Asian Journal of Andrology (2011) 13, 312–316

www.nature.com/aja

ORIGINAL ARTICLE

The number of spermatozoa collected with testicular sperm extraction is a novel predictor of intracytoplasmic sperm injection outcome in non-obstructive azoospermic patients

Giorgio Cavallini[1], Maria Cristina Magli[2], Andor Crippa[2], Silvia Resta[2], Giovanni Vitali[3], Anna Pia Ferraretti[4] and Luca Gianaroli[4]

The purpose of this study was to determine the relationships between monitors of spermatogenesis and predictors of the intracytoplasmic sperm injection (ICSI) outcome in patients with non-obstructive azoospermia (NOA) undergoing testicular sperm extraction (TESE). Seventy-nine patients with NOA (mean age: 43.6±5.2 years), each of whom yielded (97 000±3040) spermatozoa with conventional TESE, were considered in our analysis. Their partners (mean age: 35.8±5.1 years) underwent a total of 184 ICSI cycles; 632 oocytes were collected, 221 oocytes were injected, 141 oocytes were fertilized, 121 embryos were obtained, 110 embryos were transferred, 14 clinical pregnancies were achieved and only one miscarriage occurred. Multivariate regression analysis indicated relationships between the percentage of fertilized oocytes, transferred embryos and clinical pregnancies with the following variable values: female partner's age, number of spermatozoa collected, testicular volume, male partner's levels of follicle stimulating hormone (FSH), number of oocytes collected, number of oocytes injected and number of ICSI cycles. A significant inverse relationship was found between female partner's age or male partner's FSH levels and biochemical pregnancies. A significant direct relationship emerged between the number of ICSI cycles and the percentage of oocytes fertilized, embryos transferred and biochemical pregnancies, and between the number of spermatozoa collected per testicular biopsy and biochemical pregnancies. The number of spermatozoa was positively linked to the number of clinical pregnancies, independent of the number of ICSI cycles and the number of oocytes collected/injected. The number of spermatozoa collected, FSH level and testicular volume are monitors of spermatogenesis linked to ICSI success.
Asian Journal of Andrology (2011) **13,** 312–316; doi:10.1038/aja.2010.166; published online 17 January 2011

Keywords: conventional TESE; ICSI outcomes; non-obstructive azoospermia

INTRODUCTION

Azoospermia is the absence of spermatozoa in the ejaculate after two assessments of centrifuged semen;[1] its prevalence is about 1% in the general population and about 10–15% in infertile couples.[1] Azoospermia is classified as either obstructive (OA) or non-obstructive (NOA). NOA represents the clinical evidence of strongly affected spermatogenesis, whereas OA is the result of a seminal tract obstruction. Only hypogonadotrophic hypogonadism and some types of varicocele-associated NOA might yield spermatozoa in the ejaculate after treatment; however, the majority of patients are candidates for surgical sperm collection from the testicles and subsequent intracytoplasmic sperm injection (ICSI).[1,2]

The ICSI success rate with NOA spermatozoa differs from that with OA spermatozoa. The difference has been attributed to the better quality of spermatogenesis in OA than in NOA; the overall live delivery rate for couples undergoing ICSI with spermatozoa from OA is 45%, whereas it is 30% using NOA spermatozoa.[3] It may be argued that, even in the case of NOA, different degrees of erratic spermatogenesis might be linked to different ICSI offspring. A few papers have been published regarding this topic. Various authors have found the following monitors of spermatogenesis to be associated with ICSI success: sperm motility,[4] testicular histology,[5] follicle stimulating hormone (FSH) level[6] and previous testicular pathology.[6] Because each paper examined only one relationship, the following items are still unclear: (i) whether this relationship is a mathematical or a biological occurrence; (ii) whether more than one indicator of spermatogenesis is linked to ICSI offspring in a homogeneous NOA population; and (iii) the relative weight of each monitor for ICSI offspring. Zorn *et al.*[7] indicated that FSH level, the Johnsen score, sperm status and sperm motility should be considered as male-partner factors influencing the results of a first-attempt ICSI, but their paper is complicated by the inclusion of both OA and NOA. The Johnsen score is intended to be a histological method for quantifying spermatogenesis.[8,9] More recently, *ESX1* (an X-linked homeobox gene expressed in the testis) has emerged as a potentially reliable spermatogenesis molecular marker whose clinical value as a predictor of successful sperm retrieval warrants further study.[10]

[1]Andros-Italia, Outpatient Clinic of Ferrara, 44121 Ferrara, Italy; [2]Operative Unit of Laboratory of Assisted Reproduction, Società Italiana di Studi di Medicina della Riproduzione (S.I.S.ME.R.), 40138 Bologna, Italy; [3]Operative Unit of Andrology, Società Italiana di di Studi di Medicina della Riproduzione, 40138 Bologna, Italy and [4]Operative Unit of Assisted Reproduction, Società Italiana di di Studi di Medicina della Riproduzione, 40138 Bologna, Italy
Correspondence: Dr G Cavallini (giorgiocavallini@libero.it)
Received: 25 August 2010; Revised: 29 October 2010; Accepted: 3 November 2010; Published online: 17 January 2011

This retrospective study is aimed at detecting relationships between the percentage of oocytes fertilized, embryos transferred and clinical pregnancies and the following male- and female-partner variables. The male-partner variables were age, number of spermatozoa collected, testicular volume and FSH blood level. The female-partner variables were age, numbers of oocytes collected and injected and number of ICSI cycles. The number of ICSI cycles was considered a variable to be studied with the additional aim of avoiding potential biases that could be found in the analysis with multiple ICSI attempts.

MATERIALS AND METHODS

Data were retrospectively collected using Società Italiana di Studi di Medicina della Riproduzione files. All couples whose infertility was due to azoospermia were considered candidates for the study. A diagnosis of azoospermia was ascertained with at least two consecutive centrifuged sperm analyses, each performed after 3 days of sexual abstinence.[11] The Italian law on assisted reproduction[12] and Constitutional Court sentence no. 151/2009[13] strongly influenced assisted reproduction procedures and compelled us to begin data collection on 1 April 2004 and to stop on 30 April 2009. One hundred and seventy-four azoospermic patients were reviewed (mean age: 45.4±7.3 years). Exclusion criteria were smoking (three cases), female factor(s) of infertility apart from age >35 years (four cases) and female factors affecting oocyte quality (FSH >10.7 IU l^{-1} in the follicular phase[14]) (two cases). Female factors of infertility have been previously described in the literature.[15]

Assessment of male partners

The men were studied by two andrologists (GC and GV), and the patients were admitted to the study if all the following assessments were carried out: case history, sexological counselling session(s), physical examination, bilateral scrotal echo-colour Doppler scanning with testicular volume calculation (cm^3) using the three-diameter technique (length×width×height)×0.71,[16] and assessment of serum levels of FSH, luteinizing hormone, prolactin and total testosterone. Chromosomal analysis, Y microdeletion assessment and potential cystic fibrosis mutation assessment were performed.[17] Y chromosomal microdeletions were studied on the azoospermic factor (*AZF*) (*Yq11*) region using sequence-tagged site primers. The sequence-tagged site primers used were *sY81*, *sY82*, *sY84* (*AZFa*); *sY127*, *sY142*, *sY164*, *RBM1* (*AZFb*); *sY254*, *sY255*, *sY257*, *CDY*, *BPY2* (*AZFc*); and *sY145*, *sY152*, *sY153* (*AZFd*).[18] No patients withdrew from the study.

Surgical technique of testicular sperm extraction (TESE)

The (non-)obstructive aetiology of azoospermia was verified using conventional TESE, and 149 patients with NOA were identified. TESEs were performed using an open technique under optical magnification. Each testis sample was dispersed and examined in the operating room. Sequential biopsy attempts were made until sperm were visualized or until further biopsies were thought to jeopardize the testicular blood flow. If necessary, the TESEs were performed bilaterally and a maximum of three incisions were performed in each testicle: one vertical incision at the upper pole, one equatorial horizontal incision and one vertical incision at the lower pole. One sample was obtained from each incision. Surgical sections were closed with resorbable sutures.[19] The samples were placed into about 1–2 ml of sterile medium (Ham' s F10 with 10% (v/v) adult bovine serum) and were mechanically processed in a sterile environment, i.e., dispersed, minced and centrifuged at 300 *g* for at least 10 min. The specimens were assessed under an inverted microscope at ×300 for the presence of spermatozoa.[20]

Spermatozoa cryopreservation

Surgically retrieved spermatozoa were cryopreserved in multiple small aliquots, each sufficient for a single *in vitro* fertilisation attempt. This technique utilizes embryo cryopreservation straws (0.2-mm diameter) aseptically cut into 2.5-cm sections. One end was sealed with a plastic plug. A 15- to 20-μl aliquot of the sperm/test yolk buffer-glycerol cryoprotectant (Irvine Scientific, Irvine, CA, USA) mixture was carefully loaded into the ministraw using a drawn-out micropipette, and the straw was sealed with another plastic plug. Five or six ministraws were placed in conventional 1.8-ml cryovials. The cryovial containing the ministraws was held in the vapour phase for 30 min before being plunged into liquid nitrogen. For ICSI, one ministraw can be thawed at a time.[21] The spermatozoa were cryoconserved until their use.

Assessment of sperm concentration, motility and percentage of typical forms

Spermatozoa were sought and counted in supernatants using an improved double-rule Neubauer haemocytometer. Duplicate assessments of each sample were made; the difference between the two dilutions was lower than 5%. The supernatants were not diluted before counting because an extremely low number of spermatozoa was expected.[11] Sperm morphology and motility could not be considered in this study because 36 patients displayed an insufficient number of spermatozoa to assess these variables precisely.

Spermatozoa were found in 79 patients (53.0%). The adverse effects of the surgery were as follows: two haematomas (resolved with surgical drainage on day 1 after surgery) and one instance of epididymitis (diagnosed on day 8 after surgery and resolved with 14 days of therapy with ciprofloxacin 1 g per day). These patients made up the study population; their clinical and demographical characteristics are presented in Table 1.

Table 1 Clinical and demographic characteristics of the population studied.

Patient characteristics	*Values*
No. of patients	79
Bilateral testicular volume (cm^3)	18.2±7.2
FSH (normal range: 2.3–13.6 IU l^{-1})	12.3±7.6
Histology	
Hypospermatogenesis	26 cases (32.9%)
Maturation arrest	15 cases (18.9%)
Sertoli cell-only syndrome	7 cases (8.9%)*
Focal spermatogenesis	31 cases (39.3%)
Y microdeletion	6 cases (7.6%)
Male age (year)	43.6±5.2
Female age (year)	35.8±5.1
Period of time in which couples tried to achieve a pregnancy (month)	19.2±8.6
Previous testicular pathology	
Bilateral orchidopessia due to bilateral cryptorchidism	8 cases (10.1%)
Unilateral orchidopessia due to bilateral cryptorchidism	6 cases: 4 left (5.0%) and 2 right (2.5%)
Trauma	1 case (1.3%)

Abbreviation: FSH, follicle stimulating hormone.
*The histological patterns showed isolated seminiferous tubules with spermatogenetic activity observed in the field of the seminiferous tubules which were otherwise Sertoli cell-only syndrome. Data are presented as mean±s.d.

ICSI

Controlled ovarian stimulation was performed in the previously described female partners.[15] The induction of the growth of multiple follicles was achieved by administering gonadotrophins after a long desensitisation protocol with long-acting gonadotropin-releasing hormone analogues. At 34–36 h after human chorionic gonadotrophin administration (Pregnyl; Merck-Schering-Plough, Whitehouse Station, NJ, USA) (10 000 IU), the oocytes were collected transvaginally *via* ultrasound guidance.[15,22] ICSI was performed in every case. A maximum of three metaphase II oocytes were injected per patient, and all embryos generated in each ICSI cycle were transferred according to the Italian legislation on assisted reproduction, which prohibits the formation of more than three embryos[8] and prescribes the transfer of all generated embryos. Each patient received luteal phase support with progesterone in oil (Progesterone Cream; Biovea, London, UK; 50 mg per day); luteal phase support was begun 4 days after human chorionic gonadotrophin support.[15,22]

Oocytes were considered fertilized when two distinct pronuclei were present 16–18 h after sperm injection; clinical pregnancy was established by confirmation of an intrauterine sac at ultrasound or the excision of an ectopic pregnancy. The embryos were transferred 3 days after injection, i.e., at the eight-cell stage.[15]

Statistical analysis

To investigate relationships among the variables assessed, multivariate regression analysis was carried out; significance of the relationship was expressed with the regression coefficient of the dependent variables: i.e., determinig factor (t).[23] Independence of the number of spermatozoa collected and the number of ICSI cycles was assessed by calculating the marginal contribution of these variables, i.e., with analysis of variance of the partial X regressions (also called the partial test for X variables and variability share).[23] Calculations were performed using the MATLAB program (http://www.mathworks.it). Results were considered significant at $P<0.05$. The data were corrected for repeated cycles of the same donor. Multivariate analysis and marginal contribution analysis are, by definition, corrected for interdependence.[23]

Three embryologists (MCM, SR and AC), two surgeons (GC and GV) and two ICSI operators (LG and APF) were involved in the study. Individual differences in numbers of oocytes collected, oocytes injected, oocytes fertilized, embryos transferred, embryos obtained, clinical pregnancies, number of spermatozoa collected and percentage of patients who yielded spermatozoa were analysed between the different operators with the chi-square test. No significant differences emerged between the operators.

RESULTS

TESE and ICSI results

With conventional TESE, 97 000±3040 spermatozoa per patient were obtained.

A total of 184 ICSI cycles were studied (mean±s.d.=2.3±1.0 cycles/patient); 632 oocytes were collected (mean±s.d.=3.4±1.3 oocytes/cycle), 221 oocytes were injected (mean±s.d.=1.2±1.0 oocytes/cycle), 141 oocytes were fertilized (mean±s.d.=0.8±0.8 oocytes/cycle), 121 embryos were obtained, 110 embryos were transferred, 14 clinical pregnancies were achieved and one miscarriage occurred. These data are consistent with results generated from the Italian registry of assisted reproduction of the European Society of Human Reproduction and Embryology.[24] Oocytes were classified according to the morphological criteria of the cytoplasm,[25] and only fresh oocytes with normal cytoplasm were used; the other oocytes were discarded.

Results of multivariate analysis

The results of the multivariate analysis are presented in Tables 2–4.

Table 2 shows the relationships between the percentage of oocytes fertilized and age of female and male partners, number of spermatozoa collected, testicular volume, FSH blood level, number of ICSI cycles, and numbers of oocytes collected and injected. An inverse relationship ($P<0.01$) was found between female age and the percentage of fertilized oocytes. A direct relationship ($P<0.01$) was found between the percentage of fertilized oocytes and the number of ICSI cycles, oocytes collected and oocytes injected. The relationships between the number of embryos transferred and the variable values studied are presented in Table 3. Multivariate analysis detected in this table identical relationships to those in Table 2; however, no relationship was found between the percentage of oocytes fertilized and male age, number of spermatozoa, testicular volume or FSH level.

The relationships between clinical pregnancies and the variables studied are presented in Table 4. An inverse relationship ($P<0.01$) was found between clinical pregnancies and female age and FSH level. A direct ($P<0.01$) relationship emerged between clinical pregnancies and number of spermatozoa collected, testicular volume, number of ICSI cycles, and numbers of oocytes collected and injected. No relationship was found between clinical pregnancies and male age.

Results of calculating the marginal contribution of the studied variables

The results of calculating the marginal contribution of the different variables are summarized as follows:

Table 2 Relationships between the percentage of oocytes fertilized and the values of the variables studied assessed by multivariate analysis

Relationship between independent variable and dependent variables		*Regression coefficients*	*Significance of the regression coefficient of the dependent variables*	
			Determining factors (t)	P *value*
Independent variable (predictor)	Oocytes fertilized (%)	14.337	—	—
(Level of intersection on the Y axis)				
	Female age (year)	−12.122	14.200	<0.01
	Male age (year)	1.034	0.789	0.514
	No. of spermatozoa collected	2.091	0.179	0.216
Dependent variables	Testicular volume	1.114	0.098	0.215
(Level of intersection on the X axis)	FSH level (IU l^{-1})	−1.323	1.234	0.243
	No. of ICSI cycles	7.893	14.117	<0.01
	No. of oocytes collected	1.889	11.234	<0.01
	No. of oocytes injected	2.339	9.117	<0.01

Abbreviations: FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection.

Table 3 Relationships between the percentage of embryos transferred and the value of the variables studied assessed by multivariate analysis

Relationship between independent variable and dependent variables		*Regression coefficients*	*Significance of the regression coefficient of the dependent variables*	
			Determining factors (t)	P *value*
Independent variable (predictor)	Embryos transferred (%)	−4.556	—	—
(Level of intersection on the Y axis)				
	Female age (year)	−15.001	16.200	<0.01
	Male age (year)	0.989	0.614	0.434
	No. of spermatozoa collected	3.898	1.114	0.123
Dependent variables	Testicular volume	2.312	2.169	0.105
(Level of intersection on the X axis)	FSH level (IU l^{-1})	−2.559	2.134	0.137
	No. of ICSI cycles	8.109	12.117	<0.01
	No. of oocytes collected	4.112	9.114	<0.01
	No. of oocytes injected	5.880	10.545	<0.01

Abbreviations: FSH, follicle stimulating hormone; ICSI, intracytoplasmic sperm injection.

Table 4 Relationships between the percentage of clinical pregnancies and the values of the variables studied assessed by multivariate analysis

Relationship between independent variable and dependent variables		*Regression coefficients*	*Significance of the regression coefficient of the dependent variables*	
			Determining factors (t)	P *value*
Independent variable (predictor)	Clinical pregnancies (%)	8.654	—	—
(Level of intersection on the Y axis)				
	Female age (year)	−40.443	18.000	<0.01
	Male age (year)	1.656	0.545	0.543
	No. of spermatozoa collected	9.856	8.998	<0.01
Dependent variables	Testicular volume	8.966	10.002	<0.01
(Level of intersection on the X axis)	FSH level (IU l^{-1})	−21.601	8.009	<0.01
	No. of ICSI cycles	12.434	16.001	<0.01
	No. of oocytes collected	12.011	16.234	<0.01
	No. of oocytes injected	8.010	10.117	<0.01

Abbreviations: FSH, follicle stimulating hormone; ICSI, intracytoplasmic sperm injection.

1. The analysis of variance result of the partial X regressions, which analyses relationships between the number of spermatozoa collected and the number of ICSI cycles with respect to clinical pregnancies, was 2.513 (P=0.240), i.e., the number of spermatozoa collected was related to clinical pregnancy rates independently of the number of ICSI cycles.
2. The result of the partial test for X variables, which analyses relationships between the number of spermatozoa collected and the number of oocytes collected regarding clinical pregnancies, was 1.654 (P=0.436), i.e., the number of spermatozoa collected was related to clinical pregnancy rates independently of the number of oocytes collected.
3. The variability share between the numbers of spermatozoa collected and oocytes injected with respect to clinical pregnancies was 1.772 (P=0.398), i.e. the number of spermatozoa collected was related to clinical pregnancies independently of the number of oocytes injected.

DISCUSSION

These data indicate that the monitors and, consequently, the quality of spermatogenesis of NOA, are linked to ICSI pregnancy rates; even mild impairment can reduce the likelihood of pregnancy The number of spermatozoa collected, FSH level and testicular volume are, in decreasing order of strength, the factors reflecting spermatogenic activity that are linked to ICSI success. Some authors do not believe that monitors of quality of spermatogenesis are linked to the chances of fatherhood after ICSI,[26,27] but we disagree because data on ICSI results from NOA and OA were included in the same analysis[26] and the number of ICSI cycles analysed was low.[27] The low number of clinical pregnancies here was inconsistent with the requirements of variance analysis[23] and did not permit us to analyse the histological results. This study used two internal controls to assess the reliability of the methods used: female and male age. Female-partner age is the most widely accepted parameter affecting assisted reproduction outcomes; multivariate regression analysis shows its significance, whereas paternal age did not affect the ICSI results in our study and in previous studies.[4–7] Therefore, the analysis should be regarded as reliable. Multiple regression is more reliable than simple regression analysis for detecting relationships, particularly in cases such as these in which the variables studied (number of spermatozoa collected, FSH level and testicular volume) are all different expressions of affected spermatogenesis.[23]

Fewer clinical pregnancies (14 pregnancies from 184 cycles) and harvested oocytes (mean: 3.4 oocytes per patient) were obtained here than have been reported in the literature[3]. In 2004, a law regulating assisted reproduction techniques was passed in Italy. The new rules allow for the formation and transfer of a maximum of three embryos at one time, whereas embryo selection and embryo storage are prohibited. Limiting the number of treated oocytes to three per ICSI cycle significantly reduces the number of harvested oocytes and limits the chances of transferring good-quality embryos and thus achieving pregnancy in cases of severe male-factor infertility. NOA patients are particularly affected by this legal restriction.[28]

Our data show that the quality of spermatogenesis is associated with pregnancy but not with rates of embryo transfer or fertilisation. No specific study has been carried out on this topic. However, spermatozoa collected from NOA are severely aneuploid.[29] Fertilisation and two- to four-cell stage embryos might tolerate some DNA damage in the fertilizing spermatozoon by the upregulation of the DNA repair mechanism and cell cycle delay.[30] Male and female genomes are tran-

scriptionally inactive during the period that encompasses fertilisation, and mammalian embryos use maternal components and protein and transcripts inherited from the oocyte to initiate development. The embryonic genome begins to transcribe at the two-cell stage in the mouse and at the 4- to 8-cell stage in humans, after which maternal transcripts steadily degrade.[31]

The main limitation of this study is the small number of clinical pregnancies, a consequence of the Italian legal restriction. Therefore, despite the statistical significance, our conclusions should be taken with caution. In fact, a re-analysis of the data, which included only the first cycle of each couple (79 cycles, six clinical pregnancies), did not reveal any significant relationships. Furthermore, the limited number of pregnancies did not allow the calculation of reliable thresholds for testicular volume, FSH level and number of spermatozoa retrieved to enable us to predict ICSI pregnancy; however, our results indicate that caution should be exercised before offering TESE and ICSI to a couple in which a partner is >40 years old and the male partner has NOA with small (<11 cm^3 each) testicles and a high (>13.6 UI L^{-1}) serum FSH.

This paper has two strengths. The first is that it suggests a new method for monitoring spermatogenesis in cases of NOA.[32,33] The second is that it addresses the fundamental issue for the correct management of patients affected by NOA: careful evaluation of the quality of spermatogenesis for achieving an ICSI pregnancy. Microsurgical TESE is regarded as more efficient than conventional TESE for increasing sperm yield in NOA.[1,34,35] However, no study has been carried out to establish whether more successful sperm retrieval corresponds to an improved ICSI outcome. This is critical because fine-needle aspiration is regarded as less effective than conventional TESE for sperm retrieval in NOA. However, it has been suggested that spermatozoa retrieved thorough fine-needle aspiration result in higher implantation and pregnancy rates than those from TESE, owing to a lesser degree of spermatogenetic impairment in these patients.[36]

AUTHOR CONTRIBUTIONS

GC and GV wrote the paper, MCM, SR and AC collected and analysed the data, and LG and APF reviewed the manuscript, checked the data and indicated end points of the research.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

1 Donoso P, Tournaye H, Devroey P. Which is the best sperm retrieval technique for non-obstructive azoospermia? A systematic review. *Human Reprod Update* 2007; **13**: 539–49.
2 Jarvi K, Lo K, Fischer A, Grantmyre J, Zini A *et al*. CUA Guideline: the workup of azoospermic males. *Can Urol Assoc J* 2010; **4**: 163–7.
3 Lee R, Li PS, Schlegel PN, Goldstein M. Reassessing reconstruction in the management of obstructive azoospermia: reconstruction or sperm acquisition? *Urol Clin N Am* 2008; **35**: 289–301.
4 Dafopoulos K, Griesinger G, Schultze-Mosgau A, Orief Y, Schopper B *et al*. Factors affecting outcome after ICSI with spermatozoa retrieved from cryopreserved testicular tissue in non obstructive azoospermia. *Reprod Biomed Online* 2005; **10**: 455–60.
5 de Croo I, van der Elst J, Everaert K, de Sutter P, Dhont M. Fertilization, pregnancy and embryo implantation rates after ICSI in cases of obstructive and non-obstructive azoospermia. *Human Reprod* 2000; **15**: 1383–88.
6 Zitzmann M, Nordhoff V, von Schonfeld V, Nordsiek-Mengede A, Kliesch S *et al*. Elevated follicle stimulating hormone levels and the chances for azoospermic men to become fathers after retrieval of elongated spermatids from cryopreserved testicular tissue. *Fertil Steril* 2006; **86**: 339–47.
7 Zorn B, Virant-Klun I, Drobni S, Sinkovec J, Meden-Vrtovec H. Male and female factors that influence ICSI outcome in azoospermia or aspermia. *Reprod Biomed Online* 2009; **18**: 168–76.
8 Ezeh UI, Moore HD, Cooke ID. Correlation of testicular sperm extraction with morphological, biophysical and endocrine profiles in men with azoospermia due to primary gonadal failure. *Human Reprod* 1998; **13**: 3066–74.
9 Johnsen SG. Testicular biopsy score count. A method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones*1970; **1**: 2–25.
10 Bonaparte E, Moretti M, Colpi GM, Nerva F, Contalbi G *et al*. ESX1 gene expression as a robust marker of residual spermatogenesis in azoospermic men. *Hum Reprod* 2010; **25**: 1398–403.
11 World Health Organization. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press; 1999.
12 Parlamento della Repubblica Italiana. Norme in materia di procreazione medicalmente assistita. *Gazzetta Ufficiale* 2004; **45**: 5–13.
13 Corte Costituzionale della Repubblica Italiana. Giudizio di legittimità costitutuzionale in *via* incidentale. *Gazzatta Ufficiale* 2009; **109**: 6–23.
14 Nelson SM, Yates RW, Fleming R. Serum anti-Müllerian hormone and FSH: prediction of live birth and extremes of response in stimulated cycles—implications for individualization of therapy. *Hum Reprod* 2007: **22**: 2414–21.
15 Ferraretti AP, Magli MC, Kopcow L, Gianaroli L. Prognostic role of preimplantation genetic diagnosis for aneuploidy in assisted reproduction technology outcome. *Hum Reprod* 2004; **3**: 694–9.
16 Lin CC, Huang WJ, Chen KK. Measurement of testicular volume in smaller testes: how accurate is the conventional orchidometer? *J Androl* 2009; **30**: 685–9.
17 Biagiotti G, Cavallini G, Modenini F, Vitali G, Gianaroli L. Spermatogenesis and spectral echo-colour Doppler traces from the main testicular artery. *BJU Int* 2002; **90**: 903–8.
18 Balkan M, Tekes S, Gedik A. Cytogenetic and Y chromosome microdeletion screening studies in infertile males with oligozoospermia and azoospermia in Southeast Turkey. *J Assist Reprod Genet* 2008; **25**: 559–65.
19 Ostad M, Liotta D, Ye Z, Schlegel PN. Testicular sperm extraction for nonobstructive azoospermia: results of a multibiopsy approach with optimized tissue dispersion. *Urology* 1998; **52**: 692–6.
20 Baukloh V; German Society for Human Reproductive Biology. Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. *Hum Reprod* 2002; **17**: 1788–94.
21 AbdelHafez F, Bedaiwy M, El-Nashar SA, Sabanegh E, Desai N. Techniques for cryopreservation of individual or small numbers of human spermatozoa: a systematic review. *Hum Reprod Update* 2009; **15**: 153–64.
22 Hugues JN. Ovarian stimulation for assisted reproductive technologies. In: Vayena E, Rowe PJ, Griffin DP, editors. Current Practices and Controversies in Assisted Reproduction. Geneva: World Health Organization; 2002. pp102–25.
23 Camussi A, Moller F, Ottaviano E, Gorla M. Metodi statistici per la sperimentazione biologica. 2nd ed. Bologna: Zanichelli; 1995.
24 Andersen AN, Goossens V, Ferraretti AP, Bhattacharya S, Felberbaum R *et al*. Assisted reproductive technology in Europe, 2004: results generated from European registers by ESHRE. *Hum Reprod* 2008; **23**: 756–71.
25 Serhal PF, Ranieri DM, Kinis A, Marchant S, Davies M *et al*. Oocyte morphology predicts outcome of intracytoplasmic sperm injection. *Hum Reprod* 1997; **12**: 1267–70.
26 Friedler S, Raziel A, Strassburger D, Schachter M, Soffer Y *et al*. Factors influencing the outcome of ICSI in patients with obstructive and non-obstructive azoospermia: a comparative study. *Hum Reprod* 2002; **17**: 3114–21.
27 Shulman A, Feldman B, Madgar I, Levron J, Mashiach S *et al*. *In-vitro* fertilization treatment for severe male factor: the fertilization potential of immotile spermatozoa obtained by testicular extraction. *Hum Reprod* 1999; **14**: 749–52.
28 Ciriminna R, Papale ML, Artini PG, Costa M, de Santis L *et al*. Impact of Italian legislation regulating assisted reproduction techniques on ICSI outcomes in severe male factor infertility: a multicentric survey. *Hum Reprod* 2007; **22**: 2481–7.
29 Cavallini G, Crippa A, Magli MC, Cavallini N, Ferraretti AP *et al*. A study to sustain the hypothesis of the multiple genesis of oligoasthenoteratospermia in idiopathic infertile males. *Biol Reprod* 2008; **79**: 667–73.
30 Shimura T, Inohue M, Taga M, Shiraishi K, Uematsu M *et al*. p53 dependent s-phase checkpoint and pronuclear cross-talk in mouse zygotes with X irradiated sperm. *Mol Cell Biol* 2002; **22**: 2220–8.
31 Hardy K, Spanos S. Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol* 2002; **172**: 221–36
32 Foresta C, Selice R, Moretti A, Pati MA, Carraro M *et al*. Gonadotropin administration after gonadotropin releasing hormone agonist: a therapeutic option in severe testiculopathies. *Fertil Steril* 2009; **92**: 1326–32.
33 Patry G, Jarvi K, Grober ED, Lo KC. Use of the aromatase inhibitor letrozole to treat male infertility. *Fertil Steril* 2009; **92**: 829.
34 Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal with minimal tissue excision. *Hum Reprod* 1999; **14**: 131–5.
35 Tsujimura A, Miyagawa Y, Takao T, Takada S, Koga M *et al*. Savage microdissection testicular sperm extraction after failed conventional testicular sperm extraction in patients with non-obstructive azoospermia. *J Urol* 2006; **175**: 1446–9.
36 Mercan R, Urman B, Alatas C, Aksoy S, Nuhoglu A *et al*. Outcome of testicular sperm retrieval procedures in non-obstructive azoospermia: percutaneous aspiration versus open biopsy. *Human Reprod* 2000; **15**: 1548–51.