occurring to facilitate the nuclear 'transition proteins' in the chromatin remodelling process. However, here, specific enzymes are able to repair the interruptions (Boissonneault, 2002). A further DNA repair occurs also in the oocyte following fertilization (Ménézo *et al.*, 2010; Setti *et al.*, 2021); however, to our knowledge there is no similar repair mechanism for chromatin decondensation. Chromatin decondensation needs to be evaluated in couples with idiopathic infertility. In recent studies it was suggested that centrifugation be eliminated in the swim-up method, supporting our data that SU-P appears to be the best suited sperm preparation (Saylan and Erimsah, 2019; Meitei *et al.*, 2021). However, as a light centrifugation was necessary in this study to fully eliminate decapacitating factors in the seminal plasma, we provide significant evidence that SU-P with short centrifugation steps reduced SDI and increased the number and forward motility of sperm recruited. This was irrespective of the technique to be used, whether it be IUI, IVF or ICSI. In fact, for IUI, even if less spermatozoa are recruited they are superior in their maturity, and less exposed to centrifugation stress. Similarly, for ICSI, it appears mandatory to lower the SDI in the sperm population suitable for injection, as damaged chromatin packaging may contribute to the failure of sperm nucleus decondensation and fertilization (Sakkas *et al.*, 1996).

Problems of sperm genomic integrity are increasing in both animals and humans due to the chemical–physical stress in our present day environment. Sperm DNA damage may be generated by a variety of different stressors. In humans, in particular, in addition to passive stress from the environment, an incorrect life style and related diseases such as cancer, diabetes and obesity may also impair gamete quality and functionality (Babakhanzadeh *et al.*, 2020; Gallo *et al.*, 2020). Furthermore, the increase in the average age of couples enrolled in IVF programmes leads to the well known paternal effect that, by affecting the sperm quality, has serious consequences on fertility, embryo development, pregnancy and the offspring health (Simon *et al.*, 2014; Gill *et al.*, 2020; Couture *et al.*, 2021).

In conclusion, many studies have claimed that the structural organization of sperm chromatin is vital for the functioning of the spermatozoa, related fertilization success and embryo development and quality (Bungum *et al.*, 2007; Galotto *et al.*, 2019). There is a consensus associating sperm chromatin condensation test with conventional semen analyses, indicating it as a diagnostic tool to predict sperm fertilizing ability and reproductive success in the routine of the ART laboratory (Lefièvre *et al.*, 2007; Tosti and Fortunato, 2012; Kim *et al.*, 2013). New techniques for sperm selection in ART have been developed in the few last years such as motile sperm organelle morphological examination, molecular binding techniques, flow cytometry, Raman spectrometry, horizontal sperm migration, migration sedimentation and rheotaxis (Henkel, 2012; Oseguera-López *et al.*, 2019; Baldini *et al.*, 2020; Meitei *et al.*, 2021; Romero-Aguirregomezcorta *et al.*, 2021). Many of these techniques were also tested to see if they were able to decrease DNA lesions in the final sperm sample used for IVF. However, the original method, such as SU, remains the simplest, most efficient and cheapest method in clinical practice for isolating the most competent spermatozoa.

In this study, we compared three sperm selection techniques, highlighting the highly significant efficacy of SU from pellet in improving sperm number and motility and decreasing the sperm decondensation rate in different populations of spermatozoa. Other technical advantages of SU-P are the reduction in toxic components from the seminal plasma and the fact that it may require less centrifugation steps that should be avoided to reduce ROS generation.

Acknowledgements. We thank Fiammetta Formisano for art graphic and Alessandra Gallo for performing statistical analysis.

Conflict of interest. Authors declare no conflict of interest.

https://doi.org/10.1017/S0967199422000132 Published online by Cambridge University Press

Funding. No funds, grants, or other support was received.

Ethics approval. The study was approved by the Ethics Committee of the Centro Fecondazione Assistita, Naples, Italy (reference no. 1/2021). Written informed consent to participate in this study was obtained from all patients.

References

Adiga, S. K.C. and Kumar, P. (2001). Influence of swim-up method on the recovery of spermatozoa from different types of semen samples. *Journal of Assisted Reproduction and Genetics*, **18**(3), 160–164. doi: 10.1023/a:1009464121194

Agarwal, A. and Said, T. M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. *Human Reproduction Update*, **9**(4), 331–345. doi: 10.1093/humupd/dmg027

Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Molecular Reproduction and Development*, **84**(10), 1039–1052. doi: 10.1002/mrd.22871

Aitken, R. J. and Clarkson, J. S. (1988). Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *Journal of Andrology*, **9**(6), 367–376. doi: 10.1002/j.1939-4640.1988.tb01067.x

Aitken, R. J., Buckingham, D., West, K., Wu, F. C., Zikopoulos, K. and Richardson, D. W. (1992). Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *Reproduction*, **94**(2), 451–462. doi: 10.1530/jrf.0.0940451

Aitken, R. J., Jones, K. T. and Robertson, S. A. (2012). Reactive oxygen species and sperm function—In sickness and in health. *Journal of Andrology*, **33**(6), 1096–1106. doi: 10.2164/jandrol.112.016535

Amaral, A., Lourenço, B., Marques, M. and Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction*, **146**(5), R163–R174. doi: 10.1530/REP-13-0178

Babakhanzadeh, E., Nazari, M., Ghasemifar, S. and Khodadadian, A. (2020). Some of the factors involved in male infertility: A prospective review. *International Journal of General Medicine*, **13**, 29–41. doi: 10.2147/IJGM.S241099

Baldini, D., Baldini, A., Silvestris, E., Vizziello, G., Ferri, D. and Vizziello, D. (2020). A fast and safe technique for sperm preparation in ICSI treatments within a randomized controlled trial (RCT). *Reproductive Biology and Endocrinology: RB&E*, **18**(1), 88. doi: 10.1186/s12958-020-00642-8

Balhorn, R., Cosman, M., Thornton, K., Krishnan, V., Corzett, M., Bench, G., Kramer, C., Hud, N., Allen, M. and Prieto, M. (1999). Protamine mediated condensation of DNA in mammalian sperm. In C. Gagnon (Ed.), *The male gamete: From basic science to clinical applications* (pp. 55–70). Cache River Press.

Boissonneault, G. (2002). Chromatin remodeling during spermiogenesis: A possible role for the transition proteins in DNA strand break repair. *FEBS Letters*, **514**(2–3), 111–114. doi: 10.1016/s0014-5793(02)02380-3

Booze, M., Brannian, J., Von Wald, T., Hansen, K. and Evenson, D. (2019). High DNA stability in the SCSA is associated with poor embryo development and live birth outcome. In American Association of bioanalysts conference (pp. 16–18).

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J. and Giwercman, A. (2007). Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Human Reproduction*, **22**(1), 174–179. doi: 10.1093/humrep/del326

Caron, C., Govin, J., Rousseaux, S. and Khochbin, S. (2005). How to pack the genome for a safe trip. In P. Jeanteur (Ed.), Epigenetics and chromatin, 38 (pp. 65–89). Springer-Verlag.

Coughlan, C., Clarke, H., Cutting, R., Saxton, J., Waite, S., Ledger, W., Li, T. and Pacey, A. A. (2015). Sperm DNA fragmentation, recurrent implantation failure and recurrent miscarriage. *Asian Journal of Andrology*, **17**(4), 681–685. doi: 10.4103/1008-682X.144946

Couture, V., Delisle, S., Mercier, A. and Pennings, G. (2021). The other face of advanced paternal age: A scoping review of its terminological, social, public health, psychological, ethical and regulatory aspects. *Human Reproduction Update*, **27**(2), 305–323. doi: 10.1093/humupd/dmaa046

Curry, M. and Watson, P. (1995). Sperm-structure and function. In J. G. Grudzinskas & J. L. Yovich (Eds.), *Gametes: The spermatozoon* (pp. 45–69). Cambridge University Press.

Dai, X., Wang, Y., Cao, F., Yu, C., Gao, T., Xia, X., Wu, J. and Chen, L. (2020). Sperm enrichment from poor semen samples by double density gradient centrifugation in combination with swim-up for IVF cycles. *Scientific Reports*, **10**(1), 2286. doi: 10.1038/s41598-020-59347-y

Dale, B., Wilding, M., Coppola, G. and Tosti, E. (2010). How do spermatozoa activate oocytes? *Reproductive Biomedicine Online*, **21**(1), 1–3. doi: 10.1016/j.rbmo.2010.02.015

Daumler, D., Chan, P., Lo, K. C., Takefman, J. and Zelkowitz, P. (2016). Men's knowledge of their own fertility: A population-based survey examining the awareness of factors that are associated with male infertility. *Human Reproduction*, **31**(12), 2781–2790. doi: 10.1093/humrep/dew265

Edwards, R. G., Bavister, B. D. and Steptoe, P. C. (1969). Early stages of fertilization *in vitro* of human oocytes matured *in vitro*. *Nature*, **221**(5181), 632–635. doi: 10.1038/221632a0

Edwards, R. G., Steptoe, P. C. and Purdy, J. M. (1980). Establishing full-term human pregnancies using cleaving embryos grown *in vitro*. *British Journal of Obstetrics and Gynaecology*, **87**(9), 737–756. doi: 10.1111/j.1471-0528.1980.tb04610.x

Erel, C. T., Senturk, L. M., Irez, T., Ercan, L., Elter, K., Colgar, U. and Ertungealp, E. (2000). Sperm-preparation techniques for men with normal and abnormal semen analysis. A comparison. *Journal of Reproductive Medicine*, **45**(11), 917–922.

Esterhuizen, A. D., Franken, D. R., Lourens, J. G. H., Van Zyl, C., Müller, I. I. and Van Rooyen, L. H. (2000). Chromatin packaging as an indicator of human sperm dysfunction. *Journal of Assisted Reproduction and Genetics*, **17**(9), 508–514. doi: 10.1023/a:1009493824953

Esterhuizen, A. D., Franken, D. R., Becker, P. J., Lourens, J. G. H., Müller, I. I. and van Rooyen, L. H. (2002). Defective sperm decondensation: A cause for fertilization failure. *Andrologia*, **34**(1), 1–7. doi: 10.1046/j.0303-4569.2001.00423.x

Evenson, D. and Jost, L. (2000). Sperm chromatin structure assay is useful for fertility assessment. *Methods in Cell Science*, **22**(2–3), 169–189. doi: 10.1023/a:1009844109023

Evenson, D. P. and Wixon, R. (2006). Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology*, **65**(5), 979–991. doi: 10.1016/j.theriogenology.2005.09.011

Evenson, D. P., Witkin, S. S., de Harven, E. and Bendich, A. (1978). Ultrastructure of partially decondensed human spermatozoal chromatin. *Journal of Ultrastructure Research*, **63**(2), 178–187. doi: 10.1016/s0022-5320(78)80073-2

Evenson, D. P., Jost, L. K., Marshall, D., Zinaman, M. J., Clegg, E., Purvis, K., de Angelis, P. and Claussen, O. P. (1999). Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Human Reproduction*, **14**(4), 1039–1049. doi: 10.1093/humrep/14.4.1039

Evenson, D. P., Larson, K. L. and Jost, L. K. (2002). Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *Journal of Andrology*, **23**(1), 25–43. doi: 10.1002/j.1939-4640.2002.tb02599.x

Gallo, A., Boni, R. and Tosti, E. (2020). Gamete quality in a multistressor environment. *Environment International*, **138**, 105627. doi: 10.1016/j.envint.2020.105627

Galotto, C., Cambiasso, M. Y., Julianelli, V. L., Valzacchi, G. J. R., Rolando, R. N., Rodriguez, M. L., Calvo, L., Calvo, J. C. and Romanato, M. (2019). Human sperm decondensation *in vitro* is related to cleavage rate and embryo quality in IVF. *Journal of Assisted Reproduction and Genetics*, **36**(11), 2345–2355. doi: 10.1007/s10815-019-01590-y

Gandini, L., Lombardo, F., Paoli, D., Caruso, F., Eleuteri, P., Leter, G., Ciriminna, R., Culasso, F., Dondero, F., Lenzi, A. and Spanó, M. (2004). Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Human Reproduction*, **19**(6), 1409–1417. doi: 10.1093/humrep/deh233

Gellert-Mortimer, S. T., Clarke, G. N., Baker, H. W. G., Hyne, R. V. and Johnston, W. I. H. (1988). Evaluation of Nycodenz**Nyegaard and Co., Oslo, Norway. and Percoll††Pharmacia. *Fertility and Sterility*. Uppsala, **49**(2), 335–341. doi: 10.1016/s0015-0282(16)59725-8

Ghaleno, L. R., Valojerdi, M. R., Janzamin, E., Chehrazi, M., Sharbatoghli, M. and Yazdi, R. S. (2014). Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: A flow cytometric study. *Archives of Gynecology and Obstetrics*, **289**(1), 173–180. doi: 10.1007/s00404-013-2946-1

Gill, K., Jakubik-Uljasz, J., Rosiak-Gill, A., Grabowska, M., Matuszewski, M. and Piasecka, M. (2020). Male aging as a causative factor of detrimental changes in human conventional semen parameters and sperm DNA integrity. *Aging Male*, **23**(5), 1321–1332. doi: 10.1080/13685538.2020.1765330

Giwercman, A., Lindstedt, L., Larsson, M., Bungum, M., Spano, M., Levine, R. J. and Rylander, L. (2010). Sperm chromatin structure assay as an independent predictor of fertility *in vivo*: A case–control study. *International Journal of Andrology*, **33**(1), e221–e227. doi: 10.1111/j.1365-2605.2009.00995.x

Gorus, F. K. and Pipeleers, D. G. (1981). A rapid method for the fractionation of human spermatozoa according to their progressive motility. *Fertility and Sterility*, **35**(6), 662–665. doi: 10.1016/s0015-0282(16)45561-5

Haddad, M., Stewart, J., Xie, P., Cheung, S., Trout, A., Keating, D., Parrella, A., Lawrence, S., Rosenwaks, Z. and Palermo, G. D. (2021). Thoughts on the popularity of ICSI. *Journal of Assisted Reproduction and Genetics*, **38**(1), 101–123. doi: 10.1007/s10815-020-01987-0

Hammadeh, M. E., Stieber, M., Haidl, G. and Schmidt, W. (1998). Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. *Andrologia*, **30**(1), 29–35. doi: 10.1111/j.1439-0272.1998.tb01379.x

Hao, S. L., Ni, F. D. and Yang, W. X. (2019). The dynamics and regulation of chromatin remodeling during spermiogenesis. *Gene*, **706**, 201–210. doi: 10.1016/j.gene.2019.05.027

Henkel, R. (2012). Sperm preparation: State-of-the-art—Physiological aspects and application of advanced sperm preparation methods. *Asian Journal of Andrology*, **14**(2), 260–269. doi: 10.1038/aja.2011.133

Henkel, R. R. and Schill, W. B. (2003). Sperm preparation for ART. *Reproductive Biology and Endocrinology: RB&E*, **1**, 108. doi: 10.1186/1477-7827-1-108

Henkel, R. R., Franken, D. R., Lombard, C. J. and Schill, W. B. (1994). Selective capacity of glass-wool filtration for the separation of human spermatozoa with condensed chromatin: A possible therapeutic modality for male-factor cases? *Journal of Assisted Reproduction and Genetics*, **11**(8), 395–400. doi: 10.1007/BF02211725

Ihara, M., Meyer-Ficca, M. L., Leu, N. A., Rao, S., Li, F., Gregory, B. D., Zalenskaya, I. A., Schultz, R. M. and Meyer, R. G. (2014). Paternal poly (ADP-ribose) metabolism modulates retention of inheritable sperm histones and early embryonic gene expression. *PLOS Genetics*, **10**(5), e1004317. doi: 10.1371/journal.pgen.1004317

Irez, T., Sahmay, S., Ocal, P., Goymen, A., Senol, H., Erol, N., Kaleli, S. and Guralp, O. (2015). Investigation of the association between the outcomes of sperm chromatin condensation and decondensation tests, and assisted reproduction techniques. *Andrologia*, **47**(4), 438–447. doi: 10.1111/and.12286

Irez, T., Dayioglu, N., Alagöz, M., Karatas, S. and Güralp, O. (2018). The use of aniline blue chromatin condensation test on prediction of pregnancy in mild male factor and unexplained male infertility. *Andrologia*, **50**(10), e13111. doi: 10.1111/and.13111

Iwasaki, A. and Gagnon, C. (1992). Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertility and Sterility*, **57**(2), 409–416. doi: 10.1016/s0015-0282(16)54855-9

Jerre, E., Bungum, M., Evenson, D. and Giwercman, A. (2019). Sperm chromatin structure assay high DNA stainability sperm as a marker of early miscarriage after intracytoplasmic sperm injection. *Fertility and Sterility*, **112**(1), 46–53.e2. doi: 10.1016/j.fertnstert.2019.03.013

Karydis, S., Asimakopoulos, B., Papadopoulos, N., Vakalopoulos, I., Al-Hasani, S. and Nikolettos, N. (2005). ICSI outcome is not associated with the incidence of spermatozoa with abnormal chromatin condensation. *In Vivo*, **19**(5), 921–925.

Kazerooni, T., Asadi, N., Jadid, L., Kazerooni, M., Ghanadi, A., Ghaffarpasand, F., Kazerooni, Y. and Zolghadr, J. (2009). Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *Journal of Assisted Reproduction and Genetics*, **26**(11–12), 591–596. doi: 10.1007/s10815-009-9361-3

Kim, H. S., Kang, M. J., Kim, S. A., Oh, S. K., Kim, H., Ku, S. Y., Kim, S. H., Moon, S. Y. and Choi, Y. M. (2013). The utility of sperm DNA damage assay using toluidine blue and aniline blue staining in routine semen analysis. *Clinical and Experimental Reproductive Medicine*, **40**(1), 23–28. doi: 10.5653/cerm.2013.40.1.23

Krüger, T. F., Acosta, A. A., Simmons, K. F., Swanson, R. J., Matta, J. F., Veeck, L. L., Morshedi, M. and Brugo, S. (1987). New method of evaluating sperm morphology with predictive value for human *in vitro* fertilization. *Urology*, **30**(3), 248–251. doi: 10.1016/0090-4295(87)90246-9

Kutchy, N. A., Menezes, E. S. B., Ugur, M. R., Ul Husna, A., ElDebaky, H., Evans, H. C., Beaty, E., Santos, F. C., Tan, W., Wills, R. W., Topper, E., Kaya, A., Moura, A. A. and Memili, E. (2019). Sperm cellular and nuclear dynamics associated with bull fertility. *Animal Reproduction Science*, **211**, 106203. doi: 10.1016/j.anireprosci.2019.106203

Lefièvre, L., Bedu-Addo, K., Conner, S. J., Machado-Oliveira, G. S. M., Chen, Y., Kirkman-Brown, J. C., Afnan, M. A., Publicover, S. J., Ford, W. C. L. and Barratt, C. L. R. (2007). Counting sperm does not add up any more: Time for a new equation? *Reproduction*, **133**(4), 675–684. doi: 10.1530/REP-06-0332

Lewis, S. E. M. and Aitken, R. J. (2005). DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell and Tissue Research*, **322**(1), 33–41. doi: 10.1007/s00441-005-1097-5

Lewis, S. E. M., Agbaje, I. and Alvarez, J. (2008). Sperm DNA tests as useful adjuncts to semen analysis. *Systems Biology in Reproductive Medicine*, **54**(3), 111–125. doi: 10.1080/19396360801957739

Li, Z., Zhou, Y., Liu, R., Lin, H., Liu, W., Xiao, W. and Lin, Q. (2012). Effects of semen processing on the generation of reactive oxygen species and mitochondrial membrane potential of human spermatozoa. *Andrologia*, **44**(3), 157–163. doi: 10.1111/j.1439-0272.2010.01123.x

Lin, M. H., Kuo-Kuang Lee, R., Li, S. H., Lu, C. H., Sun, F. J. and Hwu, Y. M. (2008). Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in *in vitro* fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertility and Sterility*, **90**(2), 352–359. doi: 10.1016/j.fertnstert.2007.06.018

Manicardi, G. C., Bianchi, P. G., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U. and Sakkas, D. (1995). Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biology of Reproduction*, **52**(4), 864–867. doi: 10.1095/biolreprod52.4.864

Matsuura, R., Takeuchi, T. and Yoshida, A. (2010). Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian Journal of Andrology*, **12**(5), 753–759. doi: 10.1038/aja.2010.46

Meitei, H. Y., Uppangala, S., Sharan, K., Chandraguthi, S. G., Radhakrishnan, A., Kalthur, G., Schlatt, S. and Adiga, S. K. (2021). A simple, centrifugation-free, sperm-sorting device eliminates the risks of centrifugation in the swim-up method while maintaining functional competence and DNA integrity of selected spermatozoa. *Reproductive Sciences*, **28**(1), 134–143. doi: 10.1007/s43032-020-00269-5

Ménézo, Y. (2021). Evaluation of the human sperm nucleus: Ambiguity and risk of confusion with chromomycin staining. *Zygote*, **29**(4), 257–259. doi: 10.1017/S0967199420000672

Ménézo, Y. J., Hazout, A., Panteix, G., Robert, F., Rollet, J., Cohen-Bacrie, P., Chapuis, F., Clément, P. and Benkhalifa, M. (2007). Antioxidants to reduce sperm DNA fragmentation: An unexpected adverse effect. *Reproductive Biomedicine Online*, **14**(4), 418–421. doi: 10.1016/s1472-6483(10)60887-5

Ménézo, Y., Dale, B. and Cohen, M. (2010). DNA damage and repair in human oocytes and embryos: A review. *Zygote*, **18**(4), 357–365. doi: 10.1017/S0967199410000286

Mohamed, E. E. and Mohamed, M. A. (2012). Effect of sperm chromatin condensation on the outcome of intrauterine insemination in patients with male factor infertility. *Journal of Reproductive Medicine*, **57**(9–10), 421–426.

Mortimer, D. (1991). Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Human Reproduction*, **6**(2), 173–176. doi: 10.1093/oxfordjournals.humrep.a137300

Muratori, M., Maggi, M., Spinelli, S., Filimberti, E., Forti, G. and Baldi, E. (2003). Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *Journal of Andrology*, **24**(2), 253–262. doi: 10.1002/j.1939-4640.2003.tb02670.x

Muratori, M., Tamburrino, L., Marchiani, S., Cambi, M., Olivito, B., Azzari, C., Forti, G. and Baldi, E. (2015). Investigation on the origin of sperm DNA fragmentation: Role of apoptosis, immaturity and oxidative stress. *Molecular Medicine*, **21**, 109–122. doi: 10.2119/molmed.2014.00158

Oseguera-López, I., Ruiz-Díaz, S., Ramos-Ibeas, P. and Pérez-Cerezales, S. (2019). Novel techniques of sperm selection for improving IVF and ICSI outcomes. *Frontiers in Cell and Developmental Biology*, **7**, 298. doi: 10.3389/fcell.2019.00298

Palermo, G. D., O'Neill, C. L., Chow, S., Cheung, S., Parrella, A., Pereira, N. and Rosenwaks, Z. (2017). Intracytoplasmic sperm injection: State of the art in humans. *Reproduction*, **154**(6), F93–F110. doi: 10.1530/REP-17-0374

Poccia, D. (1986). Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. *International Review of Cytology*, **105**, 1–65. doi: 10.1016/s0074-7696(08)61061-x

Rahman, M. B., Schellander, K., Luceño, N. L. and Van Soom, A. (2018). Heat stress responses in spermatozoa: Mechanisms and consequences for cattle fertility. *Theriogenology*, **113**, 102–112. doi: 10.1016/j.theriogenology.2018.02.012

Rathke, C., Baarends, W. M., Awe, S. and Renkawitz-Pohl, R. (2014). Chromatin dynamics during spermiogenesis. *Biochimica et Biophysica Acta*, **1839**(3), 155–168. doi: 10.1016/j.bbagrm.2013.08.004

Rex, A. S., Aagaard, J. and Fedder, J. (2017). DNA fragmentation in spermatozoa: A historical review. *Andrology*, **5**(4), 622–630. doi: 10.1111/andr.12381

Romero-Aguirregomezcorta, J., Laguna-Barraza, R., Fernández-González, R., Štiavnická, M., Ward, F., Cloherty, J., McAuliffe, D., Larsen, P. B., Grabrucker, A. M., Gutiérrez-Adán, A., Newport, D. and Fair, S. (2021). Sperm selection by rheotaxis improves sperm quality and early embryo development. *Reproduction*, **161**(3), 343–352. doi: 10.1530/REP-20-0364

Sabeti, P. Pourmasumi, S., Rahiminia, T., Akyash, F. and Talebi, A. R. (2016). Etiologies of sperm oxidative stress. *International Journal of Reproductive Biomedicine*, **14**, 231–240.

Sakkas, D., Manicardi, G. C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P. G. and Bianchi, U. (2000). The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Human Reproduction*, **15**(5), 1112–1116. doi: 10.1093/humrep/15.5.1112

Sakkas, D., Urner, F., Bianchi, P. G., Bizzaro, D., Wagner, I., Jaquenoud, N., Manicardi, G. and Campana, A. (1996). Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Human Reproduction*, **11**(4), 837–843. doi: 10.1093/oxfordjournals.humrep.a019263

Sakkas, D., Urner, F., Bizzaro, D., Manicardi, G., Bianchi, P. G., Shoukir, Y. and Campana, A. (1998). Sperm nuclear DNA damage and altered chromatin structure: Effect on fertilization and embryo development. *Human Reproduction*, **13** Suppl. 4, 11–19. doi: 10.1093/humrep/13.suppl_4.11

Sánchez, R., Villagrán, E., Risopatrón, J. and Célis, R. (1994). Evaluation of nuclear maturity in human spermatozoa obtained by sperm-preparation methods. *Andrologia*, **26**(3), 173–176. doi: 10.1111/j.1439-0272.1994.tb00784.x

Saylan, A. and Erimsah, S. (2019). High quality human sperm selection for IVF: A study on sperm chromatin condensation. *Acta histochemica*, **121**(7), 798–803. doi: 10.1016/j.acthis.2019.07.006

Sellami, A., Chakroun, N., Ben Zarrouk, S., Sellami, H., Kebaili, S., Rebai, T. and Keskes, L. (2013). Assessment of chromatin maturity in human spermatozoa: Useful aniline blue assay for routine diagnosis of male infertility. *Advances in Urology*, **2013**, 578631. doi: 10.1155/2013/578631

Setti, A. S., Braga, D. P. A. F., Provenza, R. R., Iaconelli, A. and Borges, E. (2021). Oocyte ability to repair sperm DNA fragmentation: The impact of maternal age on intracytoplasmic sperm injection outcomes. *Fertility and Sterility*, **116**(1), 123–129. doi: 10.1016/j.fertnstert.2020.10.045

Shamsi, M. B., Imam, S. N. and Dada, R. (2011). Sperm DNA integrity assays: Diagnostic and prognostic challenges and implications in management of infertility. *Journal of Assisted Reproduction and Genetics*, **28**(11), 1073–1085. doi: 10.1007/s10815-011-9631-8

Simon, L., Murphy, K., Shamsi, M. B., Liu, L., Emery, B., Aston, K. I., Hotaling, J. and Carrell, D. T. (2014). Paternal influence of sperm DNA integrity on early embryonic development. *Human Reproduction*, **29**(11), 2402–2412. doi: 10.1093/humrep/deu228

Simon, L., Emery, B. and Carrell, D. T. (2019). Sperm DNA fragmentation: consequences for reproduction. *Advances in Experimental Medicine and Biology*, **1166**, 87–105. doi: 10.1007/978-3-030-21664-1_6

Soygur, B., Celik, S., Celik-Ozenci, C. and Sati, L. (2018). Effect of erythrocyte-sperm separation medium on nuclear, acrosomal, and membrane maturity parameters in human sperm. *Journal of Assisted Reproduction and Genetics*, **35**(3), 491–501. doi: 10.1007/s10815-017-1085-1

Sreenivasa, G., Kavitha, P., Vineeth, V. S., Channappa, S. K. and Malini, S. S. N. (2012). Evaluation of *in vitro* sperm nuclear chromatin decondensation among different subgroups of infertile males in Mysore, India. *Journal of Research in Medical Sciences: the Official Journal of Isfahan University of Medical Sciences*, **17**(5), 456–460.

Starosta, A., Gordon, C. E. and Hornstein, M. D. (2020). Predictive factors for intrauterine insemination outcomes: A review. *Fertility Research and Practice*, **6**(1), 23. doi: 10.1186/s40738-020-00092-1

Takeshima, T., Yumura, Y., Kuroda, S., Kawahara, T., Uemura, H. and Iwasaki, A. (2017). Effect of density gradient centrifugation on reactive oxygen species in human semen. *Systems Biology in Reproductive Medicine*, **63**(3), 192–198. doi: 10.1080/19396368.2017.1294214

Talebi, A. R., Vahidi, S., Aflatoonian, A., Ghasemi, N., Ghasemzadeh, J., Firoozabadi, R. D. and Moein, M. R. (2012). Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. *Andrologia*, **44** Suppl. 1, 462–470. doi: 10.1111/j.1439-0272.2011.01206.x

Tomlinson, M. J., Amissah-Arthur, J. B., Thompson, K. A., Kasraie, J. L. and Bentick, B. (1996). Prognostic indicators for intrauterine insemination (IUI): Statistical model for IUI success. *Human Reproduction*, **11**(9), 1892–1896. doi: 10.1093/oxfordjournals.humrep.a019513

Torabi, F., Binduraihem, A. and Miller, D. (2017). Sedimentation properties in density gradients correspond with levels of sperm DNA fragmentation, chromatin compaction and binding affinity to hyaluronic acid. *Reproductive Biomedicine Online*, **34**(3), 298–311. doi: 10.1016/j.rbmo.2016.11.011

Tosti, E. and Fortunato, A. (2012). Is sperm chromatin packaging relevant for IVF success? *Journal of Fertilization*, **01**(2). doi: 10.4172/2165-7491.1000e107

Tosti, E. and Ménézo, Y. (2016). Gamete activation: Basic knowledge and clinical applications. *Human Reproduction Update*, **22**(4), 420–439. doi: 10.1093/humupd/dmw014

Twigg, J., Fulton, N., Gomez, E., Irvine, D. S. and Aitken, R. J. (1998a). Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: Lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Human Reproduction*, **13**(6), 1429–1436. doi: 10.1093/humrep/13.6.1429

Twigg, J., Irvine, D. S., Houston, P., Fulton, N., Michael, L. and Aitken, R. J. (1998b). Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: Protective significance of seminal plasma. *Molecular Human Reproduction*, **4**(5), 439–445. doi: 10.1093/molehr/4.5.439

Vaughan, D. A. and Sakkas, D. (2019). Sperm selection methods in the 21st century. *Biology of Reproduction*, **101**(6), 1076–1082. doi: 10.1093/biolre/ioz032

Virro, M. R., Larson-Cook, K. L. and Evenson, D. P. (2004). Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility*, **81**(5), 1289–1295. doi: 10.1016/j.fertnstert.2003.09.063

Volpes, A., Sammartano, F., Rizzari, S., Gullo, S., Marino, A. and Allegra, A. (2016). The pellet swim-up is the best technique for sperm preparation during *in vitro* fertilization procedures. *Journal of Assisted Reproduction and Genetics*, **33**(6), 765–770. doi: 10.1007/s10815-016-0696-2

Ward, W. S. (2010). Function of sperm chromatin structural elements in fertilization and development. *Molecular Human Reproduction*, **16**(1), 30–36. doi: 10.1093/molehr/gap080

Ward, W. S. and Coffey, D. S. (1991). DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. *Biology of Reproduction*, **44**(4), 569–574. doi: 10.1095/biolreprod44.4.569

Wilding, M. and Dale, B. (1997). Sperm factor: What is it and what does it do? *Molecular Human Reproduction*, **3**(3), 269–273. doi: 10.1093/molehr/3.3.269

World Health Organization. (2010). *WHO Laboratory manual for the examination and processing of human semen*.

Zalata, A., Hafez, T. and Comhaire, F. (1995). Evaluation of the role of reactive oxygen species in male infertility. *Human Reproduction*, **10**(6), 1444–1451. doi: 10.1093/humrep/10.6.1444

Zhang, Z., Zhu, L. L., Jiang, H. S., Chen, H., Chen, Y. and Dai, Y. T. (2016). Predictors of pregnancy outcome for infertile couples attending IVF and ICSI programmes. *Andrologia*, **48**(9), 874–881. doi: 10.1111/and.12525

Zhao, Y., Vlahos, N., Wyncott, D., Petrella, C., Garcia, J., Zacur, H. and Wallach, E. E. (2004). Impact of semen characteristics on the success of intrauterine insemination. *Journal of Assisted Reproduction and Genetics*, **21**, 143–148. doi: 10.1023/b:jarg.0000031246.76666.f6

Zheng, W. W., Song, G., Wang, Q. L., Liu, S. W., Zhu, X. L., Deng, S. M., Zhong, A., Tan, Y. M. and Tan, Y. (2018). Sperm DNA damage has a negative effect on early embryonic development following *in vitro* fertilization. *Asian Journal of Andrology*, **20**(1), 75–79. doi: 10.4103/aja.aja_19_17

Zini, A. (2011). Are sperm chromatin and DNA defects relevant in the clinic? *Systems Biology in Reproductive Medicine*, **57**(1–2), 78–85. doi: 10.3109/19396368.2010.515704

Zini, A., Finelli, A., Phang, D. and Jarvi, K. (2000). Influence of semen processing technique on human sperm DNA integrity. *Urology*, **56**(6), 1081–1084. doi: 10.1016/s0090-4295(00)00770-6

Zini, A., Boman, J. M., Belzile, E. and Ciampi, A. (2008). Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis. *Human Reproduction*, **23**(12), 2663–2668. doi: 10.1093/humrep/den321

Zorn, B., Vidmar, G. and Meden-Vrtovec, H. (2003). Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional *in vitro* fertilization and intracytoplasmic sperm injection. *International Journal of Andrology*, **26**(5), 279–285. doi: 10.1046/j.1365-2605.2003.00424.x