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RESEARCH ARTICLE

Sperm DNA damage or progressive motility: which one is the better predictor of fertilization *in vitro*?

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Sperm progressive motility has been reported to be one of the key factors influencing *in vitro* fertilization rates. However, recent studies have shown that sperm DNA fragmentation is a more robust predictor of assisted reproductive outcomes including reduced fertilization rates, embryo quality, and pregnancy rates. This study aimed to compare the usefulness of sperm progressive motility and DNA damage as predictive tools of *in vitro* fertilization rates. Here, 136 couples provided 1,767 eggs with an overall fertilization rate of 64.2%. The fertilization rate *in vitro* correlated with both sperm progressive motility ($r^2 = 0.236$; $P = 0.002$) and DNA fragmentation ($r^2 = -0.318$; $P < 0.001$). The relative risk of a poor fertilization rate was 9.5 times higher in sperm of men with high DNA fragmentation (>40%) compared with 2.6 times in sperm with poor motility (<40%). Further, sperm DNA fragmentation gave a higher specificity (93.3%) in predicting the fertilization rate than progressive motility (77.8%). Finally, the odds ratio to determine fertilization rate (>70%) was 4.81 (1.89–12.65) using progressive motility compared with 24.18 (5.21–154.51) using DNA fragmentation. This study shows that fertilization rates are directly dependent upon both sperm progressive motility and DNA fragmentation, but sperm DNA fragmentation is a much stronger test.

Keywords comet assay, IVF outcome, odds ratio, progressive motility, semen analysis, sperm DNA damage

Introduction

One in six couples experiences fertility problems during their reproductive lives so assisted reproductive technologies (ART) have a major role in modern society. The success of ART varies depending on a range of male and female factors, but adequate structure and function of male and female gametes is essential in all cases [Varghese et al. 2009]. Sperm progressive motility is essential for the sperm to penetrate the zona pellucida both *in vivo* and *in vitro*, and thus this has been considered one of the most important factors in determining fertilization rates [Chiu et al. 1987; Donnelly et al. 1998; Turner 2006]. Fertilization is comprised of two major steps: the interaction between the sperm and the oocyte, and fusion of male and female gametes to form a pronucleus [Wassarman 1999]. Vigorous sperm motility is known to facilitate fertilization by enabling the sperm to penetrate the cumulus cell, corona radiate, and finally the zona pellucida [Liu et al. 1991]. Reduced motility is observed in infertile men; often associated with increased mitochondrial abnormalities [Folger et al. 1993; Kao et al. 1998] and structural deformities in the flagella [Baccetti et al. 1993; Chemes et al. 1998]. The aetiology of impaired motility is related to an increase in oxidative factors in the seminal plasma [Urata et al. 2001; Kao et al. 2008], increased age [Kidd et al. 2001], and electromagnetic radiation [Yan et al. 2007].

Recently the limitations of conventional WHO parameters such as motility to predict ART success have been highlighted [Lefievre et al. 2007; Lewis 2007]. Through many qualitative studies, sperm DNA damage has been reported to be a more robust biomarker. It has associations with all ART outcomes, but specifically with fertilization [Donnelly et al. 1998; Sun et al. 1997; Esterhuizen et al. 2000; Host et al. 2000; Tomlinson et al. 2001; Benchaib et al. 2003; 2007; Henkel et al. 2003; 2004; Saleh et al. 2003; Chohan et al. 2004; Gandini et al. 2004; Huang et al. 2005; Payne et al. 2005; Borini et al. 2006; Muriel et al. 2006; Bakos et al. 2007; Bungum et al. 2007; Frydman et al. 2008; Lin et al. 2008]. In contrast, a smaller number of studies report that sperm DNA damage does not affect fertilization rates [Tomlinson et al. 2001; Henkel et al. 2003; Chohan et al. 2004; Gandini et al. 2004; Benchaib et al. 2007; Bungum et al. 2007; Frydman et al. 2008; Lin et al. 2008]. Thus, there is a controversy in the literature as to the impact of sperm DNA damage on this early fertility check point. To compare the power of motility with DNA damage as predictors of IVF fertilization, we determined both parameters in the same semen samples and correlated them with fertilization rates *in vitro*.

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Results

Association of semen parameters with sperm DNA damage and fertilization rates

An overall fertilization rate of 64.2% was observed using the 1,767 eggs included in the study. Of the conventional semen parameters: volume, concentration, and morphology, and male and female age, there was no correlation with fertilization rate (Table 1). In contrast, there was a positive correlation with progressive motility ($r^2 = 0.236$; $P = 0.002$). There was also a negative correlation between sperm DNA fragmentation and sperm progressive motility ($r^2 = -0.214$; $P = 0.005$) but no association was found with any other semen parameter and sperm DNA damage. In the univalent and multivalent analyses, semen parameters (volume, concentration, and normal morphology) showed no statistical significance when predicting fertilization rate (Table 2).

Relationship between progressive motility and sperm DNA fragmentation with fertilization rate

A significant negative correlation was observed between fertilization rate and sperm DNA measured in both native semen ($r^2 = -0.318$; $P < 0.001$) and DGC (Density Gradient Centrifugation) sperm ($r^2 = -0.261$; $P < 0.001$). When the fertilization rate was categorized into two categories (<70% and ≥70%) there was a significant difference in mean progressive motility (49.24 ± 3.14 vs. 57.81 ± 1.22; $P = 0.006$), sperm DNA damage measured in the native semen (56.60 ± 4.43 vs. 47.03 ± 1.73; $P = 0.010$), and the DGC sperm (41.00 ± 4.16 vs. 33.02 ± 1.51; $P = 0.044$) between the two categories. When progressive motility was divided into three categories, high and moderate motility groups had good fertilization rates 71% and 67%, respectively, while low motility was associated with a lower fertilization rate of 52% (Fig. 1). As sperm DNA fragmentation in native semen increased, the fertilization rate was reduced; low (73%), moderate (62%) and high DNA damage (51%) fertilization rate (Fig. 2). The relationship was also observedfor DGC sperm. The Chi-square analysis showed a significant association among the three categories of fertilization rate with both progressive motility and sperm DNA fragmentation ($\chi^2 = 16.06$ and 35.68, respectively), with four degrees of freedom. In the univalent model, abnormal motility and high sperm DNA damage in both native and DGC sperm showed a significant decrease in fertilization rate (Table 2). The association became stronger when DNA fragmentation was included in the multivalent analysis. However, when sperm progressive motility and DNA damage were included in the model, the odds ratio to obtain a good fertilization rate (>70%) when DNA damage <40% was 6.01 (CI: 1.57–24.78) and for DNA damage >70% was 2.03 (CI: 1.38–11.22), was significant.

Table 1. Comparison Between Semen Parameters, Age, and Sperm DNA Fragmentation with Fertilization Rate.

	Fertilization rate (%)			
	≤40%	40–70%	>70%	*P* value
Semen volume (ml)	3.2 ± 0.2	2.9 ± 0.2	3.6 ± 0.2	NS
Sperm concentration (10^6/ml)	62.4 ± 7.0	73.9 ± 5.3	71.2 ± 4.5	NS
Progressive motility (%)	46.8 ± 3.3	57.9 ± 1.7	60.1 ± 2.2	0.038
Normal morphology (%)	29.1 ± 2.0	23.7 ± 1.4	25.4 ± 1.3	NS
Female age (years)	34.7 ± 0.6	34.6 ± 0.6	35.6 ± 0.4	NS
Men age (years)	37.1 ± 0.8	36.6 ± 0.8	36.9 ± 0.5	NS
DNA damage in DGC sperm (%)	44.0 ± 4.1	35.0 ± 2.7	30.9 ± 1.9	0.023
DNA damage in native semen (%)	59.9 ± 4.3	48.2 ± 3.2	42.6 ± 2.2	0.019

NS: non-significant ($P > 0.05$).

Clinical significance of progressive motility and sperm DNA fragmentation to determine in vitro fertilization success

The odds ratio to determine fertilization using sperm motility was 4.81 (1.89–12.65) while the odds ratio was 24.18 (5.21–154.51) using sperm DNA fragmentation. Sperm of men with high DNA fragmentation and low motility results in 9.5 times and 2.6 times the increased relative risk of lower fertilization (<40%), when compared with low DNA damage and poor motility. Similarly, sperm DNA damage showed a higher specificity (93.3%) in predicting fertilization rates than progressive motility (77.8%). Sperm with high progressive motility and low sperm DNA damage had a 96.0% probability of resulting in >70% fertilization rate (Table 3).

Discussion

Evaluation of semen parameters is still the gold standard in diagnosing male infertility. These conventional tests are also used to choose IVF or ICSI as the best treatment for each

Table 2. The Effect of Standard Semen Parameters and DNA Fragmentation on Fertilization Rate.

		Univalent OR (95% CI)	Model I OR (95% CI)	Multivalent Model II OR (95% CI)	Model III OR 95% CI)
Semen volume (ml)	≤2 vs. >2	1.22 (0.53–2.82)	2.76 (0.70–11.32)	0.67 (0.15–2.89)	4.50 (0.41–114.50)
Sperm concentration (10^6/ml)	≤20 vs. >20	1.49 (0.40–5.69)	0.55 (0.02–6.51)	0.45 (0.05–3.22)	1.05 (0.06–33.52)
Progressive motility (%)	≤50 vs. >50	2.84 (1.37–5.89)	6.01 (1.57–24.78)	1.65 (0.50–5.49)	2.03 (1.38–11.22)
Normal morphology (%)	≤30 vs. >30	0.90 (0.40–1.76)	0.56 (0.19–1.69)	0.91 (0.30–2.78)	2.50 (0.41–16.01)
DNA damage in DGC sperm (%)	≤40 vs. >40	2.42 (1.24–4.73)	–	–	–
DNA damage in native semen (%)	≤40 vs. >40	3.66 (1.38–9.83)	–	–	–

OR: odds ratio; CI: confidence interval.

Models I, II, and III includes DNA fragmentation <40%, 40–70%, and >70% in the multivalent analysis, respectively.

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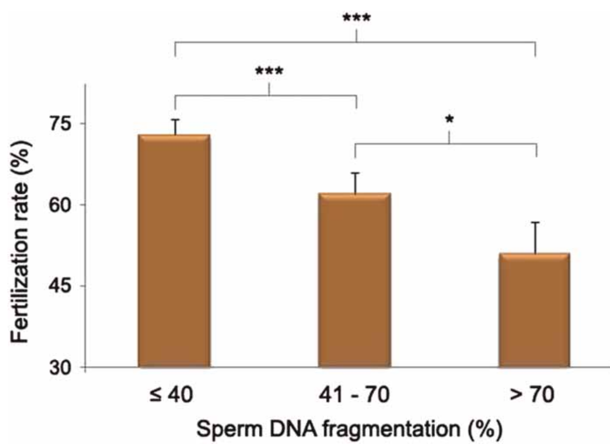

Figure 1. Relationship between sperm DNA fragmentation and fertilization rate. Bar chart showing decrease in fertilization rate with increase in DNA fragmentation in the native system. Significance at $^{*}P < 0.05$, $^{***}P < 0.001$.

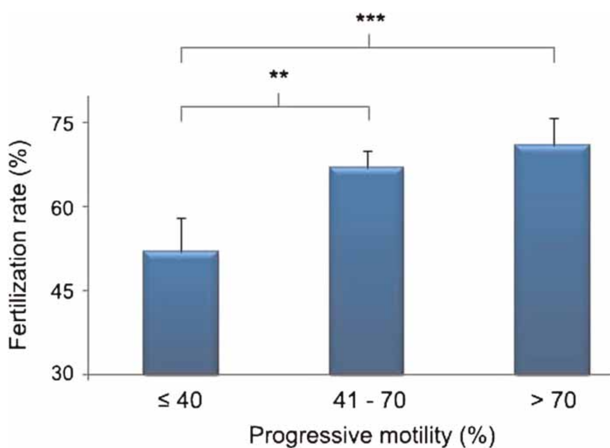

Figure 2. Relationship between progressive motility and fertilization rate. Bar chart showing increase in fertilization rate with increase in progressive motility. Significance at $^{**}P < 0.01$, $^{***}P < 0.001$.

couple [Repping et al. 2002; van Weert et al. 2004]. In addition to determining male fertility prior to ART, semen analysis is also performed to determine the chance of a spontaneous pregnancy [Wald 2008]. However, despite its continued widespread use, this conventional method of infertility diagnosis has limited use as it lacks the predictive power to determine male infertility or assisted reproductive outcomes (reviewed by [Tomlinson et al. 1999; Lewis 2007]). Microscopic analysis may remain the first stage of diagnosis but molecular testing is needed to provide a more robust prognostic tool in ART [Lopes et al. 1998; Tomlinson et al. 2001].

Table 3. Prognostic Value of Progressive Motility and Sperm DNA Fragmentation to Determine Fertilization Rate *in vitro*.

	Progressive motility (>70%)	Sperm DNA fragmentation (≤40%)
Odds Ratio (95% CI)	4.81 (1.89–12.65)	24.18 (5.21–154.51)
Sensitivity (%)	57.89	66.33
Specificity (%)	77.78	93.33
PPV (%)	89.19	97.44
NPV (%)	36.84	38.89
RR (95% CI)	2.61(1.39–4.90)	9.50 (2.47–36.49)

PPV: positive predictive value; NPV: negative predictive value; RR: relative risk; CI: confidence interval.

The most important finding in this study is that we report DNA fragmentation measured by the alkaline comet assay has a stronger prognostic ability to predict *in vitro* fertilization rate than progressive motility. The existing data regarding the relationship between sperm DNA fragmentation and fertilization rates are conflicting [Morris et al. 2002; Chohan et al. 2004; Henkel et al. 2004; Borini et al. 2006; Bakos et al. 2007; Bungum et al. 2007; Lin et al. 2008]. Our data show a strong relationship between sperm DNA fragmentation and fertilization rates in IVF both in native semen ($r^2 = -0.318$; $P < 0.001$) and DGC sperm ($r^2 = -0.261$; $P < 0.001$). When sperm DNA fragmentation is >40%, we found a significant negative relationship with fertilization rate. Again, this is in agreement with Benchaib et al. [2003] although they used a threshold value of 10% when measuring damage by the TUNEL assay. Our higher threshold is due to the sensitivity of the alkaline comet assay where all double and single strand breaks are measured throughout the entirety of relaxed chromatin. This is in contrast to other assays where perhaps only peripheral DNA damage is measurable. The correlation between sperm DNA damage and fertilization rates is consistent with when the sperm DNA fragmentation is low, the oocytes are able to repair the DNA damage [Sakkas et al. 1996; Ahmadi and Ng 1999]. However, their ability to repair is limited as the level of sperm DNA fragmentation increases. It is also postulated that with DNA fragmentation sperm may fail to decondense and thus not be able to develop to the pronuclear stage resulting in fertilization failure [Sakkas et al. 1996].

Some studies have shown relationships between semen parameters and sperm DNA damage [Tomlinson et al. 2001; Larson-Cook et al. 2003; Virro et al. 2004]. However, our study supports those of Frydman et al. [2008] and Greco et al. [2005] in showing few correlations between conventional semen parameters and sperm DNA fragmentation as we observed a significant negative correlation between sperm DNA and only one conventional parameter; that of sperm motility.

The present study has shown that IVF outcomes are not correlated with semen volume, sperm concentration, or morphology. However, the chance of fertilization increased with the progressive motile population of sperm. Several studies have shown fertilization failure when sperm motility is less than 30% and a reduction of fertilization rate in semen with motility < 50% [van Uem et al. 1985; Hirsch et al. 1986]. Similarly, our results show a good fertilization rate in semen with both good (>70%) and moderate (40–70%) motility but not with low motility <40% (Fig. 1). Our findings (also reported in another study from our group [Simon et al. 2010; 2011]) conflict with several older studies that report a decrease in fertilization rate with decrease in sperm concentration, percentage motility, or normal morphology

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[Battin et al. 1985; Hirsch et al. 1986; Matson et al. 1986]. Mahadevan and Trouson [1984] showed an influence of sperm concentration on fertilization rate when the count fell below a level of 10 million/ml, whereas variations in sperm concentration above this level had no influence on fertilization rate. However, in support of this study, sperm motility alone was shown to be the only semen parameter to influence fertilization rate [Amann 1989; Bongso et al. 1989; Donnelly et al. 1998]. Further, Battin et al. [1985] showed sperm motility after swim-up was also associated with the rate of fertilization. Abnormalities of sperm motility include flagellar abnormalities, deficient mitochondrial metabolism, failure of sperm recognition of the zona pellucida, and an inability to complete sperm-oocyte fusion [Kao et al. 2008]. Any or all of these could be the cause of reduced fertilization. Our study supports the reports by Bartoov et al. [1993] and Robinson et al. [1994] that the rate of fertilization is unaffected by an increase in abnormal morphology of the sperm.

The odds ratio to obtain a good (>70%) fertilization rate was higher using sperm DNA fragmentation then the conventional sperm motility (24.18 vs. 4.81, respectively). Although both progressive motility and DNA damage were significant to determine the fertilization rate, measurement of sperm DNA damage showed greater sensitivity (66%) and specificity (93%) in predicting good fertilization rate than progressive motility (sensitivity 58% and specificity 78%). Men with high DNA fragmentation had a higher relative risk i.e., 9.5 times, a fertilization rate of ≤40%. The strong prognostic value of sperm DNA fragmentation was also supported with a high positive predictive value (97.44%). In semen samples having both high motility and low sperm DNA fragmentation, the probability of obtaining a good fertilization rate was >96.0%. In conclusion, measurement of sperm DNA fragmentation by the alkaline comet assay has a markedly greater relative risk and specificity than the conventional measurement of sperm progressive motility in predicting *in vitro* fertilization rates.

Materials and Methods

This project was approved by the Office for Research Ethics Committees in Northern Ireland and the Royal Group of Hospitals Trust Clinical Governance Committee. The study was conducted at the Regional Fertility Centre, Royal Jubilee Maternity Services, Belfast, Northern Ireland, UK during the period April, 2008 to December, 2009. Sperm samples for research were obtained after written consent from all patients. A total of 240 couples attending for IVF were recruited and of these, 136 couples were included in this study as they met the following criteria: a) a minimum of five oocytes were retrieved and b) female partners were <40 years. Couples with failed fertilization and men with antisperm antibodies in their semen were excluded from the study.

Semen analysis and sperm preparation

Semen samples were collected by masturbation from men after 2–5 d of recommended abstinence, on the day of IVF treatment. Semen analysis was performed within 1 h of ejaculation, following a period of incubation at 37°C to allow for liquefaction. Sperm motility was measured by light microscopy, also according to WHO [1992] criteria. Semen was subjected to density gradient centrifugation (DGC) using a two-step discontinuous Puresperm gradient (90%–45%; Hunter Scientific Limited, UK). For each semen sample with a normozoospermic profile the whole sample was layered on top of 2 ml (90%) and 4 ml (45%) gradient and centrifuged at 250 x g for 20 min. For semen samples with less than normal WHO parameters, 1 ml of semen was layered on top of 1 ml (90%) and 1 ml (45%) gradient and centrifuged at 100 x g for 20 min. The resulting sperm pellets were washed twice with culture media (Vitrolife G5 sequential media series, Vitrolife Inc, Goteborg, Sweden) and concentrated by centrifugation at 250 and 100 x g, respectively, for 10 min or resuspended in fresh culture media (2 ml). After DGC, sperm motility was measured again. Hence, two populations of sperm were used to measure both motility and DNA damage: the whole population (native semen) for each patient and that with the best fertilizing potential as used for clinical treatment (DGC).

IVF treatment

All IVF cycles were performed according to routine procedures. Briefly, ovulation induction was achieved with recombinant FSH following a long protocol of pituitary desensitization with a GnRH analogue. HCG was administered when there were at least three follicles of diameter >17 mm, 36 h before oocyte retrieval. Mature, metaphase II oocytes obtained by vaginal ultrasound-guided aspiration were cultured in media Vitrolife G5 sequential media series (Vitrolife Inc, Goteborg, Sweden) at 37°C with 6% CO_2 in air. One or two embryos were transferred into the uterine cavity after an additional 24-48 h. Luteal phase support was provided by vaginally administered progesterone.

Alkaline comet assay

Sperm DNA damage was assessed using an alkaline single cell gel electrophoresis (comet) assay as previously modified by our group [Hughes et al. 1997; Donnelly et al. 1999]. Our previous study has reported an intra-assay coefficient variation of 6% for this assay [Hughes et al. 1997].

Statistical analysis

Data was analyzed using the Statistical Package for the Social Sciences (SPSS 15) for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error. The amount of fragmented DNA migrated in the comet tail was expressed as percentage of damage for native semen or DGC sperm. Sperm DNA damage was categorized into three groups: low DNA damage (0–40%), moderate DNA damage (41–70%), and high DNA damage (71–100%) [Simon et al. 2010]. The fertilization rate for each couple was calculated as the percentage of oocytes fertilized. Fertilization rates were categorized into two groups (≤70% and >70%) and three groups, poor (0–40%), moderate

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(41–70%), and good (71–100%) [Ola et al. 2001]. Similarly, progressive motility was categorized into three groups: poor (0–40%), moderate (41–70%), and good (71–100%) motility [Bonde et al. 1998; Tesarik et al. 2006]. All tests were two-sided with a probability value of less than 0.05 to be regarded as significant.

Spearman's Rank correlation coefficient was used to analyze the relationship between semen parameters with sperm DNA fragmentation. Logistic regression was used to evaluate the effect of semen parameters, male age, or female age on fertilization rate. Duncan's test for multi-group comparison was performed to analyze each category of fertilization rate with semen parameters and age. Pearson correlation was used to find any association between the variables: progressive motility, sperm DNA fragmentation, and fertilization rate. Finally, Chi-square analysis was performed comparing each of the three variables and their categories separately. We evaluated the effect of sperm DNA fragmentation on fertilization rate, in cases where all the standard semen parameters were normal and also where one semen parameter was abnormal. Each semen parameter was categorized into a normal or an abnormal category according to WHO [1992] guidelines. Univalent and multivalent analyzes were performed using sperm DNA damage as fixed variable and the odds ratios to obtain a fertilization rate (>70%) were determined. The odds ratios and their 95% CI, specificity, sensitivity, positive and negative predictive power, and relative risk in predicting good fertilization rates (>70%) was then estimated for sperm progressive motility compared to DNA damage.

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