The role of sperm aneuploidy as a predictor of the success of intracytoplasmic sperm injection?

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BACKGROUND: We present the first powered prospective study to assess whether sperm aneuploidy can predict the outcome of ICSI. METHODS: Our null hypothesis was that aneuploidy rates (AR) are identical in men who achieve successful (Group A) and unsuccessful (Group B) ICSI outcome. A power calculation yielded a sample number of 56 to achieve 80% power to reject our hypothesis at the 5% significance level. Samples for testing were obtained on the day of embryo transfer and tests were performed on raw pre-preparation samples. Sperm AR of chromosomes 13, 18, 21, X/Y were assessed using fluorescence in-situ hybridization (FISH) techniques (mean of 1223 sperm). RESULTS: There was no significant difference in any patient, seminal, cycle or laboratory characteristic between groups that may have affected outcome. Total AR (2.37 versus 1.18%, $P = 0.01$), as well as AR of chromosomes 18, X/Y and 18 + X/Y (1.48 versus 0.67%, $P = 0.005$) were significantly higher in Group B compared with Group A. Regression analysis confirmed these differences to be independent of other variables and showed a 2.6-fold change in odds of achieving a pregnancy for every 1% change in total AR. CONCLUSIONS: Our findings confirm a potential role for aneuploidy testing in the work-up of ICSI patients as a predictor of success, as well as in future genetic counselling. If confirmed, there may also be a place for a study of preimplantation genetic screening to improve ICSI success in men found to have high AR and ICSI failure.

Keywords: ART outcome; intracytoplasmic sperm injection; male infertility; sperm aneuploidy

Introduction

It has long been recognized that chromosomal abnormalities are more frequent in sperm from subfertile men than in the general population with a consistent finding in the published literature of a predominance of sex chromosomal anomalies. Reviews that pooled data from seven (De Braekeleer and Dao, 1991), six (Van Assche *et al.*, 1996) and 11 series (Johnson, 1998; $n = 9766$) of azoospermic and oligozoospermic men demonstrated rates of abnormality of 12.0, 13.7 and 4.6%, as well as an abnormality rate of 5.8% which was compared with a rate of 0.38% in a series of phenotypically normal newborn controls. Even among men with a normal karyotype, there is a considerable frequency of chromosomal abnormalities limited to the germ line is considerable as a consequence of mitotic or meiotic non-disjunction.

The first report of an association between increased sperm aneuploidy and male subfertility came in 1995 from Moosani *et al.*, who utilized both the human sperm-hamster oocyte system and FISH to demonstrate a frequency of 3.1% (chromosomes 1, 12 and X/Y) in sperm from five infertile men and of 0.84% in fertile controls. A review has identified over 30 studies that have further investigated this relationship (Tempest and Griffin, 2004) and although there is considerable variation in terms of baseline ARs, definitions of subfertility, choice of patient and control populations, number and combination of chromosomes studied, number of sperm analysed (range of 113–20 000), FISH methodology and stringency of scoring criteria, the majority confirm that men with suboptimal semen quality have a higher incidence of sperm abnormalities. Two of the studies used mathematical models to calculate total aneuploidy for the 23 chromosomes and suggested a range of between 33 and 74% for a group of oligoasthenoteratozoospermic (OAT) men compared with 4.1–7.7% for their control groups (Pang *et al.*, 1999; Pfeffer *et al.*, 1999).

As this relationship between male subfertility and the genetic characteristics of sperm is increasingly understood, the question of how this can be applied to clinical practice arises. This is of particular relevance in view of the potential effect that these abnormalities may have on ICSI offspring. The increase risk of inherited chromosomal abnormalities is

now well-documented from both studies using retrospectively matched controls (Tarlatzis and Bili, 1998; Bonduelle *et al.*, 1998) and a prospective controlled study (Aboulghar *et al.*, 2001). However, Bonduelle *et al.* demonstrated an increase not only in inherited (0.92 versus 0.47%), but also de-novo (1.66 versus 0.44%), abnormalities of which half were sex chromosomal (0.83 versus 0.19%). More recently, the same authors confirmed these findings in a larger cohort of 1586 prenatally tested ICSI fetuses and suggested a significant effect of the level of either oligozoospermia or teratozoospermia on the rate of de-novo abnormalities (Bonduelle *et al.*, 2002).

The effects of the presence of various genetic abnormalities that impact on male fertility and ICSI outcome have been studied. As long as spermatozoa are present in either the ejaculate or from sperm retrieval, the presence of Y-microdeletions (Van Golde *et al.*, 2001), or congenital absence of vas deferens as a consequence of cystic fibrosis mutations (Nicopoullos *et al.*, 2004a, b) does not impact on the outcome of assisted reproduction. Similarly, although the relationship between sperm chromatin damage and male fertility is well-documented and DNA fragmentation may impact on the chances of natural conception, intrauterine insemination (IUI) and IVF success (Bungum *et al.*, 2007), once a decision has been made to proceed with ICSI, our data (Nicopoullos *et al.*, 2007) and a meta-analysis (Evenson, 2006) suggest that DNA fragmentation has no role as a predictor of ICSI success.

In contrast, there is little data on the effect of sperm aneuploidy on ICSI outcome. This is of particular relevance in view of the key role that the injection of aneuploid sperm from euploid men may have in the development of aneuploid offspring. We therefore present the first powered prospective study to assess the effect of sperm aneuploidy on ICSI outcome and discuss future roles for such screening in clinical practice.

Materials and Methods

Power calculation

The null hypothesis was that the ARs (AR) would be identical among the successful (Group A) and unsuccessful (Group B) ICSI patients (success defined as a clinical pregnancy). This hypothesis would be rejected if the AR for the unsuccessful patients were found to be significantly higher than those for successful patients. The power of the design was the probability that the null hypothesis would be rejected, given that the alternative hypothesis would be correct. In order to calculate this probability, the details of the alternative hypothesis were first decided upon based on two previous papers who reports of data on sperm aneuploidy and ICSI outcome (Calogero *et al.*, 2001; Burrello *et al.*, 2003).

The power of the design to reject the null hypothesis was calculated to be 0.80 at the 5% level if 28 participants in each group were recruited.

Patient recruitment and preparation

Following approval (RREC 3513) by the Riverside Research Ethics Committee of Chelsea and Westminster Hospital (CWH), participants were recruited from couples undergoing treatment at the Assisted Conception Unit (ACU) once the decision was made that ICSI was required based on a full history and investigation of both partners

according to unit policy. For the purpose of this study any secondary female factors identified were classified according to the Hull and Rutherford classification of infertility (Rutherford and Jenkins, 2002).

Further preparation was in keeping with the guidelines set out by the Human Fertilisation and Embryology Authority (HFEA sixth code of practice, 2003), with couples offered the opportunity to receive counselling; a genitourinary screen on both partners was performed and treatment was not provided until account had been taken of the welfare of any child born. Men with who were either HIV, Hepatitis B/C positive or azoospermic were excluded.

Assisted reproductive techniques

The choice of stimulation protocol between a Day 1 or 21 long protocol with down-regulation using the GnRH agonist Buserelin acetate, (Suprefact®, Hoescht, Hounslow, UK) or a GnRH antagonist protocol (Cetrorelix acetate; Cetrotide®, Serono Pharmaceuticals, Middlesex, UK) and the choice of gonadotrophin (hMG; Menopur®, Ferring Pharmaceuticals, Berkshire, UK or Gonal F®, Serono Pharmaceuticals) was based on clinician preference.

The dose of gonadotrophin was individualized based on female age, FSH levels and BMI. Follicular response was assessed by transvaginal scan after 7 days of stimulation, and every two days thereafter with doses of gonadotrophin adjusted accordingly until three or more follicles had reached 18 mm. Once achieved, Ovitrelle® 6500 IU (Serono Pharmaceuticals) was administered to achieve follicular maturation and vaginal ultrasound-guided oocyte retrieval was performed 36 h thereafter.

Semen samples for ICSI were obtained by masturbation on the day of oocyte retrieval, after a period of abstinence of at least three days, and prepared using 45–90% density gradient centrifugation. The ejaculate was layered over 45% (4.5 ml PureSperm® (Nidacom International, Gothenburg, Sweden)/5.5 ml Sperm buffer (Sydney IVF Sperm Buffer: Cook®, Brisbane, Queensland, Australia) to 90% (9 ml PureSperm/1 ml Sperm buffer) density gradients warmed to room temperature and centrifuged at 1200 rpm for 20 min. The sperm pellet was aspirated, re-suspended in equilibrated culture medium (Sydney IVF Fertilisation medium: Cook®) and further centrifuged at 1000 rpm for 10 min. This procedure was repeated, the pellet was re-suspended in culture medium and the prepared samples were placed in a sperm incubator until insemination.

The oocytes were stripped of surrounding cumulus cells 30 min prior to injection using glass pipettes of decreasing diameter (170–130 μl) until the oocyte was completely cumulus-free after which it was exposed to hyaluronidase (SynVitro® Hyadase; Medicult, Jyllinge, Denmark) for a maximum of 30 s. The stripped oocytes were then transferred to fresh fertilization medium.

The ICSI procedure was performed at 41 h post-hCG injection on the heated stage of an inverted microscope at ×400 magnification (Narashige; Nikon Diaphot 200). Approximately 1 μl (dependent on final density) of the prepared sperm sample was added to a 5 μl drop of polyvinylpyrrolidone (PVP; Medicult) and a single motile, morphologically normal sperm was selected and immobilized using an injection pipette by snapping the tail region against the base of the dish. The selected sperm was aspirated tail-end first into an injection pipette and introduced into the oocyte at the 3 o'clock position and the sperm was placed in the centre of the oocyte with minimal PVP and the injection pipette was withdrawn with care. This procedure was repeated until all the oocytes were injected and the injected oocytes were transferred into fertilization medium and stored in an embryo culture incubator at 37°C.

Fertilization was assessed 16–20 h post-ICSI and the zygotes were transferred into culture dishes containing 0.5 ml of cleavage medium (Sydney IVF Cleavage medium: Cook®) and 0.3 ml of culture oil

(Sydney IVF Culture Oil: Cook®) and returned to the embryo culture incubator.

The most developmentally advanced and morphologically normal embryos were subsequently transferred under ultrasound-guidance on either Day 2 or 3 post-oocyte retrieval. The embryos were graded according to size and shape of blastomeres, and degree of fragmentation (Grade 1: evenly sized blastomeres with no fragmentation; Grade 2: evenly sized blastomeres with moderate degree of cytoplasmic fragmentation of 25%; Grade 3: uneven or indistinct blastomeres with significant fragmentation of >25%). Luteal support was administered in the form of progesterone pessaries (800 mg/day; Cyclogest®; Shire Pharmaceuticals, Basingstoke, UK) from the day of transfer and continued until the 12th week of pregnancy where appropriate. A biochemical pregnancy was detected using urinary or serum beta HCG tests taken 14 days after embryo transfer and clinical pregnancy (i.e. our definition of a successful ICSI outcome in this study) was defined as the presence of an intrauterine gestation sac at a six-week scan. Implantation rate was defined as the number of gestation sacs observed divided by the number of embryos transferred.

Sperm aneuploidy testing

Semen samples were obtained by masturbation on either the day of embryo transfer or on the day of one of the stimulation scans, following an abstinence period of at least three days. Samples were allowed to liquefy at room temperature for 30 min, analysed according to World Health Organisation criteria (WHO, 1992) and the remaining sample was frozen in cryovials by placing them directly into dry ice ($-70°C$) to await transport for FISH.

Once thawed by immersion in a 37°C water bath, the semen was prepared by adding 5 ml of 0.1% Trypsin, centrifugation for 5 min at 1000 rpm and the pellet was re-suspended in 3 ml of potassium chloride. After 20 min, 1 ml of the fixative 3:1 methanol/acetic acid was added, the sample was centrifuged as above, the supernatant was removed and a further 5 ml of fixative was added and left for 1 h at 4° before a further centrifugation and resuspension. Sperm preparations were then spread onto slides (∼60% cell density was required) and allowed to air dry.

Sperm head decondensation was performed using NaOH solution (1 mol/l) for 2 min at room temperature, followed by two 5-min washes in standard citrate solution (SSC). The sperm DNA slides were than dehydrated in an ethanol series (1 min washes in each of 70, 85 and 100%) and the slides were left to air dry.

A double- and a triple-colour FISH were carried out on each patient, using DNA probes specific for chromosome 13 (LSI 13 SpectrumGreen, 13q14) and chromosome 21 (LSI 21 SpectrumOrange, 21q22.13-q22.2) and DNA probes specific for chromosome 18 (CEP 18 SpectrumAqua, 18p11.1-q11.1), chromosome X (CEP X SpectrumGreen, Xp11.1-q11.1) and for chromosome Y (CEP Y SpectrumOrange, Yp11.1-q11.1) using the commercial kit Aneuvysion® (CEP® 18, X, Y-alpha satellite, LSI® 13 and 21, Vysis, Illinois, USA).

The probes (pre-mixed in hybridization buffer) were removed from cold storage and placed away from direct light at 37°C for 15 min, vortexed for 3 s, centrifuged at 1300 rpm for 15 s and the procedure was repeated. The sperm slides were placed at 45°C for 2 min before applying probe mixture. An amount of 3 μl of DNA probe mixture was applied to the sperm nucleus preparation, and a coverslip was applied and sealed with rubber cement once the probe mix had reached the edge. Denaturation was performed for 3 min at 73°C, and then the slides were transferred to a dark, humidified chamber at 37°C overnight (15–18 h) for hybridization.

The prepared slides were placed in 2 × SSC for 1 min to loosen the glue and coverslip, and the slides washed for 2 min in 0.4 × SSC 0.3%

NP40 solution at 73°C and then for 30 s in 2 × SSC 0.1% NP40 solution at room temperature. Thereafter, the slides were rinsed in distilled water and left to air dry in the dark. A counterstain DAPI (4, 6-diamidino-2-phenylindole), was added (20 μl) and the slides viewed by fluorescence microscopy.

The laboratory was blinded to the pregnancy outcome of the ICSI cycles from the men upon whom they were assessing sperm ARs and strict criteria were applied before a sperm head was scored or classified as abnormal; (i) only intact sperm with a similar degree of decondensation and clear hybridization signals were scored: (ii) Sperm were regarded as abnormal if they presented two (or more) distinct hybridization signals for the same chromosome, each equal in intensity and size to the single signal found in normal monosomic nuclei; the signals needed to be separated from each other by at least one signal domain and clearly positioned within the sperm head: (iii) Sperm were scored as nullisomic if no signal was demonstrated while the signal of the other chromosomes tested were positive: (iv) The absence of FISH signals in a sperm head showing regular DAPI staining was considered as failure of hybridization and this was scored separately as an estimate of hybridization efficiency.

Statistical analysis

For comparisons between continuous variables, the Shapiro–Wilk test of normality was utilized and the non-parametric Mann–Whitney *U* test was applied to test for a difference between two non-normally distributed independent samples (i.e. observations made on different subjects). Comparisons on the same subjects (i.e. comparison of individual chromosomal aneuploidy) were performed using Wilcoxon signed-ranks test. For analysis of pregnancy outcome, statistical significance was determined using Fisher's exact test.

The predictive ability of individual threshold (obtained by dividing data into inter-quartile ranges) for the chromosomes assessed was analysed using likelihood ratio analyses with values >1 suggesting an higher likelihood of achieving a clinical pregnancy. The overall predictive value of a test was compared and presented pictorially using receiver operator curve (ROC) analysis. The ROC plot shows the sensitivity on the *y*-axis against 1-specificity on the *x*-axis of the method and allows areas under the curve (AUC) to be calculated with an AUC of 1.0 if a test correlates perfectly with outcome. Normal and abnormal cases were defined as clinical pregnancy and no clinical pregnancy following ICSI. All of the above statistical analyses were performed using Analyse-It statistical software for Microsoft Excel, and a statistically significant difference was accepted when the *P*-value was ≤0.05. Binomial logistic regression analysis was used to ascertain the effect of other confounding variables using SPSS version 14.0 for Windows.

Results

Maternal characteristics

Table I outlines the characteristics of the female partners of the couples recruited in both Group 1 (i.e. successful ICSI outcome defined as a clinical pregnancy) and Group 2 (i.e. unsuccessful ICSI outcome defined as no clinical pregnancy).

There was no significant difference in maternal age ($P = 0.22$), maternal BMI ($P = 0.11$), maternal FSH levels ($P = 0.65$) or maternal parity ($P = 0.32$) between the two groups. In Group 1, 21 (75%) and 4 (14.3%) of the couples, and in Group 2, 19 (67.9%) and 7 (25.0%) of the couples were suffering from primary subfertility or had previously had a successful pregnancy outcome (live birth), respectively.

Table I. Maternal characteristics of Group 1 (successful ICSI outcome) and Group 2 (unsuccessful ICSI outcome).

	Group 1 clinical pregnancy, $n = 28$		Group 2 No. clinical pregnancy, $n = 28$	
Maternal age at oocyte collection	34.4 ± 4.4[a]	$36.0 (23–41)$[b]	36.2 ± 3.4[a]	$36.0 (27–42)$[b]
Maternal BMI (kg/m^2)	22.2 ± 2.6[a]	$21.8 (18.2–29.8)$[b]	24.7 ± 4.9[a]	$23.0 (17.9–34.8)$[b]
Maternal serum FSH (u/l)	6.2 ± 1.7[a]	$6.1 (2.0–11.0)$[b]	6.4 ± 1.6[a]	$6.4 (4.0–11.6)$[b]
Maternal parity				
Primary subfertility, %	75.0 (21/28)		67.9 (19/28)	
Secondary subfertility, %	25.0 (7/28)		22.1 (9/28)	
% with previous live birth	14.3 (4/28)		25.0 (7/28)	
% Gynae factors (Hull criteria)[c]	39.3 (11/28)		42.9 (12/28)	
Ovulatory dysfunction	4		3	
Tubal/pelvic inflammatory disease	2		3	
Endometriosis	1		5	
Uterine abnormality	2		1	
BMI <19	2		1	

[a]All values are mean $\pm$ SD; [b]all values are median (range); [c]some have greater than one factor hence totals may not sum correctly.

On detailed history, examination and fertility work-up, 11 (39.3%) and 12 (42.9%) of the 28 women in Groups 1 and 2, respectively, were found to have fertility factors as classified by Hull and Rutherford (Table I).

Paternal and seminal characteristics

Table II demonstrates the characteristics of the male partners of the couples recruited. There were no significant differences in paternal age ($P = 0.31$), smoking ($P = 0.54$) or alcohol intake ($P = 0.34$) between the two groups. There with three smokers in Group 1 (ranging 5–20 cigarettes per day) and five in Group 2 (ranging 2–10 cigarettes per day). In Group 1, 18 men (ranging 5–20 units per week), and in Group 2, 20 men (ranging from 1–28 units per week) drank alcohol.

Of the 56 men recruited, none were found to have Y-chromosome deletions and one man in each group was found to have an abnormal karyotype (balanced chromosome 13/14 Robertsonian translocation [45, XY, der (13;14) (q10;q10) in Group 1 and 47 XYY in Group 2].

Analysis of the semen samples used for aneuploidy testing demonstrated no significant difference in seminal volume ($P = 0.35$), sperm concentration ($P = 0.91$), total count ($P = 0.33$), overall motility ($P = 0.58$), progressive motility ($P = 0.41$) or abnormal morphology ($P = 0.31$)

between the groups. Overall, no single sample provided had all parameters above the WHO reference values.

Table III outlines possible aetiological factors of subfertility found in the male partners of the couples recruited. A total of 15 (53.6%) of the men recruited in Group 1 and 14 (50%) of the men recruited in Group 2 had no demonstrable cause of subfertility.

Cycle and laboratory characteristics

In Groups 1 and 2, respectively, 53.6 and 42.9% of the couples were undergoing their first cycle of assisted reproduction and only two couples in each were undergoing their third cycle or more. There was no significant difference in rank of attempt between the two groups ($P = 0.69$). The majority of the stimulation cycles in both groups used a Day 21 long protocol (21 and 25 of 28 in Groups 1 and 2, respectively) and in both groups 71.4% of the cycles (21 of 28) used Gonal F® as the gonadotrophin of choice.

Table IV outlines cycle and laboratory characteristics of the ICSI cycles undertaken. There was no significant difference in the number of days of stimulation required to achieve optimal follicular growth ($P = 0.51$), total dose of gonadotrophin required ($P = 0.58$), number of follicles available for aspiration at oocyte collection ($P = 0.93$), number of oocytes collected ($P = 0.69$) or the number injected ($P = 0.74$). The

Table II. Paternal and semen parameters of Group 1 men (successful ICSI outcome) and Group 2 men (unsuccessful ICSI outcome).

	Group 1 clinical pregnancy		Group 2 No. clinical pregnancy	
Paternal age at oocyte collection	36.9 ± 5.4[a]	$36.0 (28–56)$[b]	38.0 ± 5.2[a]	$38.0 (29–53)$[b]
Volume (ml)	3.5 ± 1.9[a]	$3.0 (0.9–8.2)$[b]	2.9 ± 1.5[a]	$2.9 (0.9–6.6)$[b]
Concentration (10^6/ml)	12.7 ± 15.4[a]	$8.6 (0.1–61.4)$b	12.8 ± 15.9[a]	$6.6 (0.4–76.0)$[b]
Total count (10^6)	37.0 ± 39.5[a]	$23.5 (0.1–150.8)$[b]	26.1 ± 25.8[a]	$16.2 (1.4–106.4)$[b]
Total motility (%)	36.9 ± 18.1[a]	$38.5 (1–69)$[b]	34.5 ± 16.6[a]	$35.0 (4–74)$[b]
Motility progession (%)				
Grade a	0.0 ± 0.0[a]	$0.0 (0–0)$[b]	0.1 ± 0.4[a]	$0.0 (0–2)$[b]
Grade b	12.1 ± 13.1[a]	$7.0 (0–50)$[b]	9.3 ± 11.1[a]	$7.0 (0–45)$[b]
Progressive motility, i.e. (Grade a + b)	12.1 ± 13.1[a]	$7.0 (0–50)$[b]	9.4 ± 11.2[a]	$7.0 (0–45)$[b]
Grade c	31.1 ± 20.5[a]	$31.0 (0–79)$[b]	36.8 ± 15.6[a]	$35.0 (4–59)$[b]
Grade d	56.9 ± 19.5[a]	$57.0 (19–99)$[b]	54.0 ± 16.5[a]	$52.0 (30–96)$[b]
Morphology (% abnormal)	90.9 ± 5.3[a]	$93.0 (77–98)$[b]	92.6 ± 3.3[a]	$94.5 (83–96)$[b]

[a]All values are mean $\pm$ SD; [b]all values are median (range).

Table III. Postulated aetiological factors of male subfertility in Group 1 (successful ICSI outcome) and Group 2 (unsuccessful ICSI outcome).

Group 1 (*n* = 28)

Idiopathic	15
Undescended testes (childhood orchidopexy)	3
Varicocoele	2
Ejaculatory dysfuncion	2
Mumps orchitis	1
Unilateral orchidectomy (Leydig cell tumour)	1
Crohns disease requiring azathioprine	1
Anti-sperm antibodies	1
Anti-sperm antibodies/epidiymo-orchitis	1
Genetic	1

Group 2 (*n* = 28)

Idiopathic	14
Hernia repair in infancy or childhood	3
Varicocoele	3
Undescended testes (childhood orchidopexy)	2
Vasectomy and reversal	2
Mumps orchitis	1
Chemotherapy	1
Workplace toxin exposure	1
Genetic	1

levels of oocyte maturity were also similar between the two groups.

Overall, the fertilization rate (FR, defined as the number of embryos/number of oocytes injected) was 69.0% in the cycles in Group 1 and 71.7% in the cycles in Group 2 (P = 0.92) and therefore a similar number of embryos were available for transfer in the two groups (P = 0.80).

All embryos were assessed on the day of transfer and in both groups there were a similar proportion of available embryos that achieved optimal cell division, i.e. $\geq$4-cell on Day 2 or $\geq$6-cell on Day 3 (36.1 and 36.9% in Groups 1 and 2, respectively) with no difference according to whether the embryos were transferred (and therefore assessed on Day 2 or 3).

Although a higher proportion of embryos transferred in Group 1 were Grade 1 (21.4 versus 10.3%) and a higher proportion of embryos transferred in Group 2 were either Grade 1 or 2 (93.1 versus 83.9%), this difference was non-significant. In each group, 21 of the 28 transfers were categorized as Grade 0 transfers (excellent).

Effect of sperm aneuploidy on ICSI outcome

FISH analysis of aneuploidy in our complete cohort of 56 subfertile men undergoing ICSI with a mean of 1223 sperm analysed per man demonstrated mean (median) rates of aneuploidy of 0.50 (0.40), 0.37 (0.20), 0.34 (0.20) and 0.71% (0.40) for chromosomes 13, 18, 21 and X/Y, respectively, and a total AR of 1.78% (1.40). The ratio of X to Y chromosomes analysed was 1.11. Aneuploidy in the sex chromosomes was significantly higher than in chromosome 18 and 21 (Wilcoxon; both P = 0.0004) and the increase in relation to chromosome 13 was of borderline significance (P = 0.08). Comparison of autosomal aneuploidy demonstrated only a significant difference between chromosome 13 and 21 (P = 0.02).

Table V demonstrates the comparison of the sperm AR between our two study groups. The X/Y ratio of the sperm analysed in the two groups (1.09 and 1.12 for Groups 1 and 2, respectively) were similar to that for the overall cohort of men.

For the primary outcome of total aneuploidy (i.e. the sum of aneuploidy found in chromosomes 13, 18, 21 and X/Y) for which the power calculation was derived, the mean AR in the failed ICSI cycles (Group 2) was found to be 2.37% (median 1.70%), which was over twice the mean total AR of 1.18% (median 1.20%) found in the successful ICSI cycles (P = 0.01).

The AR of all the individual chromosomes assessed were higher, with a significant increase in both chromosome 18 (P = 0.01) and the sex chromosomes (P = 0.05), in the samples from men who had unsuccessful ICSI cycles. When the AR of these chromosomes (18 and X/Y) are taken together as a composite figure the difference between the groups was highly significant (P = 0.005). Although the total AR within the autosomes assessed (13, 18, 21) alone was also higher in

Table IV. Cycle and laboratory characteristics of Group 1 (successful ICSI outcome) and Group 2 (unsuccessful ICSI outcome).

	Group 1 clinical pregnancy		Group 2 No. clinical pregnancy	
Days of stimulation required	13.3 ± 1.9[a]	$13.0 (11–19)$[b]	13.7 ± 2.3[a]	$13.5 (10–20)$[b]
Total dose of gonadotrophin required (IU)	2642 ± 1201[a]	$2438 (1350–7200)$[b]	3225 ± 1570[a]	$3075 (1200–7650)$[b]
No. of follicles aspirated	15.3 ± 7.6[a]	$14.5 (5–29)$[b]	15.4 ± 7.4[a]	$14.5 (5–31)$[b]
No. of oocytes collected	11.5 ± 5.8[a]	$10.5 (4–27)$[b]	11.6 ± 5.1[a]	$11.0 (4–21)$[b]
Mature, %	74.6		74.0	
Borderline, %	22.9		23.2	
Immature/post-mature	2.5		2.8	
No. of oocytes injected	10.0 ± 5.2[a]	$9.0 (2–21)$[b]	10.4 ± 5.2[a]	$10.0 (2–20)$[b]
No. of embryos	6.9 ± 3.8[a]	$6.0 (2–16)$[b]	7.5 ± 4.4[a]	$6.5 (2–16)$[b]
FR, %	69.0 (194/281)		71.7 (210/293)	
Embryo development				
% available for Day 2 transfer $\geq$4-cell	36.0		35.9	
% available for Day 3 transfer $\geq$6-cell	36.2		37.5	
Embryo transfer				
No. transferred	2.00 ± 0.27[a]	$2.0 (1–3)$[b]	2.07 ± 0.26[a]	$2.0 (2–3)$[b]
Grade 1 embryos transferred, %	21.4 (12/56)		10.3 (6/58)	
Grade 2 embryos transferred, %	62.5 (35/56)		82.8 (48/58)	
Grade 3 embryos transferred, %	16.1 (9/56)		6.9 (4/58)	

[a]All values are mean $\pm$ SD; [b]all values are median (range).

Table V. Sperm aneuploidy data of Group 1 (successful ICSI outcome) and Group 2 (unsuccessful ICSI outcome).

	Group 1 clinical pregnancy		Group 2 No. clinical pregnancy		
Chromosome 13	0.40 ± 0.21^a	$0.40 (0-0.80)^b$	0.59 ± 0.68^a	$0.20 (0-3.00)^b$	$P = 0.93$
Chromosome 18	0.19 ± 0.22^a	$0.14 (0-0.60)^b$	0.55 ± 0.60^a	$0.40 (0-1.80)^b$	$P = 0.01$
Chromosome 21	0.26 ± 0.26^a	$0.20 (0-0.80)^b$	0.41 ± 0.44^a	$0.20 (0-1.60)^b$	$P = 0.29$
X/Y Chromosomes	0.48 ± 0.33^a	$0.40 (0.12-1.40)^b$	0.93 ± 0.99^a	$0.56 (0-4.20)^b$	$P = 0.05$
Aneuploidy 18+X/Y	0.67 ± 0.40^a	$0.60 (0.14-1.80)^b$	1.48 ± 1.24^a	$0.90 (0.4-4.80)^b$	$P = 0.005$
Total autosomal aneuploidy (13+18+21)	0.87 ± 0.37^a	$1.00 (0.10-1.60)^b$	1.59 ± 1.41^a	$1.20 (0.20-5.40)^b$	$P = 0.12$
Total aneuploidy (13+18+21+X/Y)	1.18 ± 1.56^a	$1.20 (0.10-2.40)^b$	2.37 ± 1.92^a	$1.70 (0.60-7.40)^b$	$P = 0.01$

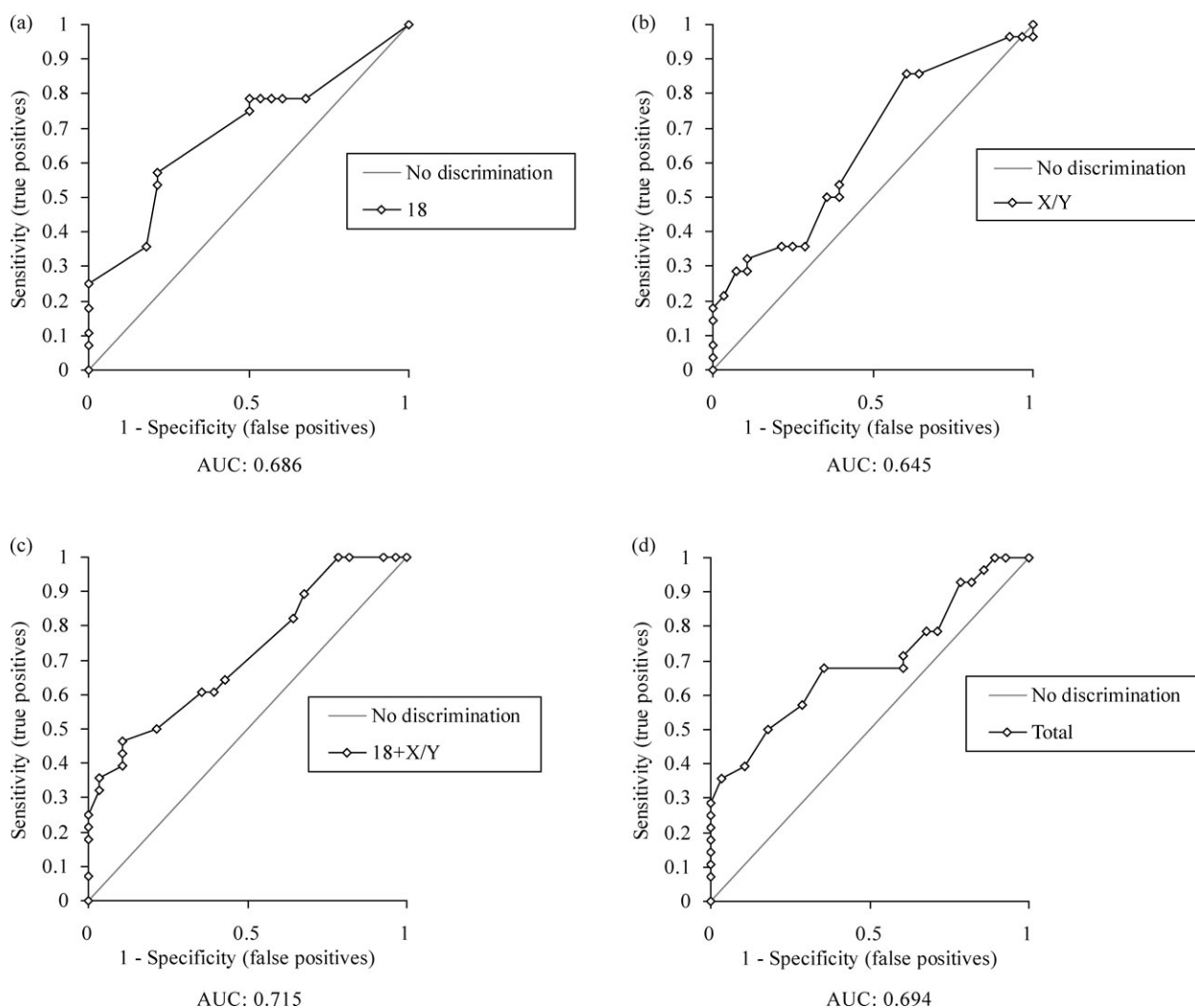[a]All values are mean percentages $\pm$ SD; [b]all values are median percentages (range).

the men with failed cycles, this difference did not achieve significance at our sample number ($P = 0.12$).

Clinical analysis

Threshold for the chromosomes for which significant differences were demonstrated were obtained by dividing the AR into interquartile ranges.

For chromosome 18, a likelihood ratio (LR) for successful pregnancy of 1.83 was demonstrated for AR of $\leq 0.20\%$, and ratios of 0.67 and 0.00 were demonstrated for AR of 0.21–0.60 and $>0.60\%$, respectively. ROC for chromosome 18 demonstrated an AUC of 0.686 (Fig. 1a).

For chromosomes X/Y, a LR of 2.50 and 1.30 for successful pregnancy was demonstrated for AR of ≤ 0.21 and $\leq 0.40\%$, respectively (0.78 for 0.21–0.40%). LR of 0.73 and 0.33

Figure 1: (**a**) ROC for chromosome 18
(**b**) ROC for chromosomes x/y. (**c**) ROC for chromosomes 18 + x/y. (**d**) ROC for total aneuploidy (13 + 18 +21 + x/y)

Table VI. Logistic regression analysis to assess the effect of confounding variables on the predictive ability of aneuploidy on ICSI outcome.

Chromosome assessed	P-value[a]	Age-adjusted P-value[b]	Odds ratio[c]	Adjusted P-value[d]
18	0.01	0.03	8.77	0.03
X/Y	0.05	0.05	3.95	0.09
18+X/Y	0.005	0.01	4.34	0.02
Total aneuploidy	0.01	0.02	2.57	0.03

[a]All values calculated with Mann–Whitney U test; [b,d]all values calculated with binomial logistic regression; [c]odds ratio refer to odds of a failed pregnancy for every 1% increase in aneuploidy.

were demonstrated for AR of >0.40 and >0.80%, respectively (1.25 for 0.41–0.80%). ROC for chromosome X/Y demonstrated an AUC of 0.645 (Fig. 1b).

For the composite of chromosomes 18 + X/Y, a LR of 3.00, 1.14, 1.14 and 0.27 was demonstrated for AR of ≤0.40, 0.41–0.64, 0.65–1.40, and >1.40%, respectively. Threshold of 0.40, 0.64 and 1.40% achieved sensitivities and specificities of 89 and 32, 61 and 61, and 36 and 96%, respectively. ROC for chromosome 18 + X/Y demonstrated an AUC of 0.715 (Fig. 1c).

For total aneuploidy (13 + 18 + 21 + X/Y), a LR of 3.00 and 1.50 for successful pregnancy was demonstrated for AR of ≤0.90 and 0.91–1.39%, respectively (2.00 for aneuploidy <1.40%). LR of 0.88 and 0.27 were demonstrated for AR of 1.40–1.90 and >1.90%, respectively. Threshold of 0.90, 1.40 and 1.90% achieved sensitivities and specificities of 79 and 32, 68 and 64, and 39 and 89%, respectively. ROC for total aneuploidy demonstrated an AUC of 0.694 (Fig. 1d). Although the AUC was highest for chromosome 18 + X/Y, this was not significantly different from that for total aneuploidy ($P > 0.05$).

Regression analysis

Table VI presents the binomial logistic regression evaluating the effect of maternal age. Following logistic regression, the difference in AR between the groups remain statistically significant (all P-values <0.05). After accounting for maternal age, logistic regression also yielded the odds of achieving or failing to achieve a pregnancy for every 1% decrease or increase in aneuploidy, respectively, for each chromosome (8.77, 3.95, 4.34 and 2.57, respectively).

The final column confirms that, following logistic regression including the variables maternal age, BMI, serum FSH, rank of attempt, maternal gynaecological factors in the history (categorized as presence or absence) and parity (classified as parous or nulliparous), the difference in AR between Groups 1 and 2 remained significant for the outcome measure for which our power calculation was produced (total aneuploidy; $P = 0.03$), as well as for chromosome 18 ($P = 0.03$), and the composite of 18 + X/Y ($P = 0.02$) and was of borderline significance for chromosomes X/Y ($P = 0.09$).

Discussion

This, to our knowledge, is the first prospective powered study assessing the effect of sperm aneuploidy on the outcome of any assisted reproductive techniques.

Our stated null hypothesis was rejected with significantly raised total aneuploidy levels (13, 18, 21 and sex chromosome; $P = 0.01$), as well as with a significant increase in ARs in chromosome 18 ($P = 0.01$), the sex chromosomes ($P = 0.05$) and the composite total of chromosomes 18/X/Y ($P = 0.005$) in sperm ejaculates from men who failed to achieve a clinical pregnancy.

Of the two studies on which a power calculation was based, one reported AR in chromosomes 8, 12, 18, X/Y in a small series of 18 unselected ICSI cycles (Calogero *et al.*, 2001) and the second used normal ranges from control men to divide 33 ICSI men into having normal and abnormal AR of similar chromosomes and assessed ICSI outcome accordingly (Burrello *et al.*, 2003). In contrast to our study, both studies analysed sperm swim-up preparations collected on the day of oocyte retrieval.

Calogero *et al.* demonstrated an increase in aneuploidy in their ICSI men compared with a group of normospermic controls and 15 of the 18 (83.3%) had total aneuploidy above the upper range of their control group (1.18%). The male partners of the seven women who achieved a clinical pregnancy showed a "slight trend towards a lower total AR which proved to be non-significant". However, the ARs in the two groups were not specified and although the raw data for the 18 men was reported, the pregnancy outcome of the individual men was not. Burrello *et al.* (2003) defined "normal" AR as the upper limit of the range of total aneuploidy demonstrated in their normospermic controls (1.55%) and demonstrated clinical pregnancy rates (CPR) of 39.1 and 81.8%, respectively, for their men defined as having "high" and "normal" ARs, respectively. These CPR are skewed in comparison to our data (CPR within our unit during the study period of 32.6% per embryo transfer) by the fact that a mean of 3.3 embryos were transferred in both groups despite median ages of 30 and 32. This Italian-based study was not regulated by the HFEA embryo transfer guidelines that regulate our practice and 57% (8/14) of the ongoing pregnancies reported were multiples. Even accounting for embryo transfer number, the CPR of 81.8% remains unusually high and may be due to the small number of patients in this group, or alternatively it may be that, in view of the relatively normal semen parameters demonstrated in this group (median volume, density, total count, total motility and normal forms of 2 ml, 20×10^6/ml, 48×10^6, 56 and 17.5%), the sparsity of information given on their history as well as the availability of sufficient sperm for both clinical and research use from a single sample, they may not be a truly reflective sample of subfertile men who genuinely require ICSI.

Other reports have indirectly linked aneuploidy with poor ICSI outcome but are limited by small numbers (Bernadini *et al.*, 1998: five ICSI-donor oocyte cycles from subfertile men with high ARs), lack of comparison groups (Pang *et al.*, 1999; Pfeffer *et al.*, 1999: poor ICSI outcome from 5 and 10 OAT men reported to have increased sperm aneuploidy) and limited information on patient characteristics, cycle characteristics and sperm parameters in view of the focus of the paper lying elsewhere (Van Dyk *et al.*, 2000: increase in diploidy in failed ICSI cycles and a correlation between sex

chromosome disomy, diploidy and FR in a study primarily focused at selection of euploid sperm) and the retrospective nature of the study (Rubio *et al*., 2001: increase in aneuploidy in recurrent miscarriage (RM) and repeated implantation failure after ICSI compared with normospermic controls). Similarly, two recent studies presented data on aneuploidy and ICSI outcome but results were presented by method of retrieval (i.e. ejaculated OAT men, MESA and TESE; Bernadini *et al*., 2000) or degree of oligozoospermia (Navgenkar *et al*., 2005) rather than clear sperm ARs.

Although retrospective, the study to most directly assess the effect of aneuploidy on outcome demonstrated increased AR in chromosomes 8, 9, 13, 18, 21, X and Y in 10 men who had failed to achieve a pregnancy after at least four attempts compared with nine men who obtained a clinical pregnancy after one to three attempts (Petit *et al*., 2005).

Interpretation of the published studies thus far is therefore limited by small numbers, methodological flaws or missing data. The diagnostic work-up required and definition of infertility are often interpreted variably between and within studies, the definition of pregnancy success remains variable between studies and assessing the effect of a any parameter on the outcome of assisted reproduction is complicated by the potential effect of other confounding factors. Our definition of subfertility, diagnostic work-up and decision that ICSI was required was consistent across all recruited couples in accordance with our unit policy and guidelines set out by the National Institute of Clinical Excellence (NICE, 2004). Our definition of pregnancy success was clearly defined at the outset as "clinical pregnancy" and was in keeping with the outcomes used in the studies from which we generated our power calculation and any potential bias was minimized by blinding the analyst scoring sperm aneuploidy from pregnancy outcome. Although we chose to recruit unselected couples undergoing a cycle under our normal protocols and unit guidelines, there was no significant difference in any other characteristic that might impact on outcome; and logistic regression analysis confirmed a 2.57-fold change in the odds of pregnancy for every 1% change in total aneuploidy and confirmed the difference in ARs demonstrated (and therefore the grounds upon which the null hypothesis was rejected) were not as a consequence of potential confounders.

Our power calculation was based on two studies that assessed three autosomes and the sex chromosomes. Approximately 50% of spontaneous abortions are associated with chromosomal abnormalities with the majority of aneuploid conceptuses thought not to reach the stage of clinical recognition (Hassold *et al*., 1996) and therefore would be classified as a failure to achieve pregnancy following assisted reproduction. Collective studies of men undergoing ICSI demonstrate a sex chromosomal abnormality rate of 0.72% (15/2084), which is 5–10 times the published population frequency (Griffin and Finch, 2005) with a consequent increase in inherited abnormalities as well as 'de-novo' abnormalities in ICSI offspring (Bonduelle *et al*., 1998). The selection of sex chromosomes alongside chromosomes 13, 18, 21 (as the commonest autosomal trisomies) allowed us to assess aneuploidy in the most clinically relevant chromosomes in terms of both potential

effect on ICSI success and assessment of long-term genetic risk and also allowed us the use of hybridization probes whose successful use has previously been established.

Our choice of raw ejaculate upon which to test our hypothesis was based firstly on the lack of consensus of the effect of sperm preparation techniques on sperm aneuploidy with reports of both a decrease (Kovanci *et al*., 2001) and no effect (Samura *et al*., 1997) following density-gradient centrifugation and both a decrease (Li and Hoshiai, 1998; Ong *et al*., 2002; Jakab *et al*., 2003) and no effect following swim-up (Samura *et al*., 1997; Pfeffer *et al*., 1999; Van Dyk *et al*., 2000). Second, the numerous reports of the association between subfertility and a rise in aneuploidy have used a variety of preparation techniques with the consistent finding of an increase in aneuploidy in subfertile men suggesting that these techniques do not select against aneuploid gametes. Third, the primary aim of our study was to assess the clinical role of sperm aneuploidy as a predictor of ICSI success and to examine a possible role for aneuploidy testing as part of the routine work-up of the subfertile man, ideally on such raw samples prior to embarking on an ICSI cycle in conjunction with routine assessment of sperm parameters. If proven to be predictive, this would be far more time and cost-effective to both patient and laboratory and be more in keeping with the role, i.e. already beginning to be adopted for DNA fragmentation as an "office" test performed on the raw sample.

The intra-individual variability in sperm ARs was not directly assessed, but we would suggest our data would be equally valid from samples in such an "office" setting taken up to 6–24 months in advance of a cycle in the absence of any significant lifestyle alterations. Although from limited numbers, this is based on Amiel *et al*. (2002) and Rubes *et al*. (2002, 2005) demonstrating no significant change in ARs over a period of six months and two years, respectively, in both normospermic and oligozoospermic men.

Two further potential limitations of our study are the number of sperm scored (1223 per sample) and the couples excluded. The number of sperm scored per patient was based on internal validity data from the laboratory from preliminary experiments that confirmed that this number ($\approx$1000) was sufficient to provide reliable information consistent with the sample as a whole. The number scored was also limited by financial constraints, as well as the labour intensity of manually scoring sperm. However, it is clear that as aneuploidy for any given chromosome occurs at a very low level, the potential for error diminishes as higher numbers of sperm are scored. The study with which we have most directly compared our findings scored an average of 1000 sperm per recruit (Petit *et al*., 2005). The introduction of validated automated counters may make the assessment of higher numbers of sperm more time and cost-effective and allow our findings to be confirmed on larger numbers.

Despite these limitations, ARs of our cohort of 56 subfertile men are similar to those found in the largest published report (Rives *et al*., 1999; $n = 50$) and our study confirms the importance of sperm aneuploidy as a predictor of the success of assisted reproduction independent of other cycle variables such as maternal age. The mechanism of effect on outcome

appears to occur post-embryo transfer with no correlation between aneuploidy in any chromosome and FR and no difference in FR, embryo development and quality at transfer between those cycles in men defined as having normal and abnormal aneuploidy sperm based on threshold established in previous studies. In contrast, and in agreement with Burrello *et al.* (38.9 and 13.3%), we demonstrated a significantly higher implantation rate of 34.7 versus 12.8% for sperm defined as having normal ploidy. This may be explained by a sperm derived influence on embryo development through the activation of the embryonic genome that occurs at day three of embryogenesis, i.e. at the 4–8 cell stage (Braude *et al.*, 1988). The activation of the paternal genome and therefore the effect of high sperm aneuploidy may only become apparent at this stage. This is supported by findings of a lower blastulation rate in ICSI compared with IVF embryos (Dumoulin *et al.*, 2000; Miller and Smith, 2001). It has been suggested that the defects in meiotic pairing and recombination observed in infertile men may lead to both meiotic arrest (azoospermia) and chromosome non-disjunction (aneuploidy; Martin *et al.*, 1996). Azoospermic men, and in particular men with non-obstructive azoospermia may well be the candidates most likely to benefit from FISH analysis of sperm in view of the high sperm ARs (Bernadini *et al.*, 2000), impaired ICSI outcome (Nicopoullos *et al.*, 2004a, b) and high incidence of aneuploid embryos derived from such men following preimplantation genetic diagnosis (PGD) (Silber *et al.*, 2003; Platteau *et al.*, 2004). This increase in aneuploid embryos in NOA is in part, we postulate, as a consequence of high sperm aneuploidy and may therefore result in less efficient implantation and lower pregnancy rates. Future research must focus on confirming our findings in azoospermic men and improving techniques to enable analysis of sufficient sperm upon which decisions can be made.

This mechanism is further supported by a higher number of abnormal and mosaic embryos in subfertile men known to have high sperm aneuploidy in comparison to a control group of fertile women undergoing PGD for sex-linked disease (Rubio *et al.*, 2005). A post-embryo transfer effect is also supported by studies that have reported an increase in sperm aneuploidy in men from couples with a history of RM of unknown cause (Rubio *et al.*, 1999; Carrell *et al.*, 2003; Al-Hassan *et al.*, 2005), with a similar mechanism impairing implantation, leading to cycle failure and early pregnancy loss.

We advocate the introduction of genetic testing of sperm (both sperm aneuploidy and DNA fragmentation) as the "fourth routine semen parameter" that should be tested in the work-up of the subfertile men alongside count, motility and morphology. A recent meta-analysis confirms that DNA Fragmentation levels impact on the chances of *in vivo* fertilization, IUI and to a lesser extent IVF success (Evenson, 2006) and may aid decision-making on the required assisted reproduction treatment modality. However, in contrast to aneuploidy, once a decision has been made to proceed with ICSI, our data suggest that DNA Fragmentation has no role as a predictor of the success of ICSI cycles (Evenson, 2006; Nicopoullos *et al.*, 2007).

A recent UK questionnaire study (Griffin *et al.*, 2003) suggested that 11% of units performed sperm aneuploidy testing routinely, although further analysis actually suggested that most positive respondents were from a single unit and the rest either used aneuploidy testing within an ongoing research study or the authors felt that a considerable number had actually misinterpreted the questionnaire and definition of sperm aneuploidy. However, over half felt there was merit in such testing. Aneuploidy testing, in association with the follow-up of both the pregnancy outcome and genetic make-up of ICSI offspring, would allow the development of a larger database from which our findings can be confirmed, the effects of individual chromosomes could be assessed, and more valid clinical threshold could be established. It would also enable us to establish risk more accurately and counsel couples on both their chances of pregnancy success and risk of abnormality. These potential benefits of routine sperm aneuploidy screening must be weighed against the potential cost and time implications, as well as the possibility that families may not benefit from such a screen as many would go ahead with ICSI regardless of the information given.

For those found to have high ARs, the options remain limited at present. At present "normal" sperm for injection at ICSI are selected on the basis of motility and morphology. Although some have demonstrated a correlation between such parameters and aneuploidy within subsets of subfertile men, there is little consistency between reports and data from our cohort demonstrates no significant correlation between any chromosome and sperm parameter. Ryu *et al.* (2001) demonstrated that 3.3% of sperm from subfertile men selected as "normal" were aneuploid for chromosomes 18, X and Y.

Burrello *et al.* (2003) demonstrated high ICSI pregnancy rates in their cohort with high ARs following transfer of multiple embryos. A logical approach in such a group, as is the case in those of an advanced maternal age where the success rate might also be lower, might be the transfer of more than the two embryos, i.e. routinely performed in the UK. However, such an option is not one that we would advocate in view of the potential obstetric and neonatal consequences of the increase in multiple pregnancy rate that would ensue, especially at a time when there is drive towards a single embryo transfer policy.

Until such time that techniques such as hyaluronic acid-sperm binding (Jakab *et al.*, 2005) to select euploid sperm are further evaluated or the postulated effect of chinese herbal medicine (Tempest *et al.*, 2003) is confirmed, the use of PGD-AS as a means of transferring genetically normal embryos appears the way forward. Such a policy is supported by the randomized trial of Staessen *et al.* (2004) that concluded that the use of PGD-AS does not improve pregnancy outcome when no restrictions are put on the number of embryos transferred, but suggested there may be place for it where limitations exist. Our advocated routine use of aneuploidy screening would also enable threshold to be established on which to base the required randomized trial of such a technique before it is introduced into routine practice.

Acknowledgements

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