

ORIGINAL ARTICLE

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Total motile sperm count has a superior predictive value over the WHO 2010 cut-off values for the outcomes of intracytoplasmic sperm injection cycles

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SUMMARY

The objective of this study was to compare (i) the intracytoplasmic sperm injection outcomes among groups with different total motile sperm count ranges, (ii) the intracytoplasmic sperm injection outcomes between groups with normal and abnormal total motile sperm count, and (iii) the predictive values of WHO 2010 cut-off values and pre-wash total motile sperm count for the intracytoplasmic sperm injection outcomes, in couples with male infertility. This study included data from 518 patients undergoing their first intracytoplasmic sperm injection cycle as a result of male infertility. Couples were divided into five groups according to their total motile sperm count: Group I, total motile sperm count $<1 \times 10^6$; group II, total motile sperm count $1-5 \times 10^6$; group III, total motile sperm count $5-10 \times 10^6$; group IV, total motile sperm count $10-20 \times 10^6$; and group V, total motile sperm count $>20 \times 10^6$ (which was considered a normal total motile sperm count value). Then, couples were grouped into an abnormal and normal total motile sperm count group. The groups were compared regarding intracytoplasmic sperm injection outcomes. The predictive values of WHO 2010 cut-off values and total motile sperm count for the intracytoplasmic sperm injection outcomes were also investigated. The fertilization rate was lower in total motile sperm count group I compared to total motile sperm count group V (72.5 ± 17.6 vs. 84.9 ± 14.4 , $p = 0.011$). The normal total motile sperm count group had a higher fertilization rate (84.9 ± 14.4 vs. 81.1 ± 15.8 , $p = 0.016$) and lower miscarriage rate (17.9% vs. 29.5%, $p = 0.041$) compared to the abnormal total motile sperm count group. The total motile sperm count was the only parameter that demonstrated a predictive value for the formation of high-quality embryos on D2 (OR: 1.18, $p = 0.013$), formation of high-quality embryos on D3 (OR: 1.12, $p = 0.037$), formation of blastocysts on D5 (OR: 1.16, $p = 0.011$), blastocyst expansion grade on D5 (OR: 1.27, $p = 0.042$), and the odds of miscarriage (OR: 0.52, $p < 0.045$). The total motile sperm count has a greater predictive value than the WHO 2010 cut-off values for laboratory results and pregnancy outcomes in couples undergoing intracytoplasmic sperm injection as a result of male infertility.

INTRODUCTION

Subfertility occurs in more than one in ten couples and reduced semen quality is implicated in approximately 50% of these cases (Maduro & Lamb, 2002). Semen analysis is recommended for the investigation of semen quality. Cut-off values have been defined by the World Health Organization (WHO) to distinguish between normal and abnormal semen samples (Cooper *et al.*, 2010). Different forms of male infertility have been described based on these cut-off values, including

oligozoospermia (O), asthenozoospermia (A), teratozoospermia (T), and the combinations of these factors (WHO 2010).

Intracytoplasmic sperm injection (ICSI) has been widely used in assisted reproductive techniques for those couples in the case of low-grade semen quality observed in male partner. Although the success rates of ICSI were thought to be independent of basic sperm parameters (Kupker *et al.*, 1995; Nagy *et al.*, 1995), reports have suggested that failures after ICSI may arise from the impact of sperm-derived factors on pre-implantation embryo

development (Tesarik, 2005; Tesarik *et al.*, 2006). Although a medical classification system would be expected to correlate with clinical outcome, such as the spontaneous pregnancy rate or the pregnancy rate after treatment, in cases of male infertility, reports suggest that the correlation between semen parameters and probability of conception is minimal, if any. Therefore, the relevance of the WHO classification for treatment prognosis is poor (Esteves *et al.*, 2012).

Individual semen parameters like volume, concentration, and motility can be combined resulting in an alternative way to express sperm quality, the total motile sperm count (TMSC), which is obtained by multiplying the volume of the ejaculate by the sperm concentration and the proportion of progressive motile spermatozoa divided by 100% (Ayala *et al.*, 1996). The TMSC can be assessed directly from the ejaculate (pre-wash TMSC) or after semen preparation (post-wash TMSC).

Several reports have shown that the TMSC has a prognostic value in couples undergoing intrauterine insemination (van Weert *et al.*, 2004; Badawy *et al.*, 2009; Nikbakht & Saharkhiz, 2011). In addition, one study demonstrated that the TMSC, together with number of follicles, can be used to predict total fertilization failure after conventional in vitro fertilization (IVF) (Rhemrev *et al.*, 2001). Recently, Hamilton *et al.* (2015) showed that the pre-wash TMSC has a better correlation with the spontaneous ongoing pregnancy rate than the WHO 2010 classification system.

Little is known about the prognostic value of the TMSC classification for ICSI outcomes. The objective of this study was to compare (i) the ICSI outcomes among groups with different TMSC ranges, (ii) the ICSI outcomes between groups with normal and abnormal TMSC, and (iii) the predictive values of WHO 2010 cut-off values and pre-wash TMSC for the ICSI outcomes, in couples with male infertility.

MATERIALS AND METHODS

Experimental design, patients, and inclusion and exclusion criteria

This cohort study included data from patients undergoing ICSI from December 2012 to April 2014 in a private fertility center located in Brazil. Inclusion criteria were as follows: Couples undergoing their first ICSI cycle with fresh embryo transfer performed on day 5 of development, as a result of male infertility as per the WHO 2010 classification system. Couples undergoing ICSI with vitrified/thawed or donated oocytes, surgical sperm retrieval, vitrified/thawed embryo transfer, donated embryo transfer, or pre-implantation genetic diagnosis or screening, as well as couples with female infertility were excluded from the analysis.

Couples were grouped according to their pre-wash TMSC, calculated by multiplying the ejaculate volume by the sperm concentration/mL by the percentage of motile spermatozoa ($a + b$) in the neat sample. Couples were divided into five groups, as previously suggested (Hamilton *et al.*, 2015): Group I, TMSC $<1 \times 10^6$ spermatozoa; group II, TMSC $1-5 \times 10^6$; group III, TMSC $5-10 \times 10^6$; group IV, TMSC $10-20 \times 10^6$; and group V, TMSC $>20 \times 10^6$, which was considered a normal TMSC value. Groups I–V were compared in terms of ICSI outcomes. Then, groups I–IV were combined to form the abnormal TMSC group,

and the ICSI outcomes in this group and the normal TMSC group (group V) were compared.

The influences of WHO cut-off points for semen analysis and the TMSC on ICSI outcomes were also investigated. Semen parameters preconized by the WHO in 2010 were individually considered as normal and abnormal (normal sperm concentration $\geq 15 \times 10^6/\text{mL}$, normal total sperm count $\geq 39 \times 10^6$, normal progressive motility $>32\%$, and normal typical morphology $\geq 4\%$) (Cooper *et al.*, 2010). Finally, as the TMSC does not take sperm morphology into account, we calculated the normal TMSC in order to investigate whether the incorporation of sperm morphology improves the predictive value of TMSC for the outcomes of ICSI. The normal TMSC was calculated by multiplying the percentage of normal sperm forms by the TMSC.

All patients signed a written informed consent form and the study was approved by the local Institutional Review Board.

All laboratorial procedures were performed by the andrology and embryology personnel, which were blinded regarding the study's experiments and groupings.

Controlled ovarian stimulation

Ovarian stimulation was achieved by the administration of recombinant follicle-stimulating hormone (r-FSH, Gonal-F, Serono, Geneva, Switzerland) on a daily basis until the visualization of at least one follicle ≥ 14 mm, at which time we began the administration of gonadotropin-releasing hormone (GnRH) antagonist, cetrorelix acetate (Cetrotide; Serono Laboratories).

Ovulation was triggered by the injection of recombinant human chorionic gonadotropin (hCG, Ovidrel, Serono) when at least three follicles ≥ 17 mm were observed. Oocyte retrieval was performed 35 h after the administration of hCG, through transvaginal ultrasonography.

Semen analysis and preparation

All semen samples were collected in the laboratory. After liquefaction for 30 min, semen samples were evaluated for sperm count, motility, and morphology. The volume of the ejaculate was determined by aspirating the liquefied sample into a graduated disposable pipette. Sperm counting and motility assessment were performed by following the instructions of the counting chamber manufacturer (Leja slide, Gynotec Malden, Nieuw-Vennep, the Netherlands). The total sperm count is the end concentration expressed as 10^6 spermatozoa/mL. Sperm motility was assessed in 100 random spermatozoa by characterizing them as (i) grade A (rapid progressive motility), grade B (progressive motility), grade C (non-progressive motility), and grade D (immotile) and the motility was expressed as a percentage. Sperm morphology was evaluated on air-dried smears, fixed, and stained using the quick-stain technique (Diff-Quick; Quick-Panoptic, Amposta, Spain). A total of 200 sperm cells were characterized as morphologically normal or abnormal and the final morphology was expressed as percentages.

Sperm samples were prepared using a two-layered density gradient centrifugation technique (50% and 90% Isolate, Irvine Scientific, Santa Ana, CA, USA).

Oocyte preparation

Retrieved oocytes were maintained in culture media (Global for fertilization, LifeGlobal, Guilford, CT, USA) supplemented with 10% protein supplement (LGPS, LifeGlobal), and covered

with paraffin oil (Paraffin oil P.G., LifeGlobal) for 2–3 h before cumulus cell removal. Surrounding cumulus cells were removed after exposure to a HEPES-buffered medium containing hyaluronidase (80 IU/mL, LifeGlobal). The remaining cumulus cells were then mechanically removed by gently pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA, USA).

Oocyte morphology was assessed using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under 400 \times magnification, just before sperm injection (5 h after retrieval). Oocytes that had released the first polar body were considered mature and were used for ICSI.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed according to Palermo *et al.* (1992), by a highly trained IVF laboratory team. Sperm selection was analyzed at 400 \times magnification using an inverted Nikon Eclipse TE 300 microscope. The injection was performed in a micro-injection dish prepared with 4- μ L droplets of buffered medium (Global w/HEPES, LifeGlobal) and covered with paraffin oil on a heated stage at 37.0 °C ± 0.5 °C in an inverted microscope.

Fertilization was confirmed by the presence of two pronuclei (PN) and the extrusion of the second polar body approximately 16 h after ICSI.

Embryo quality and embryo transfer

Embryos were morphologically evaluated on days 1, 2, 3, and 5 of development.

To evaluate the 2PN/2PB zygote morphology, the following features were recorded: the presence of a cytoplasmic halo, the size and position of the PN, and the number and distribution of nucleolar precursor bodies (NPB) in the PN. The following zygote morphological abnormalities were recorded: (i) absence of cytoplasmic halo; (ii) alteration in PN sizes and position, (iii) significant difference in the number of NPB in both pronuclei; (iv) small number of NPB without polarization in at least one pronucleus, (v) large number of polarized NPB in at least one pronucleus; (vi) very small number of NPB in at least one pronucleus; and (vii) polarized distribution of NPB in one pronucleus and non-polarized in the other (Tesarik & Greco, 1999).

To evaluate the cleavage stage morphology, the following parameters were recorded: the number of blastomeres, the percentage of fragmentation, the variation in blastomere symmetry, the presence of multinucleated blastomeres, and defects in the zona pellucida (ZP) and cytoplasm. The high-quality cleavage-stage embryos were defined as those with all of the following characteristics: four cells on day 2 or 8–10 cells on day 3, <15% fragmentation, symmetric blastomeres, the absence of multinucleation, colorless cytoplasm with moderate granulation and no inclusions, the absence of perivitelline space granularity and the absence of ZP dysmorphisms. Embryos lacking any of these characteristics were considered to be of low quality.

To evaluate the blastocyst morphology, embryos were given a numerical score from 1 to 6 based on their degree of expansion and hatching status, as follows: 1, an early blastocyst with a blastocoel that is less than half the volume of the embryo; 2, a blastocyst with a blastocoel that is greater than half the volume of the embryo; 3, a full blastocyst with a blastocoel that completely fills

the embryo; 4, an expanded blastocyst; 5, a hatching blastocyst; and 6, a hatched blastocyst (Alpha Scientists in Reproductive & Embryology, 2011).

Embryos were placed in a 50- μ L drop of culture medium (Global, LifeGlobal) supplemented with 10% protein supplement, and were covered with paraffin oil in a humidified atmosphere under 7.5% CO₂ at 37 °C for 5 days.

Embryo transfer was performed on day 5 of development using a soft catheter with transabdominal ultrasound guidance. One to four embryos were transferred per patient, depending on embryo quality and maternal age.

Clinical follow-up

A pregnancy test was performed 10 days after embryo transfer. All women with a positive test received a transvaginal ultrasound scan after 2 weeks. A clinical pregnancy was diagnosed when the fetal heartbeat was detected. Implantation rates were calculated per patient. Pregnancy rates were calculated per transfer. Miscarriage was defined as pregnancy loss before 20 weeks.

Data analysis and statistics

Data are expressed as the mean ± standard deviation for continuous variables, while percentages are used for categorical variables. For the evaluation of two groups, mean values were compared by Student's *t* parametric test or Mann–Whitney non-parametric test, according to the result obtained in the Kolmogorov–Smirnov normality test, while analysis of variance (ANOVA) was used for the evaluation of three or more groups. Percentages were compared using the chi-squared or Fisher's exact test when the expected frequency was five or lower.

Binary and linear regression analyses, adjusted for potential confounders, were used to investigate the influence of TMSC and WHO cut-off values on the ICSI outcomes. Results are expressed as odds ratios (OR) with 95% confidence intervals (CI) or regression coefficients (*r*) and *p*-values. A *p* < 0.05 was considered to be statistically significant.

Data analyses were carried out using the MINITAB version 17 statistical program (Minitab Inc., State College, PA, USA).

RESULTS

During the period covered by this study, 2155 ICSI cycles were performed in 1521 patients. After applying the inclusion and exclusion criteria of the study, 518 ICSI cycles were included in the analysis.

When the WHO criteria were used, 518 couples (100%) were diagnosed with male factor infertility as a single diagnosis. Oligozoospermia was present in 148 patients, asthenozoospermia in 106 patients, and teratozoospermia in 361 patients. The incidence of more than one sperm parameter alteration in the same patient was accounted accordingly. On the other hand, when TMSC was used, 190 men (36.7%) had abnormal spermatozoa, and 328 (63.3%) were normal.

In terms of the TMSC, 26 couples were grouped into group I, 50 couples into group II, 38 couples into group III, 76 couples into group IV, and 328 into group V.

Semen analysis

The descriptive statistics for the semen analysis are shown in Table 1.

Table 1 Descriptive analysis of semen parameters (*n* = 518)

Variables	Mean	SD	Range
<i>Neat sperm sample</i>			
Sperm volume (mL)	3.1	1.6	0.2–11.5
Sperm concentration (million/mL)	45.5	39.9	0.041–207.5
Sperm motility (%)	53.3	17.3	7–87
Progressive sperm motility (%)	42.9	16.6	5–85
Normal sperm morphology (%)	1.4	1.1	0–5
TMSC (million)	62.8	78.1	0–747.7
Normal TMSC (million)	1.1	2.1	0–22.4
<i>Processed semen sample</i>			
Sperm concentration (million/mL)	6.7	8.0	0.003–80
Sperm motility (%)	88.8	18.9	7–100
Progressive sperm motility (%)	80.7	19.6	6–100

SD, standard deviation; TMSC, total motile sperm count.

Comparison of sperm parameters and ICSI outcomes among TMSC groups

The descriptive statistics of the 518 ICSI cycles are shown in Table 2.

Mean male age was significantly higher in group III compared to groups IV and V (group III: 40.0 ± 5.7 vs. group IV: 36.5 ± 5.1 and group V: 37.4 ± 4.8 , $p = 0.007$). As expected, significant differences in sperm parameters were observed among the groups (Table 3).

Mean female age was significantly higher in group V compared to groups II, III and IV (group V: 35.3 ± 3.9 years vs. group II: 32.9 ± 4.1 , group III: 33.7 ± 4.1 , and group IV: 33.6 ± 3.9 , $p < 0.001$). Aside from the number of injected oocytes, which was higher in group I (group I: 12.9 ± 5.0 vs. group II: 9.6 ± 5.1 , group III: 9.0 ± 3.8 , group IV: 10.3 ± 4.9 , and group V: 9.4 ± 4.3 , $p = 0.002$), and the fertilization rate, which was lower in group I compared to group V (group I: 72.5 ± 17.6 vs. group V: 84.9 ± 14.4 , $p = 0.011$), there were no significant differences in the outcomes of ICSI among the TMSC groups (Table 3).

When the normality of TMSC was taken into account, 328 couples were grouped into the normal TMSC group and 190 couples into the abnormal TMSC group. Mean female age was significantly higher in the normal TMSC group compared to the abnormal group (35.4 ± 3.9 years vs. 33.5 ± 4.0 , $p < 0.001$, respectively). In addition, the normal TMSC groups showed

a significantly lower number of aspirated follicles (17.8 ± 9.7 vs. 20.8 ± 11.2 , $p = 0.002$, respectively), obtained oocytes (12.7 ± 7.2 vs. 15.1 ± 8.1 , $p = 0.001$, respectively), and mature oocytes (9.7 ± 5.5 vs. 11.2 ± 6.2 , $p = 0.003$, respectively) compared to the abnormal group. However, the normal TMSC group demonstrated a significantly higher fertilization rate (84.9 ± 14.4 vs. 81.1 ± 15.8 , $p = 0.016$) and lower miscarriage rate (17.9% vs. 29.5%, $p = 0.041$) compared to the abnormal TMSC group. The remaining ICSI outcomes did not differ significantly between the two groups (Table 4).

Influence of TMSC and WHO cut-off values on ICSI outcomes

Linear regression analysis results showed that the fertilization rate was influenced by sperm concentration (RC: 3.994, R^2 : 1.4%, $p = 0.015$), morphology (RC: 8.735, R^2 : 0.9%, $p = 0.047$) and TMSC (RC: 3.784, R^2 : 1.5%, $p = 0.013$).

Binary regression analysis results showed that the formation of high-quality zygotes on D1 was influenced by sperm concentration (OR: 1.64, CI: 1.09–2.46, $p = 0.018$) and TMSC (OR: 1.13, CI: 1.01–1.28, $p = 0.049$). The TMSC was the only parameter to affect the formation of high-quality embryos on D2 (OR: 1.18, CI: 1.03–1.35, $p = 0.013$), the formation of high-quality embryos on D3 (OR: 1.12, CI: 1.07–1.29, $p = 0.037$), the formation of blastocysts on D5 (OR: 1.16, CI: 1.04–1.26, $p = 0.011$), and the blastocyst expansion grade on D5 (OR: 1.27, CI: 1.01–1.60, $p = 0.042$). Finally, the TMSC was associated with the odds of miscarriage (OR: 0.52, CI: 0.28–0.90, $p < 0.045$) (Table 5).

Influence of paternal age on TMSC and WHO cut-off values

Binary regression analysis results demonstrated that male age was predictive of a diminished odds of TMSC normality (OR: 0.97, CI: 0.96–0.99, $p = 0.001$) and sperm progressive motility normality (OR: 0.95, CI: 0.91–0.98, $p = 0.003$). The normality of sperm concentration (OR: 0.99, CI: 0.96–1.03, $p = 0.625$), total motility (OR: 0.99, CI: 0.95–1.03, $p = 0.506$), and sperm morphology (OR: 1.00, CI: 0.90–1.10, $p = 0.961$) were not associated with male age.

DISCUSSION

Traditionally, the evaluation of male fertility potential has relied upon microscopic assessments to determine semen quality. The most important conventional parameters, such as the concentration, motility, and morphology of the spermatozoa in the ejaculate, were recommended by the WHO (WHO 2010). Recently, the pre-wash TMSC was shown to be better correlated with the spontaneous ongoing pregnancy rate than the WHO 2010 classification system (Hamilton *et al.*, 2015). However, the predictive value of TMSC for the outcomes of ICSI remains to be elucidated.

To the best of our knowledge, this is the first study to compare the ICSI outcomes in couples with different TMSC and to investigate the predictive value of TMSC and WHO cut-off values for the outcomes of ICSI in couples with male infertility. Our results showed that the fertilization rate was lower in TMSC group I (TMSC $<1 \times 10^6$) compared to TMSC group V (TMSC $>20 \times 10^6$). Moreover, the normal TMSC group demonstrated a higher fertilization rate and lower miscarriage rate compared to the abnormal TMSC group. Regarding the predictive value of TMSC and WHO cut-off values for the outcomes of ICSI, the TMSC was the only parameter that demonstrated a predictive

Table 2 Descriptive analysis of patients' demographics and ICSI outcomes (*n* = 518)

Variables	Mean	SD	Range
Paternal age (year-old)	37.6	5.3	27–63
Maternal age (year-old)	34.7	4.0	21–45
Total FSH dose administered (IU)	2370.0	600.5	800–4275
Estradiol level (pg/mL)	1860.4	1481.1	125–8437
Number of aspirated follicles	18.9	10.4	2–64
Number of obtained oocytes	13.6	7.6	1–50
Number of mature oocytes	10.2	5.8	1–39
Number of injected oocytes	9.7	4.5	1–24
Fertilization rate (%)	83.5	—	—
Number of obtained embryos	8.4	4.0	1–19
Number of transferred embryos	2.2	0.6	1–3
Implantation rate (%)	25.4	—	—
Pregnancy rate (%)	228/518 (44.0)	—	—
Miscarriage rate (%)	52/228 (22.8)	—	—

IU, international unit; SD, standard deviation.

Table 3 Comparison of semen parameters and ICSI outcomes among TMSC groups I–V

Variables	TMSC groups				
	I (n = 26)	II (n = 50)	III (n = 38)	IV (n = 76)	V (n = 328)
Paternal age (year-old)	39.1 ± 8.2 ^{ab}	38.6 ± 6.3 ^{ab}	40.0 ± 5.7 ^a	36.5 ± 5.1 ^b	37.4 ± 4.8 ^b
Sperm volume (mL)	2.662 ± 1.561 ^a	2.680 ± 1.520 ^a	2.874 ± 1.489 ^a	2.868 ± 1.540 ^a	3.264 ± 1.588 ^a
Sperm concentration (million/mL)	1.27 ± 1.30 ^c	7.38 ± 7.26 ^{bc}	15.82 ± 26.85 ^{bc}	21.04 ± 19.37 ^b	63.89 ± 37.21 ^a
Sperm motility (%)	26.08 ± 11.27 ^d	36.00 ± 15.23 ^c	43.95 ± 17.00 ^c	52.05 ± 16.99 ^b	59.53 ± 13.19 ^a
Progressive sperm motility (%)	17.62 ± 10.23 ^d	26.44 ± 13.02 ^{cd}	32.95 ± 15.38 ^c	41.47 ± 17.44 ^b	48.92 ± 12.86 ^a
Normal sperm morphology (%)	0.308 ± 0.471 ^d	0.760 ± 1.117 ^d	1.421 ± 1.407 ^d	1.395 ± 0.994 ^d	1.665 ± 1.003 ^d
TMSC (million)	0.37 ± 0.30 ^b	2.82 ± 1.07 ^b	7.42 ± 1.44 ^a	14.82 ± 3.12 ^a	94.47 ± 83.01 ^a
Normal TMSC (million)	0.002 ± 0.003 ^b	0.021 ± 0.029 ^b	0.100 ± 0.096 ^b	0.210 ± 0.161 ^b	1.745 ± 2.445 ^a
Maternal age (year-old)	34.1 ± 4.1 ^{ab}	32.9 ± 4.1 ^a	33.7 ± 4.1 ^a	33.6 ± 3.9 ^a	35.3 ± 3.9 ^{bc}
Number of aspirated follicles	20.5 ± 6.2 ^a	21.1 ± 11.5 ^a	16.5 ± 8.0 ^a	22.9 ± 6.1 ^a	17.8 ± 6.7 ^a
Number of obtained oocytes	16.6 ± 5.6 ^a	14.3 ± 8.4 ^a	12.9 ± 6.7 ^a	16.2 ± 5.1 ^a	12.7 ± 7.2 ^a
Number of injected oocytes	12.9 ± 5.0 ^a	9.6 ± 5.1 ^b	9.0 ± 3.8 ^b	10.3 ± 4.9 ^b	9.4 ± 4.3 ^b
Fertilization rate (%)	72.5 ± 17.6 ^a	82.5 ± 14.9 ^{ab}	81.5 ± 20.1 ^{ab}	82.4 ± 13.0 ^{ab}	84.9 ± 14.4 ^b
Number of obtained embryos	9.8 ± 3.7 ^a	8.3 ± 4.3 ^a	8.1 ± 3.7 ^a	8.8 ± 4.9 ^a	8.2 ± 3.8 ^a
Number of transferred embryos	2.3 ± 0.5 ^a	2.0 ± 0.3 ^a	2.2 ± 0.6 ^a	2.3 ± 0.5 ^a	2.2 ± 0.6 ^a
Implantation rate (%)	11.5 ± 18.1 ^a	32.7 ± 37.9 ^a	34.2 ± 40.8 ^a	21.9 ± 33.1 ^a	25.1 ± 36.0 ^a
Pregnancy rate (%)	12/26 (46.2) ^a	26/50 (52.0) ^a	20/38 (52.6) ^a	36/76 (47.4) ^a	134/328 (40.8) ^a
Miscarriage rate (%)	4/12 (33.3) ^a	8/26 (30.8) ^a	4/20 (20.0) ^a	12/36 (33.3) ^a	24/134 (17.9) ^a

Different letters (lowercase) on the same line represent a significant statistical difference.

Table 4 Comparison of ICSI outcomes between normal and abnormal TMSC groups

Variables	Normal TMSC group (n = 328)	Abnormal TMSC group (n = 190)	p-value
Paternal age (year-old)	37.4 ± 4.8	38.1 ± 6.1	0.187
Maternal age (year-old)	35.4 ± 3.9	33.5 ± 4.0	<0.001
Number of aspirated follicles	17.8 ± 9.7	20.8 ± 11.2	0.002
Number of obtained oocytes	12.7 ± 7.2	15.1 ± 8.1	0.001
Number of mature oocytes	9.7 ± 5.5	11.2 ± 6.2	0.003
Number of injected oocytes	9.4 ± 4.3	10.2 ± 4.9	0.067
Fertilization rate (%)	84.9 ± 14.4	81.1 ± 15.8	0.016
Number of obtained embryos	8.2 ± 3.8	8.7 ± 4.4	0.204
Number of transferred embryos	2.2 ± 0.6	2.2 ± 0.5	0.469
Implantation rate (%)	25.1 ± 36.0	25.8 ± 35.2	0.832
Pregnancy rate (%)	134/328 (40.9)	94/190 (49.5)	0.060
Miscarriage rate (%)	29/162 (17.9)	23/78 (29.5)	0.041

SD, standard deviation; TMSC: total motile sperm count.

value for the formation of high-quality embryos on D2 and D3, formation of blastocysts on D5, blastocyst expansion grade on D5, and the odds of miscarriage. Because the technical handling is a key element for the success of ICSI outcome, it is important to emphasize that our laboratorial procedures are performed by highly trained embryologists that have been working together for more than 5 years.

It could be argued that the TMSC is not a reliable outcome predictor, as it does not take sperm morphology into account. Therefore, in this study, we calculated the morphologically normal TMSC and tried to associate this parameter with the ICSI outcomes. However, the normal TMSC was not able to predict either the laboratory or clinical outcomes of ICSI.

We also observed that male age was inversely correlated with the TMSC. This finding is consistent with several previous reports affirming that male aging affects fertility (Kovac *et al.*, 2013). Generally, the number of sperm cells as well as sperm motility decreases with age, and is accompanied by the deterioration of testicular histological architecture (Zitzmann, 2013).

Ideally, the semen analysis should correlate with the chance of spontaneous pregnancy. However, in a study of 1089 infertile

couples, Polansky & Lamb (1988) failed to observe any influence of semen parameters on the chance of conception. On the other hand, in a study with 1055 infertile couples, Ayala *et al.* (1996) found a statistically significant difference in the mean TMSC values between couples who did or did not conceive, with a 6-fold higher risk ratio for conception in couples presenting with high TMSC values. The cut-off point for TMSC was 25×10^6 . The authors demonstrated that the total volume of the ejaculate has an important impact, as the TMSC had a more significant effect on the occurrence of pregnancy than sperm count alone. Recently, Hamilton *et al.* (2015) showed that the mean TMSC was significantly higher in the group with spontaneous ongoing pregnancy. Couples with a TMSC of $<5 \times 10^6$ had a significantly lower chance of spontaneous ongoing pregnancy than couples with a TMSC of $>5 \times 10^6$. The authors proposed three prognostic groups of semen quality according to the TMSC: (i) <5 , (ii) between 5 and 20, and (iii) more than 20×10^6 spermatozoa. Additionally, it was suggested that the TMSC should be the method of choice for the classification of male infertility in natural conception.

The results obtained in Hamilton's study are consistent with our findings that the TMSC predicts the ongoing pregnancy rate better than the WHO 2010 classification system. The reference values for sperm parameters established by the WHO in 2010 were determined based on the semen analysis from men with proven fertility who had recently procreated. However, it is not wise to define men with semen parameters below these limits as infertile, as pregnancy is not even guaranteed in couples with sperm parameters within or above the reference limits.

In this study, the WHO classification system was demonstrated to be much stricter than the TMSC for the definition of male infertility, as only 37% of the males with abnormal semen samples as per the WHO 2010 parameters had abnormal TMSC counts. In fact, this discrepancy may be explained by the fact that the WHO criteria dichotomize the sperm parameters, thus ignoring the effect of the slope, while the TMSC takes into consideration the absolute values of sperm volume, concentration, and progressive motility, simultaneously (van der Steeg *et al.*,

Table 5 Linear and binary regression analysis results for the influences of TMSC and WHO cut-off values on ICSI outcome

Variables	Method	OR or RC	CI or R ²	p-value
Fertilization rate	Concentration	3.994	1.4%	0.015
	Motility	0.097	0.0%	0.957
	Progressive motility	2.299	0.5%	0.163
	Morphology	8.735	0.9%	0.047
	TMSC	3.784	1.5%	0.013
	Normal TMSC	-0.253	0.1%	0.592
Formation of high-quality zygotes on D1	Concentration	1.64	1.09–2.46	0.018
	Motility	1.34	0.85–2.12	0.208
	Progressive motility	1.22	0.80–1.85	0.355
	Morphology	0.89	0.65–1.22	0.461
	TMSC	1.13	1.01–1.28	0.049
	Normal TMSC	0.99	0.97–1.02	0.629
Formation of high-quality embryos on D2	Concentration	0.93	0.76–1.09	0.101
	Motility	0.91	0.79–1.06	0.222
	Progressive motility	1.06	0.92–1.22	0.420
	Morphology	0.84	0.60–1.18	0.314
	TMSC	1.18	1.03–1.35	0.013
	Normal TMSC	0.97	0.94–1.01	0.098
Formation of high-quality embryos on D3	Concentration	0.91	0.79–1.06	0.229
	Motility	0.93	0.79–1.09	0.379
	Progressive motility	1.00	0.85–1.17	0.969
	Morphology	1.18	0.83–1.67	0.354
	TMSC	1.12	1.07–1.29	0.037
	Normal TMSC	0.98	0.95–1.02	0.319
Formation of blastocyst on D5	Concentration	1.11	0.97–1.27	0.116
	Motility	1.03	0.90–1.19	0.660
	Progressive motility	0.91	0.70–1.23	0.303
	Morphology	1.13	0.83–1.55	0.427
	TMSC	1.16	1.04–1.26	0.011
	Normal TMSC	1.00	0.97–1.04	0.802
Blastocyst expansion grade on D5	Concentration	0.83	0.66–1.05	0.120
	Motility	1.01	0.79–1.29	0.948
	Progressive motility	1.08	0.85–1.38	0.533
	Morphology	0.99	0.57–1.71	0.962
	TMSC	1.27	1.01–1.60	0.042
	Normal TMSC	1.03	0.98–1.07	0.287
Implantation rate	Concentration	2.387	0.1%	0.492
	Motility	-2.916	0.1%	0.453
	Progressive motility	-1.754	0.0%	0.616
	Morphology	6.084	0.0%	0.502
	TMSC	-0.688	0.0%	0.833
	Normal TMSC	1.705	1.0%	0.222
Pregnancy	Concentration	0.71	0.49–1.05	0.083
	Motility	0.77	0.50–1.19	0.242
	Progressive motility	0.72	0.49–1.05	0.089
	Morphology	2.17	0.78–6.07	0.132
	TMSC	1.40	0.98–2.01	0.066
	Normal TMSC	0.94	0.86–1.03	0.200
Miscarriage	Concentration	0.57	0.30–1.08	0.089
	Motility	1.32	0.61–2.85	0.478
	Progressive motility	1.05	0.54–2.05	0.886
	Morphology	0.84	0.17–4.08	0.826
	TMSC	0.52	0.28–0.90	0.045
	Normal TMSC	1.12	0.91–1.26	0.084

CI, confidence interval; OR, odds ratio; RC, regression coefficient; TMSC, total motile sperm count; WHO, World Health Organization.

2011; Hamilton *et al.*, 2015). Nevertheless, we observed that when sperm morphology was incorporated into the TMSC, the original predictive value of TMSC for the ICSI outcomes disappeared.

The prognostic value of TMSC in intrauterine insemination (IUI) and conventional IVF treatments has also been investigated. Van Voorhis *et al.* (2001), in a study involving 3479 IUI cycles and 551 IVF cycles, proposed a TMSC threshold value of 10×10^6 when choosing between IUI or IVF treatments. In a study following 820 IUI cycles, Nikbakht & Saharkhiz (2011)

showed that a TMSC of 5×10^6 to 10×10^6 is a useful prognostic factor for IUI cycles. Ok *et al.* (2013) found a significant association between the TMSC and pregnancy rate in 156 IUI cycles. Rhemrev *et al.* (2001) demonstrated that the TMSC predicts the chance of total fertilization failure in 917 IVF cycles. Similarly, in a study involving 1569 conventional IVF cycles, Repping *et al.* (2002) showed that choosing between IVF or ICSI could be assisted by a model of baseline characteristics that included the TMSC. This study is the first to have investigated the association between the TMSC and ICSI outcomes.

The TMSC can also be performed on processed samples. As the semen preparation selects morphologically normal motile spermatozoa, it has been suggested that the post-wash TMSC value also reflects the percentage of spermatozoa with normal morphology (Hall *et al.*, 1995). In fact, post-wash TMSC during fertility workup is used to assist the decision whether to treat with IVF or ICSI (Devroey *et al.*, 1998) and has been demonstrated to possess the best reproducibility of all sperm parameters (Rhemrev *et al.*, 2001).

In conclusion, the TMSC has a better predictive value than the WHO 2010 cut-off values for laboratory results and pregnancy outcomes, in couples undergoing ICSI as a result of male infertility. As these are novel findings for infertile patients undergoing ICSI treatment, prospective randomized studies should be performed to investigate (i) whether the TMSC grading is superior to the WHO classification system for classifying male infertility, and (ii) the role of sperm morphology in the outcomes of ICSI.

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