

# Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception

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**BACKGROUND:** With an increase in the use of assisted reproduction technologies the requirements of the diagnostic semen analysis are constantly changing. **METHODS:** Spermatozoa from patients undergoing IVF were analysed by examining the conventional semen parameters and DNA/chromatin integrity, using in-situ nick translation (NT) and the Chromomycin A<sub>3</sub> fluorochrome, which indirectly demonstrates a decreased presence of protamine. Samples were examined before and after preparation using discontinuous density gradient centrifugation. **RESULTS:** Density gradient centrifugation enriched samples by improving the percentage of morphologically normal forms by 138% and sperm nuclear integrity by 450%. Sperm nuclear integrity as assessed by in-situ nick translation (NT) demonstrated a very clear relationship with sperm concentration, motility and morphology. Morphology correlated with fertilization rates of patients undergoing IVF, while NT values of the spermatozoa post-preparation were significantly lower in pregnant patients. **CONCLUSIONS:** We have demonstrated that along with the classical semen parameters, the assessment of nuclear integrity improves the characterization of the semen sample and may be used as a tool for allocating patients to specific assisted reproduction treatments.

*Key words:* semen parameters/sperm chromatin/sperm morphology/sperm nuclear DNA/sperm preparation

## Introduction

Poor sperm quality remains the most common cause of subfertility amongst couples in the UK (Hull, 1992) and probably in the Western world. Traditional semen analysis is based on the estimation of sperm concentration, motility and morphology, and, providing these tests are performed with diligence and with adherence to strict guidelines (World Health Organization, 1992), useful prognostic information can be obtained (Hargreave and Elton, 1983; Kruger *et al.*, 1986; Tomlinson *et al.*, 1999). However, with the upsurge in the use of assisted reproduction technologies and especially intracytoplasmic sperm injection (ICSI), the requirements of the diagnostic semen analysis are constantly changing. The modern assisted reproduction treatment laboratory no longer concerns itself with the question 'what are the chances of achieving a natural conception?' but instead requires additional information on patient suitability to particular treatment groups, such as intrauterine insemination (IUI), IVF or ICSI. As assisted reproduction becomes more diverse in the treatments offered, we remove many of the natural selection barriers set in place to ensure that the best spermatozoa are used for fertilization. Semen analysis therefore needs to develop to the point where

we can provide patients not only with some idea of prognosis in terms of natural conception, but also whether assisted reproduction treatment will have a successful outcome both in terms of fertilization and in producing a healthy conceptus.

There is an increasing body of evidence to suggest that the sperm nuclear integrity should be routinely examined. Poor chromatin packaging has been shown to correlate with numerous reproductive outcomes: anomalies in fertilization related to ICSI (Sakkas *et al.*, 1996), poor fertilization after IVF and ICSI (Esterhuizen *et al.*, 2000; Lopes *et al.*, 1998), the fertility of couples after intercourse (Evenson *et al.*, 1999; Spano *et al.*, 2000) and a higher incidence of pregnancy loss (Evenson *et al.*, 1999). Sperm donors have also been found to exhibit lower levels of nuclear DNA damage when specifically compared to infertility patients (Irvine *et al.*, 2000). The underlying mechanisms responsible for the existence of DNA-damaged spermatozoa are unclear, although irregularities in apoptosis during spermatogenesis may be involved (Sakkas *et al.*, 1999a,b).

We and others have previously shown that sperm DNA/chromatin integrity improves after preparation by density gradient centrifugation (Colleu *et al.*, 1996; Golan *et al.*, 1997;

Angelopoulos *et al.*, 1998; Larson *et al.*, 1999; Sakkas *et al.*, 2000). Using a relatively large study population, our aim was to increase the understanding of the relationship between sperm quality, as measured by conventional semen parameters, and DNA/chromatin integrity. We present data on how these relationships alter following sperm preparation by discontinuous density gradient centrifugation and the possible implications for assisted reproduction.

## Materials and methods

### Preparation of semen samples and slides

Semen samples were obtained from a total of 140 patients (median age 36 years, range 26–50) attending the Assisted Conception Unit, Birmingham Women's Hospital, UK for diagnostic semen analysis and assisted conception. Semen analysis was carried out according to published guidelines (World Health Organization, 1999). All were in relationships which had experienced a minimum of 1 year of infertility and were attending the IVF programme. On attendance, patients were asked to fill out a questionnaire asking whether they were under medication or had experienced any serious illness in the past year. None of the patients reported any serious illness or signs/symptoms of genital tract infection at the time of treatment.

Sperm preparation was carried out for assisted conception by discontinuous density gradient centrifugation, using 0.5 ml volumes of PureSperm<sup>®</sup> (Nidacon, Gothenberg, 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 90% PureSperm<sup>®</sup> pellet were fixed in 3.5% paraformaldehyde. Three smears were then prepared on slides and left to air dry. Three smears were also prepared from the raw semen fraction. In patients undergoing IVF, smears were prepared from the semen and the prepared fraction of spermatozoa was only taken once insemination had been performed.

### In-situ nick translation (NT) assay and Chromomycin A<sub>3</sub> (CMA<sub>3</sub>) staining

We have previously shown that the CMA<sub>3</sub> fluorochrome 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; Bizzaro *et al.*, 1998). In addition to the accessibility of CMA<sub>3</sub> to mature human sperm chromatin, 'endogenous' NT experiments (that is nick translation not preceded by endonuclease treatment) indicate that the presence of DNA nicks occurs in an appreciable, even if variable, number of human ejaculated spermatozoa (Manicardi *et al.*, 1995). Although the two techniques are closely related, the CMA<sub>3</sub> is more indicative of a gross anomaly in the nucleus, i.e. the way the DNA is packaged, while nick translation represents damage in the actual DNA itself. NT 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' exonuclease activity, can catalyse movement of the nicks along the double helix. The only difference to the previously described method was that Digoxigenin-11-dUTP (Roche, Monza, Italy) was used.

For CMA<sub>3</sub> staining, slides were treated for 20 min with 100 µl CMA<sub>3</sub> solution (0.25 mg/ml McIlvaine buffer, pH 7.0, containing 10 mmol/l MgCl<sub>2</sub>) (Manicardi *et al.*, 1995). They were then rinsed in buffer, air-dried and mounted with a 1:1 mixture of 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<sub>3</sub> staining were predominantly of all-or-nothing type and the

rare cells showing ambiguous fluorescence were not considered. Fluorescence analysis was performed using a Zeiss Axioplan (Zeiss, Oberkochen, Germany).

### IVF

IVF and sperm preparation were performed in IVF-500 medium (Scandinavian IVF, Gothenburg, Sweden). Once collected, oocytes were inseminated overnight in pools of up to 5 in 1 ml IVF-500 medium under oil (OvOil, Scandinavian IVF) containing 3–4 × 10<sup>5</sup> motile spermatozoa per ml overnight. Once fertilized, embryos were placed in IVF media for 2–3 days of culture until transfer. Stimulation and luteal support were as per our standard published procedure (Sharif *et al.*, 1995). Pregnancy was assessed after 6–7 weeks under ultrasound by the observation of a fetal heartbeat.

### Statistical analyses

Seminal parameters, CMA<sub>3</sub> and NT were correlated to IVF rates using Spearman's ranked correlation. Semen parameters and DNA damage in normozoospermic individuals were compared with those of oligozoospermic and teratozoospermic men using the non-parametric Mann–Whitney test. Logistic regression was used to assess the impact of the various semen parameters plus CMA<sub>3</sub> and NT on fertilization.

## Results

### Effect of PureSperm<sup>®</sup> preparation on the semen profile

Data on pre- and post-sperm preparation were available on 140 samples. Table I shows the median and range for each parameter. It is evident from Table I that PureSperm<sup>®</sup> preparation leads to a significantly altered semen profile. Values for CMA<sub>3</sub> and NT (43 and 9%, respectively) were significantly lower (28 and 2% respectively) after preparation ( $P < 0.0001$ ), whereas normal forms were significantly higher (13 versus 18%). Post-preparation, the percentage normal forms, NT and CMA<sub>3</sub> all improved by more than 100% as seen by the enrichment index: a calculation of the percentage measured post-preparation divided by the percentage measured in the semen. Sperm concentration and motility data were not similarly examined after preparation, as all preparations for IVF were diluted to give a fixed number of motile spermatozoa (3–4 × 10<sup>5</sup> motile spermatozoa per ml) for insemination.

### Nuclear integrity and semen parameters

In agreement with previous studies, NT and CMA<sub>3</sub> were negatively correlated with sperm concentration and positively correlated with each other ( $P < 0.0001$ ) (Manicardi *et al.*, 1995; Irvine *et al.*, 2000; Sakkas *et al.*, 2000). The strongest correlations were evident between NT in the raw sample and sperm concentration ( $r = -0.24$ ,  $P = 0.01$ ), motility ( $r = -0.20$ ,  $P = 0.004$ ) and percentage normal forms ( $r = -0.28$ ,  $P = 0.01$ ) in the raw sample (Table II) but there was no such relationship after sperm preparation. There were no relationships between age of the male and any of the seminal parameters. To explore interrelationships further, patients were divided according to diagnosis, i.e. oligozoospermia (<20 × 10<sup>6</sup>/ml), teratozoospermia (<15% normal forms) and normozoospermia (>20 × 10<sup>6</sup>/ml, >14% normal forms and >50% progressive motility) and NT and CMA<sub>3</sub> data compared (Figure 1). NT ( $P < 0.01$ ) and CMA<sub>3</sub> ( $P < 0.05$ ) were

**Table I.** Semen parameters (concentration, motility and morphology), nuclear DNA damage (in-situ nick translation: NT) and Chromomycin A<sub>3</sub> (CMA<sub>3</sub>) positivity before and after density gradient preparation

	Semen parameters					Post-preparation		
	Sperm concentration	Progressive motility	Normal forms (%)	NT (%)	CMA <sub>3</sub> (%)	Normal forms (%)	NT (%)	CMA <sub>3</sub> (%)
No. of samples	140	140	122	140	140	106	140	140
Median	52	53.5	13	9	43	18*	2*	27.5*
Minimum	0.5	0	1	2	12	1	0	1
Maximum	310	95	35	31	91	42	23	85
Enrichment index <sup>a</sup>						138	450	156

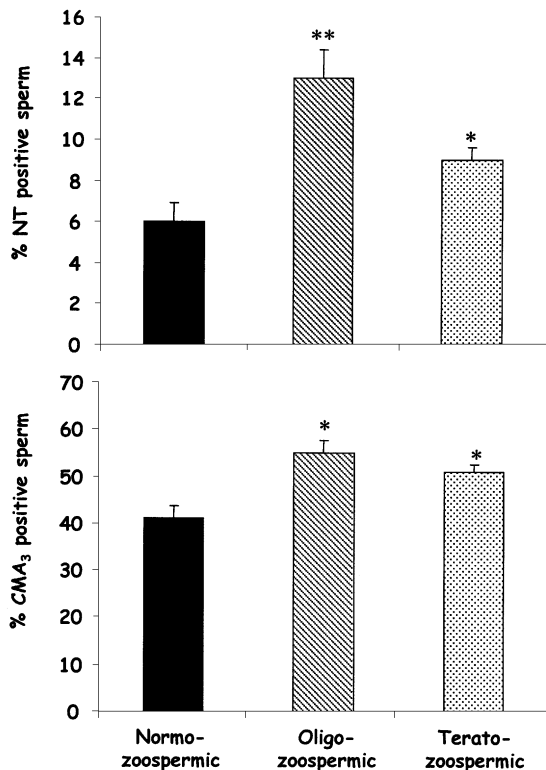
<sup>a</sup>Calculated by dividing the difference between the raw semen and prepared sample.  
\**P* < 0.0001 compared with respective raw semen parameters.

**Table II.** Correlation between nick translation and sperm concentration, motility and morphology before sperm preparation

	Sperm concentration	Motility	Normal forms
No. of samples	140	140	122
Correlation coefficient	-0.24	-0.20	-0.28
<i>P</i> -value	0.01	0.004	0.01

**Table III.** Correlation between the enrichment index (difference between the raw semen and prepared sample) and fertilization rates after IVF for nick translation (NT), Chromomycin A<sub>3</sub> (CMA<sub>3</sub>) and morphology

	Fertilization rate
NT enrichment	
Correlation coefficient	-0.02
<i>P</i> -value	0.82
CMA <sub>3</sub> enrichment	
Correlation coefficient	-0.06
<i>P</i> -value	0.54
Morphological enrichment	
Correlation coefficient	0.30
<i>P</i> -value	0.02



**Figure 1.** A comparison of sperm DNA damage (in-situ nick translation: NT) and Chromomycin A<sub>3</sub> (CMA<sub>3</sub>) levels in patients divided according to semen characteristics. Values are mean ± SEM. \**P* < 0.05 compared with normozoospermic samples. \*\**P* < 0.01 compared with normozoospermic samples.

both significantly lower in normozoospermic samples when compared to oligozoospermic samples. When compared with teratozoospermic samples, again both NT and CMA<sub>3</sub> were significantly lower (*P* < 0.05) in normozoospermic samples.

**Nuclear integrity, fertilization and pregnancy**

To compare semen parameters with the outcomes of fertilization and pregnancy, we examined both the raw semen parameters and those after preparation. The only variable to correlate directly with fertilization rates was the morphological enrichment index (percentage normal forms in the inseminate/ the percentage normal forms in the raw sample). Similar calculations using NT and CMA<sub>3</sub> were not significantly correlated with fertilization (Table III). No correlation was found between NT, CMA<sub>3</sub> and normal forms with the mean embryo score on day 2 or 3 (data not shown).

In comparing the pregnant and non-pregnant groups, the only parameter that showed a significant difference was the percentage of DNA-damaged spermatozoa in the prepared spermatozoa, which was significantly higher in those patients that did not establish a pregnancy (Table IV).

**Discussion**

The heterogeneity of a sperm population complicates the examination of interrelationships between semen parameters.

**Table IV.** Comparison of the percentage of normal morphology, nick translation (NT) and Chromomycin A<sub>3</sub> (CMA<sub>3</sub>) results in the pre- and post-preparation sample for pregnant and non-pregnant patients after IVF

Parameter	Not pregnant		Pregnant	
	Pre-preparation	Post-preparation	Pre-preparation	Post-preparation
No. of cycles	65		29	
Male age	36 ± 0.8		36.5 ± 1.1	
Female age	34 ± 0.6		35.5 ± 0.9	
Sperm concentration (×10 <sup>6</sup> /ml)	62.9 ± 5.5		67.1 ± 7.3	
% motile spermatozoa (forward progression)	50.5 ± 2.2		52.9 ± 3.2	
No. of oocytes collected	12.6 ± 1.4		13.0 ± 1.2	
Fertilization rate (%)	58.3 ± 3.3		62.4 ± 2.8	
No. embryos transferred	2.2 ± 0.1		2.2 ± 0.1	
	Not pregnant		Pregnant	
	Pre-preparation	Post-preparation	Pre-preparation	Post-preparation
Mean % spermatozoa with normal forms	12.5 ± 1.0	17.0 ± 0.4	14.5 ± 1.1	18.0 ± 0.6
Mean % of DNA damaged spermatozoa	11.5 ± 1.0	4.0 ± 0.7 <sup>a</sup>	9.3 ± 1.0	2.0 ± 0.3 <sup>a</sup>
Mean % of CMA <sub>3</sub> positive spermatozoa	42.1 ± 3.7	26.1 ± 1.9	42.6 ± 2.8	27.1 ± 2.5

Values are mean ± SEM.

<sup>a</sup>Values with the same letter are significantly different ( $P = 0.02$ ).

Furthermore, difficulties arise in relating these parameters to reproductive outcomes, such as fertilization, embryo development and pregnancy, as they are all compounded by numerous external influences, in particular female factors. In spite of complicating factors, many authors have been able to demonstrate such distinct relationships, demonstrating a paternal influence on outcome (Kruger *et al.*, 1986; Aitken *et al.*, 1991; Tomlinson *et al.*, 1992, 1993; Lopes *et al.*, 1998; Esterhuizen *et al.*, 2000).

In this study, sperm nuclear integrity as assessed by NT demonstrated a very clear relationship with sperm concentration, motility and morphology. Normozoospermics had a lower percentage of spermatozoa with DNA damage and CMA<sub>3</sub> positivity compared with oligozoospermic and teratozoospermic men. Irvine and co-workers (Irvine *et al.*, 2000) demonstrated a similar association with sperm count when they compared comet assay results from a group of sperm donors with those of infertility patients. Surprisingly, in their study, NT results did not agree with sperm count, despite a very strong correlation with comet results, and the suggestion was that the comet assay had a higher resolution than NT. It may be that the much larger study population in the present study enabled us to detect significant associations with NT. They found no associations between DNA damage and sperm morphology, whereas in the present study, an increased percentage of DNA-damaged spermatozoa was observed in teratozoospermic patients (<15% normal forms). A similar, yet much stronger, relationship has been found when comparing CMA<sub>3</sub> with normal forms using 4% as a threshold value (Esterhuizen *et al.*, 2000). The lack of a clear-cut relationship between sperm count and morphology suggests that when we examine morphology and NT or CMA<sub>3</sub> we are looking at distinct

populations of spermatozoa. We had previously shown that morphologically normal spermatozoa show differences in chromatin packaging when comparing spermatozoa from normozoospermic men with those possessing abnormal sperm parameters (Bianchi *et al.*, 1996). In addition, when we studied both NT and CMA<sub>3</sub> staining in the same sperm population we found the existence of different classes of spermatozoa in the human ejaculate. Subsequently, we proposed that in most ejaculates the majority of healthy spermatozoa contain compact chromatin (highly protaminated CMA<sub>3</sub> negative spermatozoa) and unbroken DNA. The other two main classes, however, represent sub-groups of spermatozoa that have anomalies, and contain either loosely packaged chromatin (probably underprotaminated CMA<sub>3</sub> positive spermatozoa) but unbroken DNA or contain both loosely packaged chromatin (probably underprotaminated) and nicked DNA. We concluded that the relative proportion of these classes of spermatozoa in a patient's ejaculate might have been a useful tool for assessing his fertility (Manicardi *et al.*, 1995).

What does the added assessment of DNA damage in the sperm nucleus show us? The above studies indicate that the assessment of the integrity of the sperm nucleus is highly indicative of the possible fertility status and therefore competence of human spermatozoa. NT will indicate anomalies that have occurred during the remodelling of the nuclear DNA in spermatozoa and in doing so is more likely to observe sperm anomalies not indicated by morphology. It may also be able to indicate if there is damage arising from factors such as heat exposure (Setchell, 1998; Setchell *et al.*, 1998) or the generation of reactive oxygen species following exposure to leukocytes within the male reproductive tract (Aitken *et al.*, 1989, 1991).

Others are also accumulating data, indicating that nuclear

integrity measurement, using tools such as NT, adds significantly to the diagnostic power of the semen analysis. Possibly the best reported test is that used for many years by Evenson's group. They and others have provided strong evidence of a relationship between sperm nuclear DNA integrity, as assessed using the sperm chromatin structure assay (SCSA), and fertility after both normal intercourse (Evenson *et al.*, 1999; Spano *et al.*, 2000) and assisted reproduction techniques (Larson *et al.*, 2000). The SCSA measures the susceptibility of sperm nuclear DNA to heat- or acid-induced denaturation *in situ* followed by staining with acridine orange. Evenson *et al.* found cases where the classical criteria (concentration, motility and morphology) were within the normal ranges, but the SCSA values were poor, and not compatible with good fertility after intercourse (Evenson *et al.*, 1999).

In many of the above studies, couples attempting to achieve pregnancy without the aid of assisted reproduction techniques were examined. One of the confounding problems of relating semen parameters to overall IVF outcome is that a certain population of spermatozoa is selected during the semen preparation procedure. It is likely that regardless of the initial sample, a degree of homogenization occurs after sperm preparation. This study has shown that simple density gradient centrifugation can enrich the sample both with morphologically normal forms and spermatozoa with improved nuclear integrity. This 'normalizing' effect of density gradient preparations may be the reason why sperm parameters pre-preparation had little prognostic value, in terms of fertilization and pregnancy using IVF. This indicates a need to assess spermatozoa in context, i.e. raw semen parameters in conjunction with natural conception, or intracervical insemination and prepared parameters in relation to IUI/IVF and ICSI. Concurrently, the normalizing effect led to a population of spermatozoa in the insemination drop that showed no correlation with fertilization or day 2–3 embryo development. However, morphological enrichment correlated with fertilization rates and, perhaps more importantly, NT values of the spermatozoa in the insemination drop were significantly lower in the pregnant group when compared to the non-pregnant group. The NT data support previous results using the SCSA test (Evenson *et al.*, 1999; Spano *et al.*, 2000). It seems likely that morphological sperm parameters are important up to the fertilization step, and the importance/influence of DNA integrity is realized later, becoming the most important sperm parameter related to the establishment and continuation of a pregnancy. We know that there is no difference for example in fertilization rates, when using the hamster egg penetration test after ICSI, when comparing normal and DNA-damaged spermatozoa (Twigg *et al.*, 1998). In addition, UV-irradiated mouse spermatozoa that have nuclear DNA damage are able to fertilize normally but there are significant reductions in implantation rates and birth rates when compared to control spermatozoa (Ahmadi and Ng, 1999).

We have demonstrated that along with the classical semen parameters, the assessment of nuclear integrity improves the characterization of the semen sample. However, the study needs to be expanded by performing NT or similar on a larger group of patients, eliminating as far as possible complicating influences, e.g. female factors. In doing so, we will improve

and possibly 'fine-tune' our current semen analyses for patients attempting complex assisted reproductive treatments.

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