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Predictors of pregnancy outcome for infertile couples attending IVF and ICSI programmes

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Summary

The purpose of this study was to evaluate the predictors of pregnancy outcome for infertile couples attending in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) programmes. Infertile couples attending IVF or ICSI procedures were included in this study. Related data including semen parameters and male and female age and body mass index were collected and analysed. The main outcome was clinical pregnancy, defined as an ultrasound detection of foetal heartbeat 6 weeks after embryo transfer. A total of 1316 couples who underwent IVF and 266 who underwent ICSI were recruited for this study. A multivariate logistic regression with likelihood ratio test revealed the following predictors of pregnancy outcome: female age and sperm DNA fragmentation index (DFI) and acrosomal activity in IVF procedures (chi-square of likelihood ratio = 26.42, d.f. = 3, $P < 0.005$) and female age and DFI in ICSI procedures (chi-square of likelihood ratio = 18.88, d.f. = 2, $P < 0.005$). In conclusion, our study indicated that sperm DFI, female age and acrosomal levels have a significant effect on ART pregnancy outcome.

Introduction

Male infertility is a world health problem affecting about 10–15% of couples, which accounts for half of the infertile cases (Gnoth *et al.*, 2005). Male-related factors pose a common cause of infertile couples. Assisted reproductive technology (ART), including in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), has emerged to provide a suitable solution of infertility cases.

Assessment of male infertility has traditionally been based on routine semen analyses, including sperm density, motility and morphology. Some other semen parameters, such as acrosomal levels and sperm DNA fragmentation index (DFI) and high DNA stainability (HDS), have also gained arising attention. However, the criteria for normality of the specific parameters used clinically offer limited prognostic value in predicting pregnancy outcome in IVF and ICSI programme. The male fertility assessment, or male fertility diagnostic capacities in a semen laboratory, fails to provide sufficient information in predicting pregnancy outcome in an ART programme. Furthermore, in clinical practice, the aetiology of ART failure has always been to female factors, regardless of 'semen quality' (Ludwig & Diedrich, 1999). Hence, in this study, we evaluated the relationship between the pregnancy outcome and several important semen parameters. The purpose of this study was to determine the most suitable diagnostic and prognostic factors in ART programme and to assist physicians in determining the best candidates for ART.

Materials and methods

Study population

This study complied with the ethics committee of human research and patients' written consent of Drum Tower Hospital. From November 2013 to June 2015, we recruited infertile couples from initial IVF and ICSI programmes who attended the Andrology Department and Reproductive Center of Nanjing Drum Tower Hospital. Only those men with a sperm concentration at least $1*10^6$/ml were included. The inclusion criteria for female partner were as follows: (i) female age <38 years; (ii) no obvious infertility factor like closed fallopian tubes; (iii) female baseline follicle-stimulating hormone (FSH) level <10 IU/l; (iv) has no uterine abnormalities, such as uterine synechia, adenomyosis.

Semen analyses

Routine semen analyses were conducted by one doctor according to the 4th edition of World Health Organization (WHO) laboratory manual for the examination and processing of human semen. Sperm parameters including concentration, progressive (PR%) motility (a + b%) and normal sperm morphology were collected for further analyses.

Semen chromatin structure assay (SCSA)

Semen chromatin structure assay was performed by one doctor using flow cytometry SCSA methods described previously (Larson *et al.*, 2000; Evenson, 2013). Briefly, the acid induced sperm nuclear DNA denaturation, and the semen samples were processed with acridine orange staining. Acridine orange binds to the fragmented sperm DNA that fluoresces red, while the double-strand DNA fluoresces green. The SCSA parameters included DFI defined as the percentage of the denatured sperm DNA that fluoresces red and high DNA stainability (HDS) defined as the percentage of spermatozoa with abnormally high DNA stainability.

Acrosomal activity

Semen acrosomal activity was evaluated using spectrophotometry method, using a commercial kit (Xindi Co.ltd, Nanjing, China), following the protocols provided by the manufacturer. Briefly, the acrosin amidase activity was determined by hydrolysis of N-alpha-benzoyl-DL-arginine-para-nitroanilide-HCl (BAPNA) to a chromogenic product which can be detected and analysed by spectrometer (Ball *et al.*, 1997; Cui *et al.*, 2000). Total acrosin activity (acrosin amidase activity) was expressed as μIU/10^6 spermatozoa.

IVF and ICSI procedures and outcome measurements

Women were downregulated with a gonadotrophin-releasing hormone (GnRH) agonist, using a long or a short protocol. Ovarian stimulation was performed with recombinant FSH, and the dose of recombinant FSH was monitored by ultrasound examination starting on day 8 of stimulation. ICSI treatment was used when sperm samples that fulfilled one of these criteria in our ART centre were as follows: (i) sperm PR% was <20%; (ii) sperm DFI >30%; (iii) surgically retrieved spermatozoa. Otherwise, IVF was used alternatively. All IVF and ICSI treatments were conducted in accordance with the standard protocols. Embryo transfer was performed on day 3 after oocyte retrieval. Clinical pregnancy was defined as ultrasound detection of foetal heartbeat 6 weeks after embryo transfer.

Statistical analyses

One-way Kolmogorov–Smirnov test was used to determine the normal distribution. Continuous variables were presented as mean ± standard deviation (SD) and compared by independent-sample *t*-test. The chi-square test or Fisher's exact chi-square test was used for categorical variables; quantitative data non-normally distributed were presented as median (interquartile range) and compared using nonparametric test. Multivariate logistic regression with likelihood ratio test was used to observe the significant predictors of pregnancy outcome. Receiver operating characteristic (ROC) was also performed to determine the cut-off value of the relative variables. The statistical analyses were performed using SPSS version 18.0, and a two-sided P value < 0.05 was considered significant.

Results

Comparison between the pregnant and not pregnant couples in IVF and ICSI procedures

A total of 1582 couples were included in the study: 1316 couples underwent IVF and 266 ICSI; 942 couples were pregnant and 374 not pregnant in IVF procedures. By contrast, 187 in 266 couples got pregnant, while 79 not in ICSI procedures. As shown in Table 1, conventional semen parameters as well as male body mass index (BMI) and HDS were not significantly different between couples who did and did not initiate a clinical pregnancy. In IVF procedures, the comparison between the male age, female age, female BMI and DFI and acrosomal activity yielded significant results. However, in ICSI group, we identified statistically significant differences between the pregnant and not pregnant group with regard to male age, female age and DFI.

Predictive values for pregnancy outcomes in IVF and ICSI procedures

Multiple logistic regression analyses for different parameters in predicting pregnancy in IVF or ICSI programme are shown in Table 2. Female age and DFI and acrosomal activity were the significant predictors of pregnancy outcome in IVF procedures (chi-square of likelihood ratio = 26.42, d.f. = 3, $P < 0.005$). By contrast, in ICSI procedures, female age and DFI posed significant factors for pregnancy outcome (chi-square of likelihood ratio = 18.88, d.f. = 2, $P < 0.005$).

Table 1 Comparison of relative values between pregnant subjects and not pregnant subjects in IVF or ICSI cycles (mean ± SD)

	IVF (n = 1316)			ICSI (n = 266)		
	Pregnant (n = 942)	Not pregnant (n = 374)	P value	Pregnant (n = 187)	Not pregnant (n = 79)	P value
Male age (year)	31.3 ± 5.0	35.3 ± 5.7	0.020	31.5 ± 5.1	34.1 ± 5.5	0.001
Male BMI (kg/m^2)	27.7 ± 3.1	24.5 ± 3.2	0.529	24.5 ± 3.3	24.5 ± 3.1	0.936
Female age (year)	30.3 ± 3.9	34.8 ± 4.7	0.001	29.3 ± 3.9	31.9 ± 5.0	0.001
Female BMI (kg/m^2)	21.1 ± 3.0	24.6 ± 3.4	0.018	22.2 ± 2.9	22.2 ± 3.0	0.946
Semen concentration (million/ml)	50.9 ± 22.5	49.9 ± 21.1	0.478	23.6 ± 21.1	24.2 ± 23.2	0.818
Normal morphology (%)	10.1 ± 4.1	9.9 ± 5.0	0.302	4.2 ± 3.5	5.1 ± 3.0	0.450
PR (%)	56.6 ± 15.2	55.3 ± 14.8	0.190	32.7 ± 22.5	31.2 ± 22.7	0.616
Acrosomal activity (μIU/million spermatozoa)	48.7 ± 20.2	42.7 ± 21.1	0.003	46.2 ± 26.8	45.9 ± 27.4	0.833
DFI (%)	14.9 ± 9.14	17.0 ± 9.7	0.001	26.3 ± 12.7	31.5 ± 18.7	0.010
HDS (%)	10.2 ± 5.0	10.3 ± 4.7	0.775	16.8 ± 9.6	15.0 ± 7.7	0.149

Table 2 Logistic regression analyses of relative values for pregnancy for IVF or ICSI procedures

	IVF					ICSI				
	β-coefficient	SE	OR	95% CI	P value	β-coefficient	SE	OR	95% CI	P value
Male age	−0.024	0.018	0.976	0.943–1.011	0.174	0.002	0.044	1.002	0.920–1.092	0.961
Male BMI	−0.012	0.020	0.988	0.951–1.027	0.545	−0.003	0.045	0.997	0.913–1.088	0.942
Female age	0.096	0.022	0.908	0.871–0.948	0.001	−1.132	0.053	0.876	0.790–0.972	0.012
Female BMI	0.037	0.020	1.038	0.998–1.079	0.060	−0.004	0.050	0.996	0.903–1.098	0.938
Semen density	0.000	0.003	1.000	0.993–1.006	0.875	−0.002	0.008	0.998	0.982–1.014	0.790
Normal morphology	0.001	0.002	0.998	0.966–1.010	0.430	−0.002	0.003	1.001	0.998–1.013	0.830
PR	0.016	0.018	0.984	0.980–1.054	0.381	−0.018	0.036	0.982	0.916–1.053	0.616
Acrosomal activity	0.017	0.007	1.017	1.004–1.031	0.010	0.002	0.003	1.002	0.997–1.007	0.347
DFI	−0.018	0.008	0.983	0.968–0.998	0.023	−0.035	0.012	0.966	0.943–0.989	0.004
HDS	−0.001	0.013	0.999	0.974–1.025	0.966	−0.023	0.018	0.977	0.943–1.012	0.202

Note: SE, standard error of β-coefficient; OR, odds ratio; 95% CI, 95% confidence interval.

Diagnostic accuracy of relative variables

To identify the clinical utility of the relative parameters identified by logistic regression analysis, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. When female age threshold value (31 year) determined by ROC analyses was used, the IVF group had 58.3% sensitivity and 76.3% PPV for predicting a pregnancy. The ICSI group had 63.3% sensitivity and 79.7% PPV for predicting a pregnancy (female age threshold value 31 year). In IVF, the comparison of pregnancy rate between high female age (≥31 years) and low age group (<31 years) was of significant difference (<31 year versus ≥31 year, 549/719 versus 393/597, $P < 0.005$). The comparison in ICSI achieved similar results (<31 year versus ≥31 year, 119/149 versus 68/117, $P < 0.005$). When DFI with a cut-off value

(11.3%) was used in the IVF group, the sensitivity and PPV for predicting a pregnancy outcome were 56.1% and 77.9% respectively. Of the 638 couples enrolled with a DFI ≥11.3%, 414 (64.9%) got a clinical pregnancy, while 528 (77.9%) couples with a DFI <11.3% became pregnant (414/638 versus 528/678, $P < 0.005$). By contrast, in ICSI group, the cut-off value was 30.3%, with a corresponding sensitivity and PPV of 50.6% and 79.3% respectively; 69 in 118 cycles and 118 in 148 couples achieved pregnancy when DFI exceeded 30.3% or less than 30.3% respectively (69/118 versus 118/148, $P < 0.005$). Additionally, in IVF group, the acrosomal activity cut-off value was 39.8 μIU/million spermatozoa, and the corresponding sensitivity and PPV were 58.2% and 75.5% respectively (Table 3).

Discussion

In the present study, we determined to set up a series of variables to differ 'pregnant' from the 'not pregnant' subjects before ART procedures using a multivariate logistic regression model. Three variables (female age, DFI and acrosomal activity) in IVF and two variables (female age and DFI) in ICSI were identified, and the performance characteristics corresponding to the identified variables were also observed. Although two maternal factors 'female age' and 'female BMI' were included in the overall analyses, strict criteria were established to include the female subjects in order to exclude the female factors and emphasise the male factors in predicting pregnancy outcome in ART programme. The identified parameters in correlation with pregnancy outcome in ART programme were discussed as follows.

Dfi

The relationship between sperm DFI and pregnancy outcome remains controversial. Some researchers have suggested that pregnancy is unlikely to occur when DFI is high (Evenson *et al.*, 1999; Larson *et al.*, 2000; Spano *et al.*, 2000; Collins *et al.*, 2008). Evenson & Wixon (2006) concluded in a meta-analysis that infertile couples were 2.0 and 1.6 times more likely to achieve pregnant with DFI values <30% in IVF and ICSI procedures respectively. However, the authors did not confirm its positive role of predicting value for ART outcomes of sperm DNA integrity evaluation in a guideline issued by the Practice Committee of the American Society for Reproductive Medicine (2013). Furthermore, the predication value of DFI for IVF or ICSI outcome is not confirmed in a meta-analysis by Zhang *et al.* (2015b). The clinical utility of sperm DNA integrity testing for evaluating male fertility potential remains questionable for further clinical use.

In the current study, 1% increase in DFI value was associated with 0.983 (95% CI, 0.968–0.998) and 0.966 (95% CI, 0.943–0.989) times the odds of pregnant compared to not pregnant subjects in IVF and ICSI, respectively, when adjusted for other covariates. We further analysed the proportional decrease in pregnant probability of 10% increase in DFI values. The corresponding odds ratio (OR) of pregnant versus not pregnant of 10% DFI increase was 0.849 (95% CI, 0.738–0.976, $P = 0.022$) and 0.707 (95% CI, 0.559–0.893, $P = 0.004$) in IVF and ICSI programme respectively.

DFI <15% is applied of clinical utility as DFI normal range and 15% ≤ DFI < 30% as moderate elevation. Several DFI cut-off values of previous studies have also been established to best predict pregnant outcome. Bungum *et al.* (2007) adopted 30% as cut-off value using SCSA as detection method, a threshold point to differ the 'fertile' and 'infertile' ones in IVF and ICSI procedures. By contrast, Payne *et al.* (2005) identified 27% as the cut-off value using SCSA by performing ROC analysis. Furthermore, 36.5% and 24.3% were also identified as the threshold value for IVF and ICSI treatments using terminal deoxynucleotidyl transferase-mediated dUDP nick-

Table 3 Performance characteristics for relative values and pregnancy

	IVF			ICSI	
	Female age	DFI	Acrosomal activity	Female age	DFI
Cut-off value	31 year	11.3%	39.8 μIU/million sperm	31 year	30.3%
Sen	58.3%	56.1%	58.2%	63.3%	50.6%
Spe	54.4%	60.0%	55.4%	61.8%	68.8%
PPV	76.3%	77.9%	75.5%	79.7%	79.3%
NPV	34.1%	35.1%	35.9%	41.6%	37.0%
Area under ROC (95% CI)	0.588 (0.553–0.623)	0.574 (0.541–0.607)	0.561 (0.484–0.639)	0.648 (0.572–0.724)	0.567 (0.487–0.647)

Note: Sen, sensitivity; Spe, specificity; PPV, positive predictive value; NPV, negative predictive value; 95% CI, 95% confidence interval.

end labelling assay (TUNEL) for sperm DNA integrity evaluation (Henkel *et al.*, 2003). In the present study, DFI cut-off values for IVF and ICSI treatments were also determined by conducting ROC analyses. High DFI was found to be correlated with low pregnancy rate. In the present study, sperm DFI thresholds significantly predicted pregnancy outcome in couples attempting pregnancy via ART. The possible explanation for the difference in cut-off values of IVF and ICSI procedures identified in this study was the criteria used for IVF and ICSI procedures in our ART centre. Semen samples with 'poor quality' were selected for ICSI treatment. The DFI % value in IVF and ICSI was 15.51 ± 9.34 and 27.87 ± 14.87 respectively.

Hds

In contrast to the strong predictive value of the DFI for pregnancy outcome, the predictive value of HDS was not confirmed in the present study. HDS, known as high DNA stainability, is a measure of an area of green fluorescence above the whole population of spermatozoa. HDS represents the immature form of spermatozoa that are not fully condensed that acridine orange can bind to (Zini *et al.*, 2009). Unlike DFI, published data on sperm HDS and reproductive success possibility are limited and this correlation remains unclear. Virro *et al.* (2004) found that an HDS value that exceeded 15% correlated with poor IVF fertilisation rate. Menezo *et al.* (2007) found that HDS value above 28% correlated with a lower pregnancy rate. Furthermore, Speyer *et al.* (2015) stated a statistically negative relationship between HDS and pregnancy loss. In the present study, however, the HDS value posed a nonsignificant parameter for the prediction of pregnancy outcome. HDS would not contraindicate the use of spermatozoa in ART procedures.

Acrosomal levels

Acrosome reaction involves in the early stages of fertilisation process, which acts as a crucial reaction of the recognition of semen and oocyte and the binding of spermatozoa to zona pellucida. The acrosomal activity in evaluating male semen 'fertilising' capacity and IVF outcome was confirmed in several clinical observations. Bartoov *et al.* (1994) reported a statistically comparable difference in acrosin level in the positive fertilisation IVF group and negative IVF group. Furthermore, the authors stated a cut-off value of acrosomal level 54 μIU/million spermatozoa to predict fertilisation success. A strong positive correlation was found between acrosomal level and IVF rates of inseminated ova in another prospective study. A cut-off value of 18 μIU/million spermatozoa was also established for IVF rates of ≥50% with sensitivity and specificity of 100% in this study (Menkveld *et al.*, 1996). The clinical value of acrosomal level evaluation has been further confirmed in several other studies (Kruger *et al.*, 1988; De Jonge *et al.*, 1993; Sharma *et al.*, 1993). These findings were partially consistent with those of the present study. The acrosomal activity could solely predict pregnancy outcome in a multivariate logistic regression model in IVF treatment (chi-square of likelihood ratio = 14.30, d.f. = 1, $P < 0.005$); 39.8 μIU/million spermatozoa was also identified as acrosomal activity with a sensitivity of 58.2% and specificity of 55.4%. By contrast, the predictive value of acrosomal level in ICSI treatment was not confirmed in the current study. A favourable explanation is that ICSI process is devoid of sperm–egg recognition and acrosomal reaction.

Bmi

Male BMI and female BMI were also studied in the current study. Schliep *et al.* concluded in a prospective cohort study that neither male BMI nor female BMI was associated with fertilisation rate, pregnancy rate or live birth in IVF (Schliep *et al.*, 2015). However, several published articles have confirmed the correlation between female BMI and ART outcomes (Machtinger *et al.*, 2012; Moragianni *et al.*, 2012; Zander-Fox *et al.*, 2012; Zhang *et al.*, 2015a). Given the limited and conflicting research on female and male age and BMI and ART outcome, further research augmented to include this area is warranted.

Routine semen parameters and male age

Routine semen parameters including semen density and normal morphology and PR% spermatozoa were analysed. The semen laboratory adopted 15% as the normal range in the 4th edition of WHO Laboratory Manual for the Examination and Processing of Human Semen. Although the clinical utility of routine semen analyses has been confirmed in limited observations (Kruger *et al.*, 1988; Menkveld *et al.*, 1996), the results of the majority of published data (Larson *et al.*, 2000; Spano *et al.*, 2000; Bungum *et al.*, 2004; Virro *et al.*, 2004; Payne *et al.*, 2005) failed to identify a positive role of routine semen parameters, which was consistent with the present study. Furthermore, although the male age in pregnant group was significantly lower than that in nonpregnant group, its predictive value was not confirmed in logistic regression model. On the other hand, there were inclusive results regarding the possible relationship between advanced paternal age and semen parameters. One parameter, known as DFI, has been proposed in male ageing process. DFI increases with advancing male age

(Moskovtsev *et al.*, 2006; Wyrobek *et al.*, 2006; Schmid *et al.*, 2007), which may constitute a potential explanation for age-related decline of male fertility. Elevated oxidative stress may also be a mechanism for advanced paternal ageing impacting male fertility potential (Pasqualotto *et al.*, 2000). Furthermore, DNA methylation, an epigenetic process, has been also implicated in age-mediated impairment of male fertility and pregnancy outcome. Houshdaran *et al.* (2007) reported DNA methylation elevated from poor-quality spermatozoa. Benchaib *et al.* (2003, 2005) also reported that sperm DNA methylation level correlated with pregnancy outcome. Alternations of epigenetic process may result in impaired spermatogenesis and male fertility.

Female age

The maternal factors, as embryos carriers in IVF and ICSI programmes, have always achieved more attention over male factors. The age of female attending ART procedures has always been set an upper limit, such as less than 40 years (Morris *et al.*, 2002; Bungum *et al.*, 2004, 2007; Niu *et al.*, 2011) or 38 years (Zander-Fox *et al.*, 2012). Kresowik *et al.* (2012) reported a higher clinical pregnancy rate and live birth in parallel with a younger female age in IVF programme. Female age itself is one of the strongest predictive factors for pregnancy chances in IVF (van Loendersloot *et al.*, 2010). There is a natural decline in female fertility potential with increasing age (Spandorfer *et al.*, 2007). The possible explanation for age-related decline in female fertility lies in the diminished ovarian reserve or the degeneration of oocytes. Diminished ovarian reserve results in poor response to gonadotrophin treatment and decreased the chance of conceiving subsequently (Ulug *et al.*, 2003). Advanced ageing is also associated with a decrease in the quantity and quality of oocytes, which limited the chance of pregnancy (Lim & Tsakok, 1997; Broekmans *et al.*, 2007). Hull *et al.* (1996) also suggested that ageing is correlated with the increase in chromosome fragmentation and oxidative stress and aneuploidy in oocytes, which could impair the quality of oocyte and the embryo development. In conclusion, our findings indicate that sperm DFI decreases the odds of pregnancy in IVF or ICSI cycles. Our findings suggest that DFI testing is especially essential for couples with low acrosomal levels in IVF or high female age in IVF or ICSI. However, further research is warranted to determine this usefulness of these tests.

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