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GAMETE BIOLOGY

Sperm chromatin condensation defects, but neither DNA fragmentation nor aneuploidy, are an independent predictor of clinical pregnancy after intracytoplasmic sperm injection

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Abstract

Purpose The impact of sperm DNA damage on intracytoplasmic sperm injection (ICSI) outcomes remains controversial. The purpose of the study was to evaluate the prognostic value of several types of sperm nuclear damage on ICSI clinical pregnancy.

Methods Our retrospective study included a total of 132 couples who consulted for male or mixed-factor infertility that benefited from ICSI cycles from January 2006 to December 2015. All infertile males presented at least one conventional semen parameter alteration. Sperm nuclear damage was assessed using the Motile Sperm Organelle Morphological Examination for sperm head relative vacuolar area (RVA), aniline blue staining for chromatin condensation, terminal deoxynucleotidyl transferase dUTP nick-end labeling for DNA fragmentation, and fluorescence in situ hybridization for aneuploidy.

Results Infertile males who achieved pregnancy after ICSI had fewer chromatin condensation defects than did males who did not achieve any pregnancy (15.8 ± 12.0% vs. 11.4 ± 7.9%, respectively, $P = 0.0242$), which remained significant in multivariate regression analysis (RR = 0.40 [0.18 to 0.86], $P = 0.02$). RVA, DNA fragmentation, and aneuploidy were not predictive factors of ICSI outcomes. The pregnancy rate was significantly decreased by number of progressive motile spermatozoa with normal morphology after migration ($P = 0.04$). In female partners, 17β estradiol of less than 2000 pg/mL on the day of ovulation induction significantly reduced the occurrence of clinical pregnancy ($P = 0.04$).

Conclusion Sperm chromatin condensation defects were more frequently observed in couples with ICSI failure and should be considered a negative predictive factor for the occurrence of clinical pregnancy.

Keywords ICSI outcomes · MSOME · Sperm aneuploidy · Sperm condensation · Sperm DNA fragmentation

Introduction

Intracytoplasmic sperm injection (ICSI) has been proposed as an assisted reproductive technique (ART) in the case of male factor infertility [1]. The selection of a spermatozoon before injection is primarily based on motility and morphology, which are not indicative of sperm nuclear genome integrity [2, 3].

Several female or male factors, such as maternal age [4], body mass index (BMI) [4], ovarian reserve and response to ovarian stimulation [5, 6], endometrium thickness [7], paternal age [4], conventional sperm parameters [7], and sperm nuclear alterations [8], have been proposed as predictors of ICSI outcomes.

Published data concerning the impact of sperm nuclear damage on ART outcomes suggest that paternal nuclear damage could jeopardize fertilization and embryo quality, as well as embryo viability and pregnancy outcomes. Several reviews have summarized the literature regarding this subject [9–12], and the conclusions remain controversial. Indeed, some authors who have explored the impact of sperm nuclear quality on IVF and ICSI attempts found no correlation between the fertilization rate and sperm DNA single-strand breaks [13], DNA fragmentation [14, 15], or chromatin compaction

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abnormalities [16]. By contrast, other studies showed a significant decrease in the fertilization rate with sperm DNA single-strand breaks [17, 18] or sperm DNA fragmentation [18–22]. No correlation has been found between embryo quality and the rate of sperm DNA single-strand breaks or DNA fragmentation. However, a significant decrease in embryo quality has been reported in cases of sperm DNA single-strand breaks [17, 18], sperm DNA fragmentation [18, 23], or sperm aneuploidy [24]. In addition, a high sperm head relative vacuolar area (RVA) detected by the motile sperm organelle morphology examination (MSOME) had a negative prognostic impact on the pregnancy rate in ICSI [25–28] and a close relationship with acrosomal abnormalities [29] or sperm nuclear damage [30–32].

Based on both IVF and ICSI data, no correlation has been established between the pregnancy rate and sperm DNA single-strand breaks [33–35] or sperm DNA fragmentation [22, 33], while a significantly reduced pregnancy rate has been observed in the case of sperm DNA single-strand breaks or DNA fragmentation [15, 35]. When several sperm nuclear alterations were explored simultaneously, a significantly reduced pregnancy rate was reported in cases of sperm aneuploidy, DNA breaks, and chromatin condensation abnormalities [36]. Nonetheless, no correlation has been established between the pregnancy rate, sperm DNA fragmentation, and chromatin condensation abnormalities [37].

Controversial data have also been reported concerning the impact of sperm DNA damage on ART outcomes, according to the type of ART techniques used (IVF or ICSI) and whether these techniques were assessed separately. Taking into account ICSI attempts only, a significantly lower pregnancy rate has been observed in cases of sperm DNA fragmentation [38] or chromatin condensation abnormalities [39]. Nevertheless, some studies showed a negative correlation between sperm DNA single- or double-strand breaks and the pregnancy rate in IVF but not in ICSI [40, 41]. Alternatively, a negative correlation has been reported between sperm DNA fragmentation and the pregnancy rate in ICSI but not in IVF [21]. Furthermore, taking into account studies exploring several sperm nuclear abnormalities simultaneously, a reduced pregnancy rate has been observed in IVF in cases of sperm DNA fragmentation and chromatin condensation abnormalities but not in ICSI [42]. Therefore, the Practice Committee of the American Society for Reproductive Medicine concluded that the impact of sperm DNA fragmentation on the pregnancy rate in IVF or ICSI has insufficient evidence (level C) [43]. Many hypotheses have been proposed to explain the controversy, including variability in nuclear damage and the mechanisms responsible for this damage and the lack of homogeneity between studies concerning the different tests used to explore sperm DNA damage [44]. Indeed, each test identifies a specific type of damage with its own biological and clinical influence. Finally, most studies have included a low number of patients, which might explain their lack of power and statistical significance. No study has clearly demonstrated the predictive value of conventional sperm parameters or sperm genome integrity on ART outcomes.

To address this issue, we retrospectively examined the clinical pregnancy rate after ICSI in univariate and multivariate analyses according to several types of sperm DNA damage (sperm head RVA, chromatin condensation, DNA fragmentation, and aneuploidy) that were explored simultaneously in couples with male factor or mixed infertility.

Materials and methods

Study population

This retrospective cohort study was conducted at the Reproductive Biology Laboratory—CECOS of Rouen University Hospital from January 2006 to December 2015. The couples included in the study consulted for male or mixed-factor infertility after a 12-month trial period to achieve a spontaneous pregnancy. The clinical and biological data reported in the study were collected during routine clinical and biological procedures to explore the couple infertility. The present study was approved by the Institutional Ethical Committee for Non-Interventional Research.

For this retrospective study, infertile males presented at least one conventional semen parameter alteration [sperm concentration (10^6/ml), total sperm number (10^6/ejaculate), progressive motility (WHO grades a + b combined, %), and vitality (%) defined according to the World Health Organization criteria [45] and morphology (sperm normal morphology, %)] were selected [46]. Semen parameter alterations were confirmed using two samples collected at 3-month intervals. Their blood karyotype was normal. Data collected included age (years), body mass index (BMI) (kg/m^2), duration of infertility (months), urological history that might affect male fertility, lifestyle parameters (tobacco, alcohol, or cannabis consumption) (yes/no), and professional toxic exposure (yes/no). Sperm nuclear damage was assessed using MSOME for sperm head RVA, aniline blue (AB) staining for sperm chromatin condensation, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) for DNA fragmentation, and fluorescence in situ hybridization (FISH) for sperm numerical chromosomal abnormalities.

Conventional semen parameters and sperm nuclear exploration (aniline blue staining (AB), TUNEL, and FISH assays) were also explored in a control population comprising thirty healthy males aged between 24 and 45 years. These males had proven fertility, no history of chronic illness or toxic exposure, were candidates for sperm donation during the study period, and gave their informed consent for the research. Their semen characteristics and constitutional karyotypes were normal.

In the female partners, the following demographic data were collected: age (years), BMI (kg/m^2), gynecological history that might affect female fertility, tobacco use (yes/no), antral follicle count (AFC), basal serum follicle-stimulating hormone (FSH) (UI/L), basal serum 17β estradiol (E_2) (pg/mL), and anti-Müllerian hormone (AMH) (ng/mL).

Sperm and egg donation cycles, cycles with cryopreserved spermatozoa, and cycles with obstructive and non-obstructive azoospermia in which spermatozoa were recovered by means of surgical sperm extraction were excluded from this study.

Semen analysis and spermatozoa preparation for nuclear exploration

Semen samples were directly collected by masturbation into a sterile container (Clinisperm®, CML, Nemours, France) at the Reproductive Biology Laboratory—CECOS after sexual abstinence varying between 3 and 5 days. After liquefaction at room temperature, semen analysis was performed and interpreted according to the standard methodology proposed by the WHO guidelines [45]: sperm concentration was measured by hemocytometer on diluted and fixed samples, sperm vitality was assessed by the eosin–nigrosin procedure, and morphology was evaluated on slides stained by a Shorr method. Furthermore, normal sperm morphology (%) was explored according to David's modified classification [46]. In addition, for each sample, sperm selection was performed after discontinuous density gradient using a 2-layer gradient of 70 and 90% fractions of PureSperm® (PureSperm 100®, JCD, La Mulatière, France) diluted in IVF medium® (Origio, Limonest, France). The semen sample (1 mL) was layered on top of the gradient before centrifugation at 1400 rpm for 20 min. Then, the 90% fraction was washed with IVF medium® (Origio, Limonest, France) by centrifugation at 2000 rpm for 10 min. The final pellet was resuspended in 0.5 mL of IVF medium® (Origio) and incubated at 37 °C with 5% CO_2. Progressive motility after migration (%), the number of progressive motile spermatozoa with normal morphology after migration (×10^6), survival rate 17 h after migration (% of progressive motile spermatozoa after 17 h), and the number of progressive motile spermatozoa 17 h after migration (×10^6) were assessed.

The types of sperm nuclear damage were explored in a fraction of the whole semen sample. This fraction of whole semen (0.5 to 1 mL) was washed twice with 1 mL phosphate-buffered saline (PBS) 1×. For AB staining, a pellet was fixed with 3% glutaraldehyde (Glutaraldehyde® Sigma, St. Louis, MO, USA) before being spread on two slides (SuperFrost Plus®, Menzel Gläser, Braunschwein, Germany). For TUNEL and FISH assays, a second pellet was fixed in methanol (3v)/acetic acid (1v) before being spread onto four slides (Polylysine Slides®, Menzel Gläser, Braunschwein, Germany). In addition, 10 μL of the motile fraction of spermatozoa obtained after migration was evaluated on head morphology (MSOME) only in infertile males.

Motile sperm organelle morphology examination

Two 10 μL droplets were placed into a glass-bottomed dish (WillCo Sterile®, BioSoft International, Amsterdam, The Netherlands): a first drop of polyvinylpyrolidone (PVP) (PVP Medium®, Origio, Limonest, France) and a second drop with motile spermatozoa obtained after gradient density preparation. The drops were covered with sterile mineral oil (Ovoil®100, Vitrolife, Göteborg, Sweden). A small bridge was created between the two drops to allow motile spermatozoa to swim to the PVP drop. A total of twenty-five motile spermatozoa were randomly photographed and separately analyzed using digital imaging system software (Leica Application Suite Interactive Measurement version 3.4.0, Leica, Solms, Germany) [31]. The relative vacuole area to sperm head (RVA (%) = [vacuole area (μm^2) / head area (μm^2)] × 100) was assessed [47].

Aniline blue staining

After fixation, the slides were rinsed twice with distilled water and stained with AB at 5% pH 3.5 for 5 min (Gurr®, BDH Laboratory Supplies, England). Then, the slides were quickly rinsed twice with distilled water and dehydrated in alcohol baths (70, 90, and 100%, 1 min each), before being immersed in xylene for 3 min and mounted in Eukitt (Eukitt®, EUK 100, CML, Nemours, France). A total of 500 spermatozoa were counted under a light microscope (Leitz DMRD®, Leica, Solms, Germany). Sperm nuclei were considered positive for AB staining and to have abnormal chromatin condensation, if the dark blue staining occupied more than 50% of the head area. The percentage of spermatozoa with abnormal chromatin condensation was calculated per patient by the ratio between the number of sperm nuclei positive for AB staining and the total number of explored spermatozoa.

TUNEL assay

After fixation, the slides were rinsed once for 10 min in PBS and for 2 min in acetone (Carlo Erba Reagents, Val de Reuil, France) and twice for 10 min in PBS. TUNEL assays were performed according to the manufacturer's recommendations (In Situ Cell Death Detection Kit POD®, Roche, Mannheim, Germany). Each slide was rinsed twice with PBS for 10 min and dehydrated in alcohol baths (70, 90, and 100%, 1 min each). The negative control was obtained through the incubation of slides with the substrate without adding the enzyme. The positive control was obtained after incubation of spermatozoa with DNAse I (1 mg/mL) for 15 min in a humidified chamber at 37 °C. The slides were counterstained with

propidium iodide diluted 1:1000 in anti-fading mounting medium (Antifade®, MP-QbioGene, Illkirch, France). For each patient and control, DNA fragmentation was characterized in 500 spermatozoa at × 1000 magnification using an epifluorescence microscope (DMRB®, Leica, Solms, Germany). Sperm nuclei were considered TUNEL positive and to present DNA fragmentation, if more than 50% of the head area showed green fluorescence. The percentage of spermatozoa with DNA fragmentation was calculated per patient by the ratio between the number of sperm nuclei positive for TUNEL assay and the total number of explored spermatozoa.

FISH analysis

Three-color FISH was performed to evaluate the frequency of sperm numerical chromosomal abnormalities. A probe coupled to a red fluorophore (CEP Y Sat III Spectrum Orange™, Abbott, Rungis, France) recognized the Y chromosome, a probe coupled to a green fluorophore (CEP X Spectrum Green™, Abbott, Rungis, France) recognized the X chromosome, and chromosome 18 was blue (CEP 18 Spectrum Blue, Abbott, Rungis, France). After hybridization, the slides were counterstained with 4′,6-diamidino-2-phenylindole (Counterstain I®, Adgenix) diluted 1:5 in anti-fading mounting medium. A minimum of 5000 sperm nuclei was examined at × 1000 magnification using an epifluorescence microscope equipped with a triple bandpass filter set, DAPI/FITC/Texas Red (DMRD®, Leica, Solms, Allemagne), as previously described [47]. A sperm nucleus was considered haploid if two spots with equivalent intensity but different colors (blue and red or blue and green) were observed. A sperm nucleus with three separate spots, two green or red associated with a blue spot, was considered disomic for chromosome X or Y. A sperm nucleus with three spots of different colors (red–green–blue) was considered hyperhaploid. A sperm nucleus with four spots of different colors (two blue–red–green) was considered diploid. The rate of diploidy was calculated as the sum of rates of presumed XX, YY, and XY diploid spermatozoa. The rate of aneuploidy was calculated as the sum of rates of presumed disomic XX, YY, 1818, and hyperhaploid XY spermatozoa. The rate of total chromosome abnormalities was considered as the sum of rates of aneuploidy and diploidy.

ICSI procedure

Female partners were stimulated either by a long-acting gonadotropin-releasing hormone agonist (GnRHa) or an antagonist protocol. Once pituitary desensitization was achieved and no ovarian cyst was revealed by pelvic ultrasound examination, ovarian stimulation was started using doses of recombinant FSH (Puregon®, MSD, France; Gonal-F®, Merck Serono, France), purified hMG (Menopur® Ferring, France), or purified urinary FSH (Fostimon®, Genevrier, France) ranging between 75 and 450 IU/day depending on age, ovarian reserve, BMI, the size and number of follicles, and 17β E2 plasmatic levels. Transvaginal oocyte retrieval was scheduled 35–36 h after human chorionic gonadotropin (hCG) (Ovitrelle®, Merck Serono, France) injection. Oocytes for ICSI underwent mechanical and enzymatic decoronization (Hyadase®, Origio, France).

After discontinuous density gradient performed as mentioned previously, spermatozoa were selected for ICSI into a drop of polyvinylpyrrolidone (PVP®, Origio, France) before being immobilized by a flagellum lesion using an injection pipette (ICSI Micropipets®, Origio, France). Mature oocytes were microinjected using micromanipulators (Eppendorf, Germany) mounted on an inverted microscope (DMIRB1, Leica, France). The oocytes were cultured at 37 °C with 5% CO_2 with IVF® culture medium (Origio, France). Normal fertilization was determined 16–20 h after ICSI by the presence of two pronuclei. The fertilization rate (%) was estimated by the ratio between the number of fertilized oocytes and the number of microinjected mature oocytes. Embryo morphology on day 2 (D2) was assessed on the basis of the number of blastomeres, blastomere size, fragmentation rate, and the presence of multinucleated blastomeres, according to the classification of the European Society of Human Reproduction and Embryology (ESHRE) [48]. Embryos with four regular blastomeres and less than 20% fragmentation were defined as top-quality embryos (type A). The cleavage rate (%) was estimated by the ratio between the number of embryos on D2 and the number of fertilized oocytes with two pronuclei. The number of embryos transferred on D2 after ICSI depended on their morphology and the age and antecedent of the female partner. After transfer, remaining embryos exhibiting good morphology were cryopreserved using a controlled slow freezing protocol with propanediol as a cryoprotectant agent and a seeding step according to the supplier's instructions (Freeze-Kit CLEAVE®, Vitrolife, Sweden). The β-HCG plasmatic level was assessed 14 days after embryo transfer to diagnose pregnancy. An ongoing clinical pregnancy was defined by visualization of an intrauterine gestational sac with an embryo presenting cardiac activity on ultrasound examination performed at 8 weeks of amenorrhea. The implantation rate (%) was estimated by the number of intrauterine gestational sac per number of transferred embryos on D2.

Statistical analysis

The data were collected into an Excel file, and statistical analysis was performed using STATVIEW software (for Windows, Abacus Concepts, Inc., Copyright 1992–1996 Version 4.55) and SAS (version 9.3, Statistical Analysis System Institute, Cary, NC). Data are described using the following usual descriptive parameters: mean, standard

deviation, and median for quantitative variables and frequencies for qualitative variables. The sperm parameters are expressed per infertile male or fertile male belonging to the control group. The ICSI parameter attempts are expressed per couple. The main ICSI outcome parameter explored in our study was the occurrence of a clinical pregnancy.

First, the infertile male population was compared to the control male population using Student's *t* test. Second, the patients were divided into two groups depending on the occurrence of a clinical pregnancy and third, depending on the occurrence of spontaneous abortion. Statistical analysis comparing the quantitative variables of both groups of patients according to the occurrence of a pregnancy or a spontaneous abortion was performed using Student's *t* test and non-parametric Mann and Whitney test, respectively. Moreover, the Spearman's rank correlation coefficient (*r*) was calculated to assess the correlation between the different sperm nuclear alteration and the ICSI fertilization and cleavage rates, respectively. A chi-squared test (X^2) was also performed to show independence between male tobacco exposure and pregnancy occurrence. To determine the prognostic value of sperm nuclear variables on the occurrence of clinical pregnancy, we performed step-wise univariate and multivariate logistic regressions using an automatic variable selection procedure. For the univariate and the multivariate analyses, cut-off values were selected according to the following: (a) the WHO criteria for conventional sperm parameters [45]; (b) published data for sperm chromatin condensation [49]; (c) RVA [47]; (d) endometrial thickness [69]; (e) internal standard values obtained after validation of methods according to the ISO 15089 quality standard for medical laboratories for sperm aneuploidy, total chromosome abnormalities, and DNA fragmentation; (f) internal standard values obtained for our ART center after national evaluation performed by the Biomedicine Agency for number of ICSI cycles, 17β E2 of hCG day, number of collected oocytes, number of injected oocytes, number of quality A embryos, female BMI, and male age. Only factors that maintained a *P* value less than 0.05 were retained in the final model of the multivariate logistic regression analysis. Relative risks (RRs) and the corresponding confidence intervals (CI 95%) were calculated on the basis of the final model. In our study, a *P* value of < 0.05 was considered statistically significant.

Results

Comparison of conventional semen parameters between infertile males and fertile donors

A total of 132 couples with male factor or mixed infertility was included in the study. Table 1 shows the comparative data between the 132 infertile males and the 30 fertile donors. Age did not differ significantly between the two groups. Compared with the control males, infertile males had a significantly lower sperm concentration ($P < 0.0001$), total sperm number ($P = 0.0001$), progressive motility ($P = 0.0001$), and normal sperm morphology ($P < 0.0001$). Compared with the fertile donors, the patients had a significantly higher rate of sperm chromatin condensation defects ($P = 0.0002$) and sperm DNA fragmentation ($P = 0.0007$). We did not observe any significant difference between the two groups regarding the rate of sperm numerical chromosomal abnormalities.

Table 1 Comparison of conventional semen parameters and sperm nuclear alterations in infertile males and control group. The values are expressed as the means ± standard deviations. A *P* value of < 0.05 is considered significant

Characteristics	Infertile males ($n = 132$) mean ± sd	Control group ($n = 30$) mean ± sd	*P*
Age (years)	33.7 ± 4.43	34.8 ± 6.14	0.3304
Volume (mL)	3.9 ± 1.86	3.5 ± 1.64	0.2678
Concentration ($\times 10^6$/mL)	18.1 ± 20.12	55.6 ± 22.38	< 0.0001
Total sperm number ($\times 10^6$/ejaculate)	64 ± 75.33	186.4 ± 122.37	0.0001
Sperm progressive motility (a + b, %)	26 ± 10.58	40.2 ± 6.63	0.0001
Normal sperm morphology (%)	21.9 ± 14.35	38.7 ± 14.21	< 0.0001
Abnormal chromatin condensation rate (%)	14 ± 10.73	6.5 ± 4.56	0.0002
DNA fragmentation rate (%)	9.1 ± 7.35	4.3 ± 3.87	0.0007
Aneuploidy rate (%)	0.55 ± 0.43	0.47 ± 0.22	0.3429
Diploidy rate (%)	0.35 ± 0.62	0.27 ± 0.23	0.5162
Total chromosome abnormality rate (%)	0.9 ± 0.99	0.74 ± 0.39	0.4087

Aneuploidy rate, sum of rates of presumed disomic XX, YY, 1818, and hyperhaploid XY spermatozoa

Diploidy rate, sum of rates of presumed XX, YY, and XY diploid spermatozoa

n, population size; sd, standard deviation

Total chromosome abnormality rate, sum of rates of aneuploidy and diploidy

Characteristics of infertile couples

Among the 132 infertile couples, 104 (78.8%) consulted for primary infertility and 28 (21.2%) consulted for secondary infertility. Two thirds of the couples had mixed infertility, and one third had male factor infertility only. The general characteristics of the male and female populations are shown in Figs. 1 and 2 and Tables 1 and 2. A total of 48 (37%) males had a urological history, mainly comprising unilateral or bilateral cryptorchidism in 18 (14%) males (Fig. 1). Exposure to one or more lifestyle or professional toxic factors was observed in 72 (55%) males, with tobacco consumption alone in 58 out of 72 (80%) males. The main conventional semen parameter alterations observed were astheno-teratozoospermia in 31 (24%) males and oligo-astheno-teratozoospermia (OAT) in 53 (40%) males. Figure 2 shows the distribution of gynecological history in the female population that might affect female fertility. Eighty-five females (65%) had one or more issues in gynecological history that mainly comprised dysovulation in 25 (19%) and proven polycystic ovary syndrome (PCOS) in 18 (14%). Tobacco consumption was observed in 48 (36%) females.

Table 2 presents the clinical and biological characteristics of the couples according to the occurrence of a clinical pregnancy. Males who achieved pregnancy after ICSI were younger and had fewer chromatin condensation defects than did men who did not achieve any pregnancy ($P = 0.0475$ and $P = 0.0242$, respectively). However, no significant difference was observed in the sperm head RVA ($P = 0.1552$), DNA fragmentation ($P = 0.1643$), or numerical chromosome abnormalities ($P = 0.2724$). In addition, the clinical and biological characteristics of the female population did not differ significantly between the "positive clinical pregnancy" group and the "negative clinical pregnancy" group.

The clinical and biological parameters, including sperm nuclear abnormalities of the infertile male population, did not differ significantly between the "positive miscarriage" group and the "negative miscarriage" group. In addition, the clinical and biological characteristics of the female population did not differ significantly between the "positive miscarriage" group and the "negative miscarriage" group except for the number of ICSI cycles that were significantly higher in the "positive miscarriage" group compared to the "negative miscarriage" group (2.7 ± 0.91 vs. 1.9 ± 0.90, respectively, $P = 0.002$).

ICSI attempts

Table 2 also presents the ICSI attempt parameters according to the occurrence of a clinical pregnancy. A total of 266 ICSI cycles were performed in 132 couples, resulting in 216 cycles of fresh embryo transfers. A total of 52 clinical pregnancies were obtained after fresh embryo transfers. The clinical pregnancy rate was 19.55% (52/266) per cycle, 24.07% (52/216) per fresh embryo transfer, and 39.40% (52/132) per couple.

The cleavage rate ($P = 0.0024$) and the number of type A embryos ($P = 0.0274$) were significantly higher in couples who obtained a clinical pregnancy than in couples who did not obtain any pregnancy. The implantation rate per fresh embryo transfer was 17.1%. Eighteen women (13.6%) who started a clinical pregnancy had spontaneous abortion during their ICSI attempt. The spontaneous abortion rate was 34.62% (18/52).

Predictive factors of clinical pregnancy

Step-wise logistic regression analysis is shown in Table 3. Univariate analysis identified eight male or female variables associated with the occurrence of a clinical pregnancy.

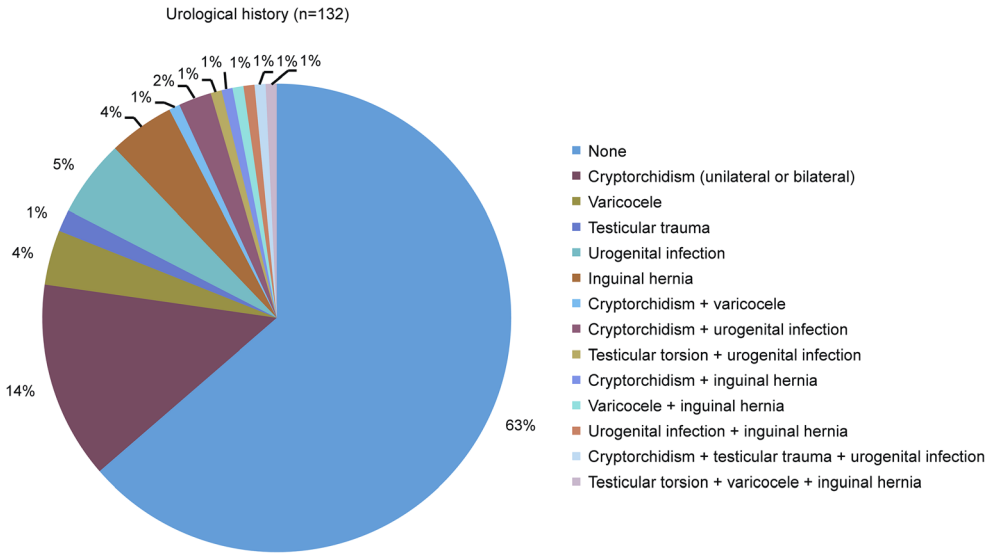

Fig. 1 Distribution of urological history in the infertile male population. *n*, population size

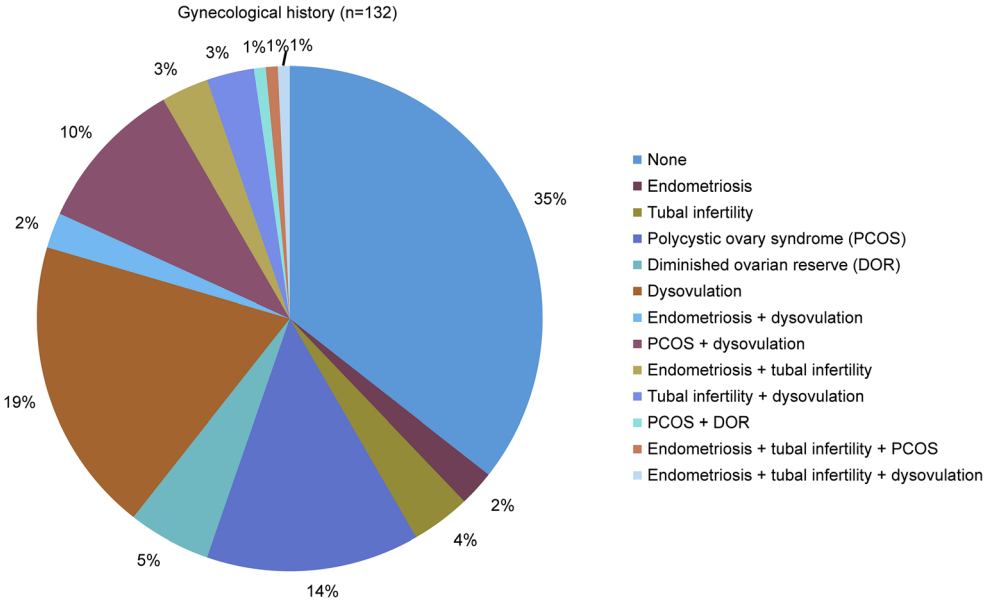

Fig. 2 Distribution of gynecological history in the infertile female population. DOR, diminished ovarian reserve; *n*, population size; PCOS, polycystic ovary syndrome

For the male variables, chromatin condensation defects concerning more than 20% of spermatozoa reduced significantly the occurrence of clinical pregnancy after ICSI (RR = 0.32 [0.13 to 0.81], $P = 0.02$). The same observation was considered for male age more than 30 years (RR = 0.40 [0.18 to 0.86], $P = 0.02$), total sperm number per ejaculate less than 39×10^6/ejaculate (RR = 0.43 [0.21 to 0.88], $P = 0.02$), number of progressive motile spermatozoa with normal morphology less than 2×10^6 after migration (RR = 0.43 [0.21 to 0.87], $P = 0.02$), and number of progressive motile spermatozoa less than 1×10^6 17 h after migration (RR = 0.48 [0.23 to 0.99], $P = 0.04$). Tobacco exposure in our male population had no negative impact on the clinical pregnancy rate (43% (25/58) in smoking males vs. 36.7% (27/74) in non-smoking males, $P = 0.62$).

For the female partner variables, a cut-off E_2 level and endometrial thickness below which a decreased clinical pregnancy rate could be expected was estimated to be less than 2000 pg/mL (RR = 0.34 [0.16 to 0.70], $P = 0.004$) and less than 10 mm (RR = 0.38 [0.19 to 0.79], $P = 0.01$) on the day of ovulation induction, respectively. In addition, the number of type A embryos (RR = 0.37 [0.16 to 0.83], $P = 0.01$) had also a negative impact on the clinical pregnancy rate.

In addition, a multivariate analysis was performed to determine independent predictors in the occurrence of clinical pregnancy with the set of eight parameters that met the significance criterion following the univariate analysis: chromatin condensation defects, male age, total sperm number per ejaculate, number of progressive motile spermatozoa with normal morphology less than 2×10^6 after migration, number of progressive motile spermatozoa 17 h after migration, E_2 level and endometrial thickness on the day of ovulation induction, and the number of type A embryos. Finally, three biological and clinical variables have a significant detrimental impact on clinical pregnancy rate: chromatin condensation defect (RR = 0.30 [0.11 to 0.83], $P = 0.02$), number of progressive motile spermatozoa with normal morphology after migration (RR = 0.43 [0.20 to 0.95], $P = 0.04$), and E_2 level on the day of ovulation induction in female partner (RR = 0.42 [0.18 to 0.96], $P = 0.04$) significantly decreased the pregnancy rate. Therefore, these three parameters can be considered negative predictors of clinical pregnancy occurrence in cases of ICSI.

Correlation between sperm nuclear alteration parameters with ICSI fertilization and cleavage rates

Additionally, we performed a Spearman's correlation test and we analyzed the fertilization and the cleavage rates according to the different types of sperm nuclear alterations. No statistically significant correlation was found between the rates of abnormal sperm chromatin condensation, aneuploidy, diploidy, total chromosome abnormalities, and the fertilization rate. However the fertilization rate decreased significantly when the rate of sperm DNA fragmentation ($r = -0.196$, $P = 0.0325$) or the RVA ($r = -0.206$, $P = 0.0242$) increased. In addition, the different rates of sperm nuclear alterations were not correlated with the embryo cleavage rate.

Discussion

To the best of our knowledge, no prior studies have used multivariate analysis to explore the impact of four different types of sperm nuclear damage on clinical pregnancy after ICSI associated with other conventional semen parameters, in addition to male and female clinical characteristics. Our study specifically

Table 2 Comparison of clinical and biological characteristics of infertile couples and ICSI attempt parameters according to the occurrence of a clinical pregnancy. The values are expressed as the means ± standard deviations. A *P* value of < 0.05 is considered significant

Characteristics mean ± sd	Couples (*n* = 132)	Clinical pregnancy		*P*
		Positive (*n* = 52)	Negative (*n* = 80)	
Male characteristics				
Age (years)	33.7 ± 5.43	32.6 ± 4.71	34.5 ± 5.78	0.0475
BMI (kg/m^2)	25.5 ± 8.71	26.4 ± 12.53	24.09 ± 4.05	0.4329
Duration of infertility (months)	42.8 ± 27.03	41.6 ± 26.63	43.3 ± 27.54	0.7369
Volume (mL)	3.9 ± 1.86	4 ± 1.81	3.8 ± 1.91	0.6481
Concentration (×10^6/mL)	18.1 ± 20.12	19.9 ± 16.48	17.1 ± 22.26	0.4353
Total sperm number (×10^6/ejaculate)	64 ± 75.33	78.8 ± 84.50	55 ± 67.75	0.0774
Vitality (live spermatozoa, %)	70.9 ± 11.04	72.5 ± 10.52	69.8 ± 11.32	0.1885
Sperm progressive motility (a + b, %)	26 ± 10.58	27.5 ± 10.73	25.2 ± 10.45	0.2331
Normal sperm morphology (%)	21.9 ± 14.35	24.3 ± 15.91	20.6 ± 13.03	0.1473
Multiple anomaly index	1.7 ± 0.28	1.67 ± 0.24	1.70 ± 0.31	0.5188
Progressive motility after migration (%)	52.8 ± 21.9	54.5 ± 21.1	21.8 ± 22.6	0.4953
Number of progressive motile spermatozoa with normal morphology after migration (×10^6)	5.1 ± 8.72	6.9 ± 10.36	4.1 ± 7.36	0.0788
Survival rate 17 h after migration (%)	23.7 ± 19.6	25.9 ± 20.6	22.6 ± 18.8	0.3508
Number of progressive motile spermatozoa 17 h after migration (×10^6)	3 ± 6.73	4.1 ± 7.84	2.4 ± 5.91	0.1752
Sperm head relative vacuolar area (RVA) (%)	7.8 ± 2.6	7.4 ± 2.6	8 ± 2.6	0.1552
Abnormal chromatin condensation (%)	14 ± 10.7	11.4 ± 7.9	15.8 ± 12.0	0.0242
DNA fragmentation (%)	9.1 ± 7.4	10.2 ± 8.9	8.4 ± 6.2	0.1643
Aneuploidy rate (%)	0.55 ± 0.43	0.48 ± 0.23	0.59 ± 0.52	0.1481
Diploidy rate (%)	0.35 ± 0.62	0.30 ± 0.19	0.39 ± 0.79	0.4498
Total chromosome abnormality rate (%)	0.90 ± 0.99	0.78 ± 0.36	0.98 ± 1.25	0.2724
Female characteristics				
Age (years)	31 ± 4.67	31 ± 4.85	31.1 ± 4.60	0.9707
BMI (kg/m^2)	24.1 ± 4.23	23.6 ± 4.24	24.5 ± 4.25	0.2665
Basal serum FSH (UI/L)	6.7 ± 1.88	6.7 ± 1.87	6.8 ± 1.91	0.7181
Basal serum 17β-E2 (pg/mL)	43.6 ± 19.88	42.1 ± 17.85	44.9 ± 21.21	0.4379
AMH (pg/mL)	4.3 ± 3.41	4.1 ± 2.17	4.4 ± 4.13	0.6496
AFC	14.8 ± 8.10	14.6 ± 8.76	15 ± 7.67	0.8592
ICSI attempt parameters				
Number of ICSI cycles/couple	2 ± 0.93	1.8 ± 0.80	2.1 ± 1	0.0782
Duration of stimulation (days)	10.5 ± 2.10	10.6 ± 1.49	10.4 ± 2.48	0.5401
Total FSH administered doses (IU)	1861 ± 790	1822 ± 691	1890 ± 873	0.6529
Number of follicles > 15 mm of hCG day	7.5 ± 3.87	7.7 ± 3.31	7.2 ± 4.26	0.5331

Table 2 (continued)

Characteristics mean ± sd	Couples ($n = 132$)	Clinical pregnancy		P
		Positive ($n = 52$)	Negative ($n = 80$)	
Endometrial thickness of hCG day (mm)	10.7 ± 3.23	11.3 ± 2.63	10.1 ± 3.62	0.0555
17β E_2 of hCG day (pg/mL)	1936 ± 798	2088 ± 692	1822 ± 865	0.0791
Number of collected oocytes	11 ± 5.52	11.7 ± 6.02	10.5 ± 5.17	0.2306
Number of injected oocytes	7.8 ± 4.21	8.3 ± 4.49	7.4 ± 4	0.2171
Fertilization rate (%)	51.2 ± 21.7	49.7 ± 20.1	52.5 ± 23.0	0.493
Cleavage rate (%)	79.9 ± 31.5	89.9 ± 17.6	73 ± 36.7	0.0024
Number of embryos	4 ± 3	4.2 ± 2.99	3.9 ± 3.03	0.5119
Number of top-quality embryos (type A)	1.1 ± 1.82	1.5 ± 1.83	0.8 ± 1.77	0.0274
Number of transferred embryos per cycle	2.3 ± 1.98	2.5 ± 1.84	2.1 ± 2.07	0.3097
Number of frozen embryos per cycle	0.8 ± 1.91	0.9 ± 1.75	0.8 ± 2.02	0.6179

AFC, antral follicle count

AMH, anti-Müllerian hormone

Aneuploidy rate, sum of rates of presumed disomic XX, YY, 1818, and hyperhaploid XY spermatozoa

BMI, body mass index

Cleavage rate, ratio between the number of embryos on D2 with the number of fertilized oocytes with two pronuclei

Diploidy rate, sum of rates of presumed XX, YY and XY diploid spermatozoa

E_2, 17β-estradiol

Fertilization rate, ratio between the number of fertilized oocytes with the number of microinjected mature oocytes

FSH, follicle-stimulating hormone

Implantation rate, ratio between the number of intrauterine gestational sac with the number of transferred embryos on day 2

n, sample size

sd, standard deviation

Total chromosome abnormality rate, sum of rates of aneuploidy and diploidy

Table 3 Univariate and multivariate analysis exploring the association between female or male clinical or biological parameters and clinical pregnancy. A *P* value of < 0.05 is considered significant

Variables	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
17β E_2 of hCG day				
> 2000 pg/mL	1		1	
≤ 2000 pg/mL	0.34 (0.16–0.70)	0.004	0.42 (0.18–0.96)	0.04
Endometrial thickness				
> 10 mm	1		1	
≤ 10 mm	0.38 (0.19–0.79)	0.01	0.48 (0.21–1.06)	0.07
Number of quality A embryos				
> 1	1			
≤ 1	0.37 (0.16–0.83)	0.01		
Male age				
≤ 30 years	1		1	
> 30 years	0.40 (0.18–0.86)	0.02	0.47 (0.2–1.10)	0.08
Abnormal chromatin condensation				
≤ 20%	1		1	
> 20%	0.32 (0.13–0.81)	0.02	0.30 (0.11–0.83)	0.02
Total sperm number				
≥ 39×10^6/ejaculate	1			
< 39×10^6/ejaculate	0.43 (0.21–0.88)	0.02		
Number of progressive motile spermatozoa with normal morphology after migration				
> 2×10^6	1		1	
≤ 2×10^6	0.43 (0.21–0.87)	0.02	0.43 (0.20–0.95)	0.04
Number of progressive motile spermatozoa 17 h after migration				
> 1×10^6	1			
≤ 1×10^6	0.48 (0.23–0.99)	0.04		
Urological history				
No	1			
Yes	0.50 (0.23–1.06)	0.07		
Total chromosome abnormalities				
≤ 1.5%	1			
> 1.5%	0.25 (0.05–1.18)	0.08		
Number of ICSI cycles				
> 2	1			
≤ 2	1.90 (0.84–4.27)	0.12		
Number of injected oocytes				
≥ 10	1			
< 10	0.59 (0.27–1.26)	0.18		
DNA fragmentation				
≤ 10%	1			
> 10%	1.54 (0.74–3.22)	0.25		
Female body mass index (BMI)				
< 30 kg/m^2	1			
≥ 30 kg/m^2	0.42 (0.08–2.08)	0.29		
Sperm vitality				
≥ 58%	1			
< 58%	0.61 (0.22–1.72)	0.35		
Normal sperm morphology				
≥ 30%	1			
< 30%	1.40 (0.59–3.29)	0.44		
Number of collected oocytes				
≥ 10	1			
< 10	0.77 (0.38–1.56)	0.48		
Sperm head relative vacuole area (RVA)				
≤ 12%	1			
> 12%	0.64 (0.16–2.56)	0.53		
Gynecological history				
No	1			
Yes	0.81 (0.39–1.69)	0.58		
Male toxic exposures				
No	1			
Yes	0.84 (0.42–1.69)	0.62		
Aneuploidy				
≤ 0.5%	1			
> 0.5%	0.85 (0.41–1.72)	0.64		

CI, confidence interval

E_2, estradiol

Aneuploidy, sum of rates of presumed disomic XX, YY, 1818, and hyperhaploid XY spermatozoa

Total chromosome abnormalities, sum of rates of aneuploidy and diploidy

highlights the prognostic value of sperm chromatin condensation defects, which were identified by AB staining, on pregnancy outcomes after ICSI. Sperm chromatin condensation defects were significantly higher in the non-pregnant group than in the pregnant group of infertile males.

In our study, infertile males had more spermatozoa with chromatin condensation defects and DNA fragmentation than did fertile males, as previously reported [41, 42, 50–52]. However, our univariate and multivariate analyses confirmed that only sperm chromatin condensation defects were a negative predictive factor of ICSI outcomes. The current study confirmed that sperm DNA fragmentation is not a good predictive factor of ICSI outcomes. Surprisingly, the sperm aneuploidy rate was comparable between infertile and fertile males, and the sperm head RVA was close to the normal value observed in semen samples with normal semen parameters (~ 6%) [47]. Indeed, our infertile males had a moderate decrease in sperm concentration compared to the WHO normal value, which might explain our data. We have previously reported that sperm aneuploidy [53] and RVA [47] have been negatively correlated with sperm concentration.

Protamination defects observed in spermatozoa with abnormal chromatin condensation reduced the fertilization rate and

embryo quality after ICSI [54]. The altered expression of protamines has also been associated with a decrease in sperm and embryo quality among couples using ART [41, 55]. Normal ejaculate should contain at least 75% negative spermatozoa after AB staining [56]. In addition, a rate of chromatin condensation abnormalities greater than 30% appears to render spontaneous pregnancy impossible [57]. According to our study, a chromatin condensation defect above 20% is a risk factor for not having a pregnancy after ICSI. An increase in the level of protamine 1 and 2 mRNA has been reported in spermatozoa obtained from patients with successful IVF compared to that obtained from patients in which IVF failed. A positive correlation was also found between the level of protamine 1 and 2 mRNA and embryo quality, which suggested that protamines contribute to the success of fertilization and have a major impact on the development of preimplantation embryos. The inability of sperm chromatin to unpack appears to be involved in fertilization defects [58].

Our study also highlighted that the number of progressive motile spermatozoa with normal forms after migration is another male predictive factor of pregnancy after ICSI. Indeed, having less than 2×10^6 progressive motile spermatozoa with normal forms after migration reduced the chance of pregnancy after ICSI. Our data agree with a study by Irez et al. [39] in which chromatin condensation assessed by AB staining was positively correlated with sperm morphology, motility and ongoing pregnancy. Our value of 2×10^6 progressive motile spermatozoa with normal forms after migration appears to be particularly high for ICSI, but this value was calculated and extrapolated from whole ejaculate that was potentially suitable to perform sperm selection.

In univariate analysis, male age was significantly higher in the group of infertile males with ICSI failure (with the same maternal age in both groups). A cut-off of 30 years appears to be a predictive factor of ICSI outcomes. Sperm DNA integrity decreased with paternal age [59, 60]. Although the type of DNA damage observed in aging males is not completely characterized, the risk of morbidity in offspring appears to increase with paternal age [61]. Indeed, advanced paternal age is associated with accumulated damage to sperm DNA and mitotic and meiotic quality control mechanisms during spermatogenesis, which are responsible for sperm chromosomal abnormalities, increased sperm DNA fragmentation and single gene mutations. An increase in related abnormalities in offspring has been described, including miscarriage, fetal loss, and single-gene disorders [62]. However, the correlation between the pregnancy rate in IVF or ICSI and paternal age is controversial [4, 63, 64]. Sperm DNA fragmentation and chromatin condensation abnormalities increase with age in infertile men [63–66]. Therefore, analysis of sperm DNA integrity may be an important predictor for infertile couples, especially in cases of young women with unexplained repeated failures in ART or advanced paternal age [44, 64, 67, 68].

Our study also showed that a plasmatic level of 17β E2 less than 2000 pg/mL on the day of ovulation induction is a negative predictive factor of pregnancy. In a study by Foroozanfard et al. [69], 128 IVF cycles were performed, and an increased pregnancy rate was related to the 17β E_2 level at the onset of ovulation induction, with more oocytes collected at the puncture and more embryos for transfer. Furthermore, in univariate analysis, endometrial thickness with a cut-off of 10 mm appears to be a predictive factor of ICSI outcomes. A previous study that enrolled 8690 women in IVF or ICSI cycles confirmed that endometrial thickness is a predictor of clinical pregnancy (OR = 1.097), birth (OR = 1.078), miscarriage (OR = 0.948), and ectopic pregnancy (OR = 0.851) [70]. However, this result was not confirmed in our multivariate analysis.

In conclusion, our study demonstrates the prognostic value of sperm chromatin condensation status on ICSI outcomes for infertile males. Developing novel assessment approaches for sperm chromatin quality, including sperm nucleoprotein composition, and its impact on ICSI outcomes seems necessary. The World Health Organization recommends a nuclear sperm evaluation in cases of abnormal semen analysis (one or more abnormalities) [71]. The AB test is an inexpensive, potentially accessible routine test for the exploration of sperm chromatin condensation abnormalities. However, to the best of our knowledge, systematic assessment of sperm nuclear damage in infertile males remains highly uncommon in ART centers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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