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The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies

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Human semen is heterogeneous in quality, not only between males but also within a single ejaculate. Differences in quality are evident, both when examining the classical parameters of sperm number, motility and morphology and in the integrity of the sperm nucleus. The aim of this study was to determine the efficiency of the PureSperm®, Percoll® and swim-up preparation techniques to eliminate spermatozoa with nuclear anomalies. Semen samples were collected, washed and one part of the semen spread on a slide, the remainder was prepared using the swim-up, PureSperm® or Percoll® techniques. Spermatozoa from different fractions were fixed on slides and assessed. Sperm samples (*n*) from different men were stained using the chromomycin A_3 (CMA_3) fluorochrome, which indirectly demonstrates a decreased presence of protamine ($n = 31$ for swim-up; $n = 45$ for PureSperm®; $n = 39$ for Percoll®). Spermatozoa prepared using PureSperm® ($n = 35$) and Percoll® ($n = 37$) were also examined for the presence of endogenous DNA nicks. Good quality spermatozoa should not possess DNA nicks and not stain (i.e. fluoresce) with CMA_3. When prepared using the swim-up technique the spermatozoa recovered showed no significant improvement with the CMA_3 staining. When spermatozoa were prepared using the PureSperm® and Percoll® techniques, a significant ($P < 0.001$) decrease in both CMA_3 positivity and DNA strand breakage was observed. These results indicate that both the PureSperm® and Percoll® techniques can enrich the sperm population by separating out those with nicked DNA and with poorly condensed chromatin.

Key words: Percoll®/PureSperm®/sperm chromatin/sperm nuclear DNA/sperm preparation

Introduction

Sperm quality is traditionally defined by the parameters measured in a diagnostic semen analysis, which is largely based on concentration, motility and morphology. However, hidden anomalies (chromosomal, nuclear, organelle and membrane) not currently detectable by standard WHO (World Health Organization, 1992) semen analysis may escape our attention and have implications when the spermatozoa are used for assisted reproduction techniques. For example, abnormal sperm nuclei in both human and animal studies have been associated with abnormal fertilization and embryo development (Evenson *et al.*, 1980, 1999; Trasler *et al.*, 1985; Qiu *et al.*, 1995a,b; Sakkas *et al.*, 1996). This brings to light the question of whether, during assisted reproduction techniques, sperm preparation is able to isolate spermatozoa of 'real' quality or whether spermatozoa with undetectable anomalies are being used. Examples of anomalies that would be missed in 'real' quality spermatozoa would include anomalies at the chromosomal, nuclear, organelle and/or membrane level.

The vast majority of assisted reproduction clinics use one of the following techniques for isolating 'functionally normal' spermatozoa: (i) swim-up or sperm migration, which separates the sample into motile and non-motile fractions or (ii) density gradient centrifugation (e.g. Percoll® and PureSperm®), which separates spermatozoa according to their density and favours the isolation of the motile and normal morphological spermatozoa (Mortimer, 1999).

Aberrant chromatin packaging and DNA damage in the nucleus of spermatozoa are examples of anomalies that are presently not detectable by standard semen analysis. A number of studies have shown the existence of spermatozoa in the ejaculate possessing anomalies in the nucleus (Ballachey *et al.*, 1986; Evenson, 1986, 1990; Gorczyca *et al.*, 1993). The presence of such spermatozoa is more likely to occur in men that are being treated using various assisted reproductive techniques, in particular intracytoplasmic sperm injection (ICSI) (Bianchi *et al*., 1996a; Sun *et al*., 1997). The group of Evenson has shown that the sperm preparation techniques, density gradient centrifugation and glass wool filtration, can enrich sperm chromatin integrity when spermatozoa are assessed using the sperm chromatin structure assay (SCSA) (Larson *et al*., 1999). The SCSA measures the susceptibility of spermatozoa to denaturation *in situ*. We have previously shown that the chromomycin A_3 fluorochrome (CMA_3) is a useful tool for assessing the packaging quality of the chromatin in spermatozoa and may allow an indirect visualization of protamine deficiency (Bianchi *et al*., 1993). In addition to the accessibility of CMA_3 to mature human sperm chromatin, 'endogenous' in-situ nick translation experiments (that is nick translation not preceded by endonuclease treatment) indicates that the presence of DNA nicks is in an appreciable, even if variable, number of human ejaculated spermatozoa (Manicardi *et al*., 1995). Although the two techniques are closely related, the CMA_3 is more indicative of a gross anomaly in the nucleus,

Table I. The mean (±SD) percentage of spermatozoa positive to chromomycin A_3 in the initial sample (wash) and various fractions after isolation using swim-up, Percoll® and PureSperm® techniques. All samples tested were from different men

Number of samples tested	Fraction of sperm preparation			Paired *t*-test (wash versus swim-up or 90%)
	Swim-up			
	Wash	Sediment	Swim-up	
31	21.5 ± 9.5	19.6 ± 9.7	22.0 ± 9.5	$P = 0.6$
	Percoll®			
	Wash	45%	90%	
39	29.9 ± 15.5	30.72 ± 18.3	18.1 ± 12.1	$P < 0.001$
	PureSperm®			
	Wash	45%	90%	
45	33.9 ± 21.2	25.0 + 19.8	12.4 ± 12.6	$P < 0.001$

i.e. the way the DNA is packaged, while nick translation represents damage in the actual DNA itself. The presence of these abnormal spermatozoa in our insemination medium may have consequences during the fertilization process and for the developing embryo (Sakkas *et al*., 1996; Sun *et al*., 1997; Lopes *et al*., 1998). Therefore, the ability of sperm preparation techniques to remove spermatozoa with such anomalies has important implications for assisted reproduction techniques outcome. This study aims to determine the efficacy of the swim-up and density gradient centrifugation techniques in the removal of spermatozoa with nuclear aberrations.

Materials and methods

Preparation of semen samples and slides

Semen samples were obtained from men attending the Clinic of Sterility, University Hospital of Geneva, Geneva, Switzerland and the Assisted Conception Unit, Birmingham Women's Hospital, Birmingham, UK. All men attending the clinics were used. The only limiting parameter was whether we could isolate over 10 000 spermatozoa to prepare the slides. Patient semen characteristics ranged from a concentration of 1×10^6 spermatozoa per ml and 10% progressive motility to 289×10^6 spermatozoa per ml and 58% progressive motility (WHO, 1999). All samples were prepared using the same techniques and products in both laboratories and once slides were prepared they were sent to the same reader using a code, so that the reader did not know the type of preparation. The semen sample from the same man was not used to test all parameters. Samples were prepared according to the WHO (WHO, 1999) manual using 1 ml starting volumes. Density gradient centrifugation was carried out using 0.5 ml volumes of either Percoll® (Sigma Pharmaceutical, Buchs, Switzerland) or PureSperm® (Nidacon International, Gothenburg, Sweden). Briefly, 0.5 ml of a 45% suspension was layered over 0.5 ml of 90% and centrifuged for 20 min (300 *g*). Spermatozoa in the upper (0.5 ml) layer of the swim-up and both PureSperm® and Percoll® fractions (45 and 90%) were fixed in 3.5% paraformaldehyde. Three smears of each were then prepared on slides and left to air dry. It should be noted that the manufacturers have now withdrawn Percoll® from use in human assisted reproduction techniques.

In-situ nick translation assay and CMA_3 staining

In-situ nick translation was performed as previously described (Manicardi *et al*., 1995) by omitting the endonuclease treatments since, in the presence of pre-existing DNA endogenous nicks, the DNA polymerase I, by virtue of its 5′-3′ exonucleotic activity, can catalyse movement of the nicks along the double helix. The only difference with the previously described method was that Digoxigenin-11-dUTP (Boehringer Mannheim, Rotkrenz, Switzerland) was used.

For CMA_3 staining slides were treated for 20 min with 100 µl of CMA_3 solution (0.25 mg/ml McIlvaine buffer, pH 7.0, containing 10 mmol/l $MgCl_2$) (Manicardi *et al*., 1995). They were then rinsed in buffer, air-dried and mounted with a 1:1 mixture of phosphate buffered saline (PBS) and glycerol. In nearly all cases an operator, working blind, examined at least 500 spermatozoa on each coded slide. The nick translation and CMA_3 staining were predominantly of all-or-nothing type and the rare cells showing ambiguous fluorescence were not considered. We have previously shown that a strong correlation exists between chromatin packaging, as revealed by CMA_3 positivity, and the presence of nicks in sperm DNA (Manicardi *et al*., 1995). Fluorescence analysis was performed using a Zeiss Photomikroskop® III (Zeiss, Oberkochen, Germany). Statistical analysis was performed using SPSS 9.0 for Windows and the mean values of each sample compared using the paired *t*-test.

Results

When examining the different fractions prepared after using the swim-up technique, no significant difference was found between the percentage of spermatozoa recovered from the upper swim-up layer that were positive to the CMA_3 fluorochrome when compared to those spermatozoa in the initial wash sample and the sediment (Table I).

When prepared using Percoll®, the mean percentage (±SD) of spermatozoa positive to the CMA_3 fluorochrome was significantly lower (paired *t*-test, $P < 0.001$) in the 90% fraction compared to those remaining in the 45% fraction and in the initial washed sample (Table I). Similarly, using PureSperm®, the percentage of spermatozoa positive to the CMA_3 fluorochrome was again significantly lower (paired *t*-test, $P < 0.001$) in the 90% fraction compared to those

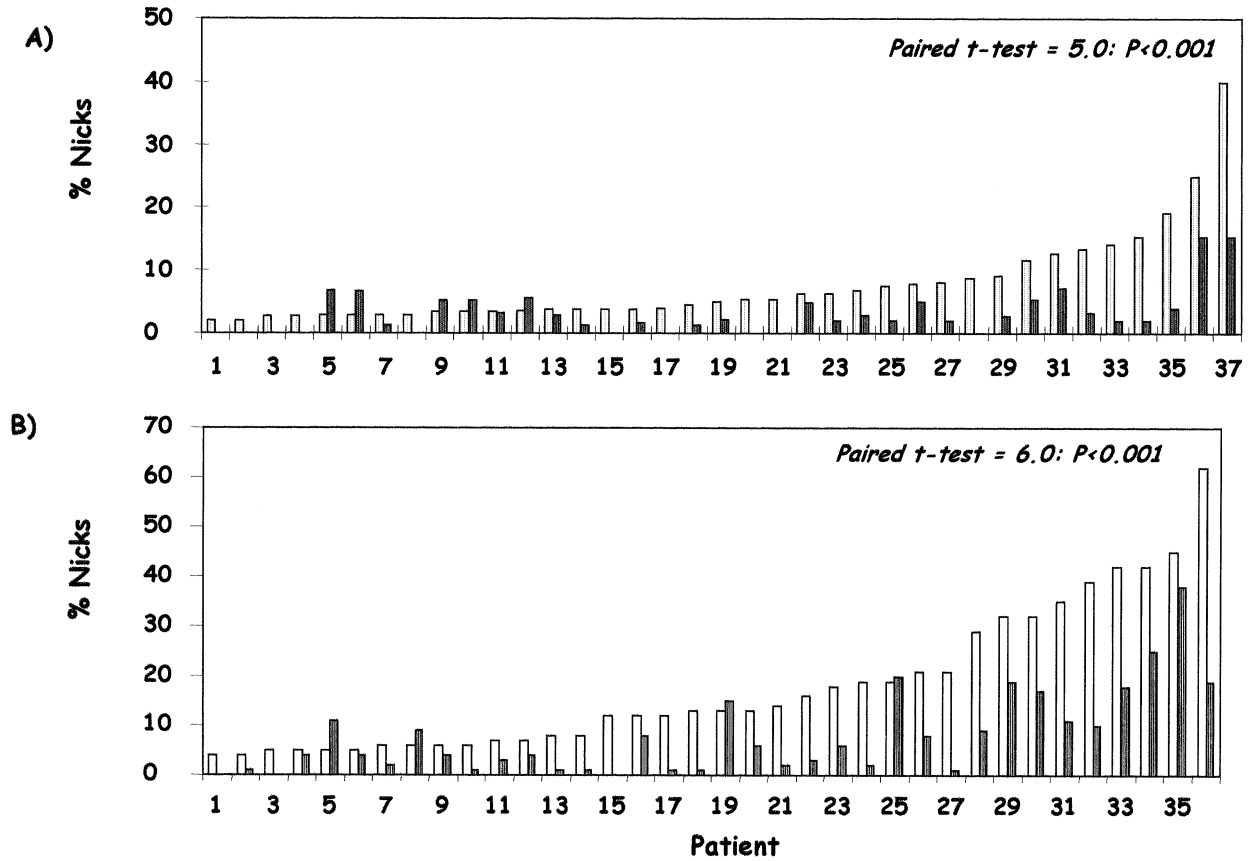

Figure 1. The percentage of human spermatozoa exhibiting endogenous DNA nicks in the 45% (white columns) and 90% (dark columns) fractions after preparation using (**A**) Percoll® and (**B**) PureSperm®. The patients used in **A** and **B** are not the same. Statistical comparisons using the paired *t*-test are included in the figure.

remaining in the 45% fraction and in the initial washed sample (Table I).

When examining for the percentage of spermatozoa with DNA damage we found a similar pattern as for the CMA_3 fluorochrome. Spermatozoa isolated in the 90% fractions of both Percoll® and PureSperm® possessed a significantly lower percentage of DNA damage when compared to the 45% fraction (Figure 1).

Discussion

In this study we have shown that the different standard sperm preparation techniques used in the routine assisted reproduction techniques laboratory vary in their ability to separate spermatozoa possessing nuclear anomalies from those which are normal. Sperm preparation using the swim-up technique does not appear to be as efficient at isolating a population of spermatozoa with a low percentage of nuclear anomalies. This is in contrast to previous reports (Angelopoulos *et al.*, 1998; Spano *et al.*, 1999) where post-rise spermatozoa prepared using the swim-up technique represented a subpopulation characterized by a general increase of the green head sperm percentage when using acridine orange. Spano and co-workers (Spano *et al.*, 1999) also reported that this subpopulation exhibited improved chromatin structure properties as assessed using the sperm chromatin structure assay (SCSA). The SCSA utilizes the metachromatic properties of acridine orange to distinguish between low-pH or heat-denatured (red fluorescence = single-stranded) and native (green fluorescence = double stranded) DNA in sperm chromatin. We have found however, using the CMA_3 fluorochrome, that although the swim-up technique is adequate for the isolation of a highly motile sperm population, overall sperm quality may be compromised, with a similar percentage of CMA_3 positive spermatozoa found in the swim-up to those found in the sediment. Interestingly, Colleu and co-workers (Colleu *et al.*, 1996) found that Percoll® gradients appeared to enrich for spermatozoa with less intermediate proteins, and more mature nucleoproteins of the protamine 2 family, a feature not observed with the swim-up spermatozoa. In contrast, Larson *et al.* (1999) reported that glass wool filtration produced sperm suspensions with improved chromatin integrity (Larson *et al.*, 1999), as measured with the SCSA, compared to density gradient centrifugation using Enhance-S plus™ (Conception Technologies, San Diego, CA, USA). To clarify the differences between the results observed using acridine orange and CMA_3 a direct comparison of the two stains using the different preparation techniques is needed.

When examining the ability of Percoll® to isolate spermatozoa of normal chromatin structure both Angelopoulos *et al.* (1998) and Golan *et al.* (1997) again found that the percentage of green fluorescent spermatozoa improved in the 90% fraction (Golan *et al.*, 1997; Angelopoulos *et al.*, 1998). In the current study, sperm preparation using density centrifugation techniques with either the now largely obsolete Percoll® or

one of its licensed replacements, PureSperm®, is able to reduce significantly the percentage of spermatozoa with nuclear abnormalities. We have assessed two forms of nuclear anomalies: firstly, using CMA_3 we have shown that spermatozoa with a more compacted chromatin are more likely to be present in the 90% fraction. We have previously reported that CMA_3 can be used to distinguish populations of spermatozoa with differing levels of protamination (Bizzaro *et al.*, 1998) and that there is a strong relationship between CMA_3 accessibility and the presence of endogenous DNA nicks in human spermatozoa (Manicardi *et al*., 1995). Our current findings with both Percoll® and PureSperm® therefore substantiate the work of Colleu (Colleu *et al*., 1996). More importantly, both Percoll® and PureSperm® significantly reduced the percentage of spermatozoa with nuclear DNA damage. This was clearly evident in the majority of patients who had high levels of DNA damage.

The patients who exhibit high levels of CMA_3 positivity and nuclear DNA damage are likely to have poor semen parameters (Manicardi *et al*., 1995; Bianchi *et al*., 1996a,b; Sun *et al*., 1997; Esterhuizen *et al*., 2000). This brings to light the question of what the benefits are of isolating a fraction of spermatozoa with lower levels of nuclear anomalies? A number of studies now clearly indicate that an abnormal sperm nucleus may have a detrimental effect on fertilization, embryo development and pregnancy outcome. We have previously shown that spermatozoa from men with high levels of DNA damaged spermatozoa are more likely to exhibit anomalies in sperm decondensation after ICSI (Sakkas *et al*., 1996). In addition, Lopes *et al*. (1998) showed that DNA damage in spermatozoa may contribute to fertilization failure after ICSI. More importantly, the group of Robaire has shown that damage to rat sperm DNA may be linked to an increase in early embryo death (Qiu *et al*., 1995a,b). Recently, Evenson showed that the SCSA could be used as a prognostic factor for human fertility (Evenson *et al*., 1999), stating that men who have an SCSA value of greater than 30% would have difficulties in achieving pregnancy. Clearly, isolating spermatozoa of a better nuclear consistency will increase the likelihood of achieving pregnancies with normal embryos.

Sperm preparation for assisted reproduction should aim to minimize the risk of abnormal spermatozoa being used for fertilization. By performing techniques such as ICSI, we remove many of the barriers set in place to select the best spermatozoa for fertilization. Unique checkpoints have already been observed after ICSI in the rhesus monkey (Hewitson *et al*., 1999) showing that both gametes can attempt to maintain reproductive capabilities even under abnormal conditions. It has also been shown that human spermatozoa possessing DNA damage have the same potential as control spermatozoa to decondense and form pronuclei when micro-injected into hamster oocytes (Twigg *et al*., 1998). The possible detrimental effects that an abnormal paternal genome may have on the fate of the human embryo after ICSI have been highlighted by a number of authors including ourselves (Sakkas *et al*., 1998; Sakkas, 1999).

Sperm preparation for assisted reproduction techniques should therefore aim to minimize the risk that abnormal spermatozoa can have on outcome. A reduction in the number of spermatozoa possessing nuclear anomalies in the final insemination preparation should be a priority, in particular as little is known about the relative influences spermatozoa with abnormal nuclei can have on the embryo and ensuing offspring. Therefore, density gradient centrifugation techniques using approved separating agents should be the preferred method.

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