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Revisiting the impact of ejaculatory abstinence on semen quality and intracytoplasmic sperm injection outcomes

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ABSTRACT

Background: Regulatory bodies recommend inconsistent ejaculatory abstinence lengths before semen analysis. The literature exploring the effect of ejaculatory abstinence length on the outcomes of intracytoplasmic sperm injection is scarce.

Objective: To study the influence of ejaculatory abstinence length on semen quality and intracytoplasmic sperm injection outcomes.

Materials and methods: This prospective cohort study included 818 patients undergoing conventional semen analysis from October 2015 to October 2016, in a private university-affiliated IVF centre. Generalized linear models adjusted for potential confounders were used to investigate the associations between ejaculatory abstinence length and seminal parameters and intracytoplasmic sperm injection outcomes.

Results: Increasing ejaculatory abstinence length was positively correlated with semen volume, sperm concentration, total sperm count, total motile sperm count and sperm DNA fragmentation index. Significant inverse correlations were observed between ejaculatory abstinence length and fertilization rate, blastocyst formation rate, implantation rate and pregnancy rate. A discriminant analysis showed a mean ejaculatory abstinence length in the positive pregnancy group of 3.14 ± 1.64 days and 4.83 ± 3.66 days in the negative pregnancy group. A cut-off point was established halfway between ejaculatory abstinence length averages, at 4 days. The ejaculatory abstinence ≤ 4 days group showed significant lower semen volume, sperm concentration, total sperm count and total motile sperm count compared to ejaculatory abstinence > 4 days group. The ejaculatory abstinence ≤ 4 days group showed significant lower sperm DNA fragmentation index, and higher rates of fertilization, high-quality embryos on day 3, blastocyst development, implantation and pregnancy compared to ejaculatory abstinence > 4 days group. The implantation rate was significantly higher and the pregnancy rate tended to be higher with one day of ejaculatory abstinence, compared to 2–4 days of ejaculatory abstinence.

Conclusions: Ejaculatory abstinence periods of >4 days have a detrimental effect on sperm DNA and intracytoplasmic sperm injection outcomes. One day of ejaculatory abstinence significantly improves implantation rate and tends to increase pregnancy rate, compared to 2, 3 and 4 days of ejaculatory abstinence.

INTRODUCTION

The World Health Organization (WHO) guidelines recommend an ejaculatory abstinence (EA) of 2–7 days before semen analysis (2010), while a narrower range of 3–4 days has been recommended by the European Society of Human Reproduction and Embryology (ESHRE) and the Nordic Association for Andrology (NAFA) (Kvist & Björndahl, 2002). However, the scientific evidences behind these recommendations are limited (Ayad *et al.*, 2018a).

The effect of EA length on seminal parameters and sperm quality has been extensively studied. Nevertheless, controversial reports have emerged and the issue remains under debate. There is common agreement that prolonged EA increases semen volume and sperm concentration, but it can have a negative impact on sperm motility and viability. Previous studies have shown that lengthening EA increases sperm concentration (Sauer *et al.*, 1988; Check *et al.*, 1991; Blackwell & Zaneveld, 1992; Pellestor *et al.*, 1994; De Jonge *et al.*, 2004) and decreases sperm motility (Magnus *et al.*, 1991; Pellestor *et al.*, 1994; De Jonge *et al.*, 2004). Sperm morphology seems to be independent of EA length (Magnus *et al.*, 1991; Pellestor *et al.*, 1994). Sperm nuclear maturity and DNA status have also been shown to be associated with EA

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length. A short EA length positively influenced chromatin quality (De Jonge *et al.*, 2004) and sperm DNA integrity (Gosalvez *et al.*, 2011; Sanchez-Martin *et al.*, 2013; Agarwal *et al.*, 2016).

The literature exploring the overall effect of EA length on assisted reproductive techniques (ART) clinical outcomes is scarce. Although shorter EA lengths have been associated with the intrauterine insemination of fewer motile sperm cells, higher pregnancy rates have been observed (Jurema *et al.*, 2005; Marshburn *et al.*, 2010). Pregnancy rates were also higher in intracytoplasmic sperm injection (ICSI) in couples with shorter EA length (Sanchez-Martin *et al.*, 2013). To our knowledge, there are few studies addressing the influence of EA length on the outcomes of ICSI, and those studies deal with a limited number of subjects.

In light of the inconsistent EA length recommended by different regulatory bodies, and the necessity for better elucidation on the issue for couples undergoing ART, this study aims to investigate the influence of EA length on (i) basic and advanced semen parameters and (ii) the outcomes of ICSI in couples undergoing conventional semen analysis for routine infertility workup followed by ICSI.

MATERIALS AND METHODS

Experimental design, patients, and inclusion and exclusion criteria

This prospective cohort study included 818 male patients undergoing conventional semen analysis for routine infertility workup in a private university-affiliated in vitro fertilization centre between October 2015 and October 2016. The influence of EA length on semen quality was investigated in all participants.

For the investigation of the influence of EA length on ICSI outcomes, only couples with isolated male infertility undergoing their first ICSI cycle, with fresh embryo transfer performed on day 5 of development, were included in the analysis. Couples undergoing ICSI with vitrified/thawed or donated oocytes, surgical sperm retrieval, vitrified/thawed embryo transfer, donated embryos, or preimplantation genetic diagnosis or screening, as well as couples with female infertility, were excluded from the analysis.

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

All laboratory procedures were performed by the andrology and embryology personnel, who were blinded regarding the study's experiments.

Controlled ovarian stimulation

Ovarian stimulation was achieved by the administration of recombinant follicle-stimulating hormone (r-FSH, Gonal-F®, Serono, Geneva, Switzerland) and gonadotropin-releasing hormone (GnRH) antagonist, cetrorelix acetate (Cetrotide, Serono, Geneva, Switzerland). Ovulation was triggered with recombinant human chorionic gonadotrophin (hCG, Ovidrel™, Serono, Geneva, Switzerland).

Semen analysis and preparation

Semen samples were collected in the laboratory by masturbation. Prior to semen sample collection, all patients filled a form regarding the EA length. It was emphasized to all patients that ejaculatory abstinence was related to the act of ejaculation, resulting either from sexual intercourse or masturbation. The EA was recorded in days for each patient.

None of the patients underwent any intervention prior to providing the semen sample that was examined.

Semen samples were analysed according to the WHO guidelines (World Health Organization 2010). After liquefaction for 30 min, semen samples were evaluated for sperm count, motility and morphology. Sperm count and motility assessment were performed by following the instructions of the count chamber manufacturer (Leja® slide, Gynotec Malden, Nieuw-Vennep, the Netherlands). The total sperm concentration is the number of spermatozoa in the ejaculate. Prewash total motile sperm count (TMSC) was calculated by multiplying the ejaculate volume by the sperm concentration/mL by the percentage of motile spermatozoa in the neat sample (Hamilton *et al.*, 2015).

Sperm motility was assessed in 100 random spermatozoa by characterizing them as (i) progressive motility, (ii) non-progressive motility and (iii) 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 a percentage.

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

Motile sperm organelle morphology examination

A total of 200 spermatozoa of each sample were analysed at high magnification using an inverted Nikon Diaphot microscope equipped with high-power differential interference contrast optics (DIC/Nomarski). The total calculated magnification was $\times 6600$.

The sperm cells were graded into four groups according to the presence or size of the vacuoles. Grade I: normal form and no vacuoles; grade II: normal form and ≤ 2 small vacuoles; grade III: normal form and >2 small vacuoles or at least one large vacuole; and grade IV: large vacuole and abnormal head shapes or other abnormalities (Vanderzwalmen *et al.*, 2008).

Sperm DNA fragmentation

Two hundred sperm cells (Lim *et al.*, 2013) were examined for sperm DNA fragmentation (SDF) with the sperm chromatin dispersion test (Halosperm®, Halotech, Madrid, Spain). Briefly, a semen aliquot of 60 μL, previously diluted to 10 million/mL, was added to the agarose containing Eppendorf tube, provided in the kit and mixed. A 20 μL aliquot of the mix was transferred to the agarose pre-coated slide, provided in the kit and covered with a coverslip. The slide was refrigerated for 5 min. The coverslip was removed and the slide immersed in an acid solution, and incubated for 7 min. The slide was immersed in 10 mL of the lysing solution for 25 min, washed with distilled water, dehydrated in ethanol baths and air-dried. The slide was stained using the rapid panoptic and examined under light microscopy for enumeration of spermatozoa with and without halos. Results were interpreted as SDF index and considered normal when $\leq 20\%$.

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ICSI

Mature oocytes were used for ICSI. Fertilization was confirmed approximately 16 h after ICSI. Embryos were morphologically evaluated on days 1, 2, 3 and 5 of development. The high-quality cleavage-stage embryos were defined as those with all of the following characteristics: four cells on day 2 or eight to 10 cells on day 3, <15% fragmentation, symmetric blastomeres, the absence of multinucleation, colourless cytoplasm with moderate granulation and no inclusions, the absence of perivitelline space granularity and the absence of zona pellucida dimorphisms. Embryos lacking any of these characteristics were of low quality.

The luteal phase was supported by intravaginal progesterone 200 mg (Utrogestan) twice a day. Embryo transfers were performed on day 3 or 5 of embryo development. Up to three embryos were transferred per patient, depending on maternal age and embryo quality. A pregnancy test was performed 10 days after embryo transfer. All women with a positive test had a transvaginal ultrasound scan 2 weeks after the positive test. A clinical pregnancy was diagnosed when the foetal heartbeat was detected. Pregnancy rates were calculated per transfer. Implantation rate was calculated by dividing the number of gestational sacs with foetal heartbeat by the number of transferred embryos. Miscarriage was defined as clinical pregnancy loss before 20 weeks.

Data analysis and statistics

The sample size calculation revealed that a sample of at least 327 subjects had 95% power to detect a 20% effect with a significance level (α) of 5% (two-tailed). The calculation was performed using G*Power 3.1.7. Data are expressed as the mean ± standard deviation for continuous variables, while percentages are used for categorical variables. Generalized linear models (GzLM) with adjustment for potential confounders were used to investigate the associations between ejaculatory abstinence length and:

1 Semen quality (semen volume, sperm concentration, total sperm count, sperm motility, progressive sperm motility, TMSC, sperm morphology, incidences of sperm grade I–IV observed by motile sperm organelle morphology examination (MSOME) and SDF) – adjusted for male age and smoking habit;
2 Laboratory ICSI outcomes (fertilization rate, high-quality embryos rate on day 3, blastocyst formation rate on day 5) – adjusted for maternal and paternal ages, smoking habits and body mass index, seminal parameters, total dose of FSH administered, estradiol levels on the day of hCG administration, and number of oocytes and mature oocytes;
3 Clinical ICSI outcomes (implantation rate, pregnancy rate and miscarriage rate) – adjusted for the same variables cited in item ii, as well as for number of transferred embryos.

Potential confounders were selected when a strong association between the variable and the dependent variable was noted.

In a further step, a discriminant function analysis was performed to determine which variables discriminate between pregnancy groups (positive and negative), using maternal and paternal ages, smoking habits and body mass index, seminal parameters, total dose of FSH administered, estradiol levels on the day of hCG administration, number of oocytes and mature oocytes, and number of transferred embryos as covariates, and the pregnancy outcome as the dependent categorical variable. A cut-off point was established halfway between EA length averages in both pregnancy groups. Then, the data were grouped according with established cut-off for EA length and the reanalysed by GzLM followed by Bonferroni post hoc test, adjusted for the confounders variables described above. Finally, to investigate the influence of shorter EA lengths, patients with EA length below the cut-off point were split into four groups (Group 1, EA length of one day; Group 2, EA length of 2 days; Group 3, EA length of 3 days; and Group 4, EA length of 4 days), and data were reanalysed by GzLM followed by Bonferroni post hoc test, adjusted for the confounders variables described above.

The results are expressed as standardized regression coefficients (B) for continuous dependent variables, or exponentiation of the B coefficient [Exp(B)] for dichotomous dependent variables, with 95% confidence intervals (CI) and p-values. A $p < 0.05$ was considered statistically significant. Data analyses were conducted using the SPSS Statistics 21 (IBM, New York, NY, USA).

RESULTS

Semen quality

The mean male age was 38.17 ± 6.39 years. Seven hundred and seventy-two out of 818 men (94.4%) had abnormal semen parameters, defined as having at least one of the following: sperm count <15 million sperm/mL, sperm motility <40% or normal sperm morphology <4%. Mean ejaculatory abstinence length was 4.15 ± 2.72 days. General seminal profile is shown in Table 1.

The GzLM results for the correlations between sperm parameters and EA length are shown in Table 2. Increasing EA length was positively correlated with semen volume (B: 0.097, CI: 0.040–0.154, $p = 0.001$), sperm count/mL (B: 2.267, CI: 0.308–4.226, $p = 0.023$), total sperm count (B: 13.813, CI: 7.270–20.356, $p < 0.001$) and TMSC (B: 6.808, CI: 3.130–10.486, $p < 0.001$). Sperm motility, progressive sperm motility and sperm morphology were not significantly correlated with EA length. Increasing EA length was not correlated with the incidences of sperm cells grade I–IV observed by MSOME, but was positively influenced SDF index (B: 0.598, CI: 0.230–0.966, $p = 0.001$).

Table 1 General seminal profile of men undergoing conventional semen analysis for infertility investigation ($n = 818$)

Variable	Mean	SD	Min–max
Male age (years)	38.17	6.39	16.00–63.00
EA length (days)	4.15	2.72	0.00–20.00
Semen volume (mL)	3.04	1.62	0.01–18.00
Sperm concentration ($\times 10^6$/mL)	61.27	51.57	0.01–505.00
Total sperm count ($\times 10^6$)	177.76	172.15	0.01–2.05
Total sperm motility (%)	56.92	18.53	0.00–92.00
Progressive sperm motility (%)	48.81	18.66	0.00–91.00
TMSC ($\times 10^6$)	97.60	102.00	0.00–724.15
Morphology (%)	1.32	1.28	0.00–6.00
MSOME grade I (%)	1.44	1.98	0.00–12.00
MSOME grade II (%)	4.73	4.86	0.00–27.00
MSOME grade III (%)	4.44	4.22	0.00–25.00
MSOME grade IV (%)	70.22	35.10	0.00–98.00
SDF (%)	17.58	9.41	3.00–57.70

EA: ejaculatory abstinence; TMSC: total motile sperm count; MSOME: motile sperm organelle morphology examination; SDF: sperm DNA fragmentation; SD: standard deviation; Min: minimum; Max: maximum.

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Table 2 GzLM results for the association between sperm parameters and EA length (n = 818)

Semen parameters	B	95% CI	p-value
Semen volume (mL)	0.097	0.040–0.154	0.001
Sperm concentration ($\times10^6$/mL)	2.267	0.308–4.226	0.023
Total sperm count ($\times10^6$)	13.813	7.270–20.356	< 0.001
Total sperm motility (%)	0.033	−0.535–0.601	0.911
Progressive sperm motility (%)	0.128	−0.473–0.729	0.676
TMSC ($\times10^6$)	6.808	3.130–10.486	< 0.001
Morphology (%)	−0.004	−0.053–0.044	0.857
MSOME grade I (%)	0.037	−0.056–0.131	0.432
MSOME grade II (%)	0.047	−0.182–0.277	0.685
MSOME grade III (%)	−0.003	−0.202–0.196	0.977
MSOME grade IV (%)	−1.250	−2.897–0.397	0.137
SDF (%)	0.598	0.230–0.966	0.001

Adjusted for paternal age and smoking habit. GzLM: generalized linear model; EA: ejaculatory abstinence; B: unstandardized regression coefficient; CI: confidence interval; TMSC: total motile sperm count; MSOME: motile sperm organelle morphology examination; SDF: sperm DNA fragmentation.

ICSI outcomes

After the application of inclusion criteria for the association between EA intervals on ICSI outcomes, 483 couples were included in the analysis. The mean male age was 38.28 ± 5.74 years. The mean ejaculatory abstinence length was 4.21 ± 2.88 days. The general characteristics of seminal profiles and ICSI cycles are shown in Tables 3 and 4, respectively.

The GzLM results for the association between ICSI outcomes and EA length are shown in Table 5. Negative correlations were observed between increasing EA length and fertilization rate (B: −0.983, CI: −1.954 to −0.011, p = 0.047), blastocyst formation rate on day 5 (B: −2.384, CI: −4.552 to −0.216, p = 0.031), implantation rate (B: −3.299, CI: −5.388 to −1.260, p = 0.002) and pregnancy rate (Exp(B): 0.506, CI: 0.290–0.882, p = 0.016). EA length was not associated with high-quality embryos rate on day 3 or miscarriage rate.

A discriminant analysis was conducted to predict whether the cycles had resulted in positive or negative pregnancy. The discriminate function correctly classified 67.7% of original cases, best predicting negative pregnancy (84.4%). The cross-validated classification showed that overall 73.5% were correctly classified.

Table 3 General seminal profile of men undergoing ICSI as a result of isolated male infertility (n = 483)

Variable	Mean	SD	Min–max
Male age (years)	38.28	5.74	24.00–58.00
EA length (days)	4.21	2.88	1.00–20.00
Semen volume (mL)	3.09	1.55	0.50–12.80
Sperm concentration ($\times10^6$/mL)	66.30	50.42	1.10–505.00
Total sperm count ($\times10^6$)	194.98	174.58	1.35–2048.00
Total sperm motility (%)	59.95	15.29	5.00–92.00
Progressive sperm motility (%)	51.47	16.36	0.00–91.00
TMSC ($\times10^6$)	107.80	99.72	0.00–675.84
Morphology (%)	1.41	1.24	0.00–6.00
MSOME grade I (%)	1.00	1.22	0.00–3.00
MSOME grade II (%)	6.00	4.95	2.00–14.00
MSOME grade III (%)	4.20	3.63	1.00–9.00
MSOME grade IV (%)	88.80	8.64	76.00–97.00
SDF (%)	17.63	9.48	3.00–57.70

ICSI: intracytoplasmic sperm injection; EA: ejaculatory abstinence; TMSC: total motile sperm count; MSOME: motile sperm organelle morphology examination; SDF: sperm DNA fragmentation; SD: standard deviation; Min: minimum; Max: maximum.

Table 4 General characteristics of ICSI cycles and ICSI outcomes (n = 483)

Variable	Mean	SD	Min-max
Female age (years)	34.51	4.49	20.00–38.00
Total dose of FSH administered (IU)	2521.40	616.50	1050.00–5500.00
Number of follicles	14.04	10.93	1.00–63.00
Number of retrieved oocytes	10.15	8.10	1.00–43.00
Fertilization rate (%)	82.23	21.25	10.00–100.00
High-quality embryos rate on day 3 (%)	45.68	33.14	0.00–100.00
Blastocyst formation rate on day 5 (%)	44.15	30.28	0.00–100.00
Number of transferred embryos (%)	1.97	0.80	1.00–3.00
Implantation rate (%)	30.09	32.42	0.00–100.00
Transferred cycles (%)	310/483 (64.18)	*	*
Pregnancy rate (%)	101/310 (32.58)	*	*
Miscarriage rate (%)	12/101 (11.88)	*	*

ICSI: intracytoplasmic sperm injection; IU: international unit; SD: standard deviation; Min: minimum; Max: maximum; *: not applicable.

Table 5 GzLM results for the association between ICSI outcomes and EA length (n = 483)

Continuous ICSI outcomes	B	95% CI	p-value
Fertilization rate[a]	−0.983	−1.954 to −0.011	0.047
High-quality embryos rate on day 3[a]	−0.090	−0.207 to 2.284	0.131
Blastocyst formation rate on day 5[a]	−2.384	−4.552 to −0.216	0.031
Implantation rate[ab]	−3.299	−5.388 to −1.260	0.002
Continuous ICSI outcomes	**Exp(B)**	**95% CI**	***p*-value**
Pregnancy rate[ab]	0.506	0.290–0.882	0.016
Miscarriage rate[ab]	0.736	0.458–1.185	0.207

GzLM: generalized linear model; ICSI: intracytoplasmic sperm injection; EA: ejaculatory abstinence; B: unstandardized regression coefficient; CI: confidence intervals; Exp(B): exponentiation of the B coefficient. [a]Adjusted for maternal and paternal ages, smoking habits and body mass index, seminal parameters, total dose of FSH administered, estradiol levels on the day of hCG administration, number of oocytes and mature oocytes; [b]Adjusted for number of transferred embryos.

In this model, mean EA length in the positive pregnancy group was 3.14 ± 1.64 days and 4.83 ± 3.66 days in the negative pregnancy group (p = 0.043). A cut-off point was established halfway between EA length averages, at 4 days. Cycles were then split into two groups according to the established EA length cut-off point (EA ≤ 4 days and EA > 4 days groups). The EA ≤ 4 days group showed lower semen volume (2.9 ± 0.1 ml vs. 3.4 ± 0.1 ml, p = 0.002), lower sperm concentration (60.1 ± 3.9 $\times$ 10^6/mL vs. 73.9 ± 4.2 $\times$ 10^6/mL, p = 0.015), lower total sperm count (156.1 ± 12.8 $\times$ 10^6 vs. 244.6 ± 13.8 $\times$ 10^6, p < 0.001) and lower TMSC (87.9 ± 7.7 $\times$ 10^6 vs. 136.2 ± 7.7 $\times$ 10^6, p < 0.001) compared to EA > 4 days group. On the other hand, the EA ≤ 4 days group showed lower SDF index than the EA > 4 days group (16.8 ± 0.7% vs. 19.2 ± 0.8%, p = 0.028). Regarding ICSI outcomes, higher rates of fertilization (85.5 ± 2.2% vs. 77.3 ± 2.7%, p = 0.021), high-quality embryos on day 3 (56.8% vs. 41.6%, p = 0.022), blastocyst formation on day 5 (50.2 ± 4.7% vs. 35.6 ± 4.9%, p = 0.046), implantation (24.8 ± 4.1% vs. 7.3 ± 4.3%, p = 0.005) and pregnancy (40.0% vs. 10.0%, p = 0.016) were observed in EA ≤ 4 days compared to EA > 4 days group (Table 6).

When patients with EA length below the cut-off point were split into four groups according to the EA length, significant differences were observed between groups 1 and 4 in semen volume

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Table 6 Descriptive analysis of semen quality and ICSI outcomes by EA length cut-off

	EA ≤ 4 days	EA > 4 days	*p*-value
Semen parameters[a]			
Semen volume (mL)	2.9 ± 0.1	3.4 ± 0.1	0.002
Sperm concentration (×10^6/mL)	60.1 ± 3.9	73.9 ± 4.2	0.015
Total sperm count (×10^6)	156.1 ± 12.8	244.6 ± 13.8	<0.001
Total sperm motility (%)	59.4 ± 1.1	60.9 ± 1.2	0.360
Progressive sperm motility (%)	50.7 ± 1.2	53.4 ± 1.3	0.126
TMSC (×10^6)	87.9 ± 7.7	136.2 ± 7.7	<0.001
Morphology (%)	1.4 ± 0.1	1.5 ± 0.1	0.669
SDF (%)	16.8 ± 0.7	19.2 ± 0.8	0.028
ICSI outcomes			
Fertilization rate [b]	85.5 ± 2.2	77.3 ± 2.7	0.021
High-quality embryos rate on day 3 [b]	56.8	41.6	0.022
Blastocyst formation rate on day 5 [b]	50.2 ± 4.7	35.6 ± 4.9	0.046
Implantation rate [bc]	24.8 ± 4.1	7.3 ± 4.3	0.005
Pregnancy rate [bc]	40.0	10.0	0.016
Miscarriage rate [bc]	10.8	8.8	0.548

ICSI: intracytoplasmic sperm injection; EA: ejaculatory abstinence; TMSC: total motile sperm count; SDF: sperm DNA fragmentation. Values are means ± standard deviations unless otherwise noted. [a]Adjusted for male age and smoking habit; [b]adjusted for maternal and paternal ages, smoking habits and body mass index, seminal parameters, total dose of FSH administered, estradiol levels on the day of hCG administration, number of oocytes and mature oocytes; [c]adjusted for number of transferred embryos.

(2.3 ± 0.4 ml vs. 3.6 ± 0.2 ml, $p < 0.001$, respectively) and in total sperm count ($137.4 \times 10^6 \pm 35.5 \times 10^6$ vs. $221.4 \times 10^6 \pm 20.2 \times 10^6$, $p = 0.001$, respectively). The differences observed among the four groups in fertilization, high-quality embryos on day 3, blastocyst development and miscarriage rates were not statistically significant. Implantation rate was significantly higher in Group 1 compared to the other groups (Group 1: 45.4% ± 11.9% vs. Group 2: 13.2% ± 7.3% vs. Group 3: 16.2% ± 5.8% vs. Group 4: 24.9% ± 6.6%, $p = 0.015$). Pregnancy rate tended to be higher in Group 1 compared to the other groups; however, statistical significance was not reached, probably due to a small sample size (Group 1: 69.0% vs. Group 2: 24.0% vs. Group 3: 27.0% vs. Group 4: 35.0%, $p = 0.062$) (Table 7).

DISCUSSION

The results of this study demonstrate that increasing EA length correlates positively with semen volume, sperm concentration, total sperm count and TMSC. However, it negatively affects SDF, and the rates of fertilization, blastocyst formation, implantation and pregnancy.

Our results concerning semen quality corroborate those from previous studies. The length of EA positively influenced sample volume (Padova *et al.*, 1988; Pellestor *et al.*, 1994; Carlsen *et al.*, 2004; De Jonge *et al.*, 2004; Marshburn *et al.*, 2010; Agarwal *et al.*, 2016), sperm count (Oldereid *et al.*, 1984; Pellestor *et al.*, 1994; Carlsen *et al.*, 2004; De Jonge *et al.*, 2004; Jurema *et al.*, 2005; Marshburn *et al.*, 2010; Agarwal *et al.*, 2016) and TMSC (Jurema *et al.*, 2005; Marshburn *et al.*, 2010). The numbers of progressive motile spermatozoa (Carlsen *et al.*, 2004; Jurema *et al.*, 2005) and the percentage of morphologically normal spermatozoa were not affected by EA periods (Carlsen *et al.*, 2004). Several studies have suggested extremely short EA lengths (i.e. <1–4 h) due to their positive influence on semen parameters (Gosalvez *et al.*, 2011; Valsa *et al.*, 2013; Bahadur *et al.*, 2016; Mayorga-Torres *et al.*, 2016; Ayad *et al.*, 2018b). A recent systematic review of 28 studies suggested that longer EA is associated with increased semen volume and sperm count, which is also in agreement with our findings. On the other hand, the study reported that effect of EA on sperm motility, morphology and DNA fragmentation rates is controversial, despite a trend towards improvements in semen parameters with shorter abstinence appears to exist (Hanson *et al.*, 2018). Long periods of EA may induce senescence of spermatozoa, producing damage at a functional level that may not be recognized by conventional semen analysis. For instance, a decrease in sperm acrosin activity, which is implicated in many reproductive functions, such as sperm–egg interaction and sperm zona pellucida binding and penetration, has been previously observed with longer EA periods (Blackwell & Zaneveld, 1992).

Our study did not reveal an association between EA and the incidence of sperm cells graded I–IV by MSOME. This could be explained by the fact that lengthy EA periods do not seem to be

Table 7 Descriptive analysis of semen quality and ICSI outcomes by EA length groups

	Group 1 (*n* = 31)	Group 2 (*n* = 104)	Group 3 (*n* = 205)	Group 4 (*n* = 123)
Semen parameters[1]				
Semen volume (mL)	2.3 ± 0.4[a]	3.2 ± 0.2[ab]	3.4 ± 0.2[ab]	3.6 ± 0.2[b]
Sperm concentration (×10^6/mL)	59.1 ± 14.0[a]	54.9 ± 8.1[a]	58.9 ± 6.5[a]	66.4 ± 8.2[a]
Total sperm count (×10^6)	137.4 ± 35.5[a]	159.9 ± 19.9[ab]	174.4 ± 16.0[ab]	221.4 ± 20.2[b]
Total sperm motility (%)	60.5 ± 4.1[a]	60.5 ± 2.3[a]	59.1 ± 1.9[a]	62.4 ± 2.4[a]
Progressive sperm motility (%)	52.6 ± 4.3[a]	50.7 ± 2.5[a]	49.1 ± 2.0[a]	53.8 ± 2.5[a]
TMSC (×10^6)	81.8 ± 22.5[a]	94.8 ± 13.0[a]	98.1 ± 10.4[a]	129.0 ± 13.2[a]
Morphology (%)	1.3 ± 0.3[a]	1.6 ± 0.2[a]	1.4 ± 0.2[a]	1.4 ± 0.2[a]
SDF (%)	16.6 ± 2.7[a]	16.3 ± 1.6[a]	18.2 ± 1.3[a]	19.8 ± 1.6[a]
ICSI outcomes				
Fertilization rate [2]	74.6 ± 6.6[a]	85.9 ± 3.7[a]	88.3 ± 2.9[a]	83.9 ± 3.6[a]
High-quality embryos rate on day 3 [2]	40.2 ± 10.8[a]	47.6 ± 6.0[a]	51.6 ± 4.5[a]	47.2 ± 5.7[a]
Blastocyst development rate on day 5 [2]	37.2 ± 13.4[a]	33.3 ± 6.6[a]	39.1 ± 5.4[a]	40.0 ± 6.0[a]
Implantation rate [2,3]	45.4 ± 11.9[a]	13.2 ± 7.3[b]	16.2 ± 5.8[b]	24.9 ± 6.6[b]
Pregnancy rate [2,3]	70.0[a]	30.0[a]	25.0[a]	30.0[a]
Miscarriage rate [2,3]	0.0[a]	0.0[a]	0.0[a]	0.0[a]

ICSI: intracytoplasmic sperm injection; EA: ejaculatory abstinence; TMSC: total motile sperm count; SDF: sperm DNA fragmentation. Values are means ± standard deviations unless otherwise noted. [1]Adjusted for male age and smoking habit; [2]adjusted for maternal and paternal ages, smoking habits, and body mass index, seminal parameters, total dose of FSH administered, estradiol levels on the day of hCG administration, number of oocytes and mature oocytes; [3]adjusted for number of transferred embryos. Different letters in the same line represent significant statistical difference ($p < 0.05$).

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related to sperm morphological alterations (Carlsen *et al.*, 2004). On the other hand, we noted a positive association between EA and the incidence of SDF. The role of EA on sperm DNA fragmentation has been studied, and inconsistent reports have emerged. De Jonge *et al.* (2004) reported increased rate of sperm with immature chromatin after 1 day of EA, while Pons *et al.* (2013) observed a 90% reduction in sperm DNA fragmentation after the same period of EA. Similar studies have shown that short EA periods result in lessened incidence of SDF (Marshburn *et al.*, 2010; Gosalvez *et al.*, 2011; Sanchez-Martin *et al.*, 2013; Mayorga-Torres *et al.*, 2015; Agarwal *et al.*, 2016).

Lengthening of EA intervals may be associated with SDF because, in the absence of ejaculation, spermatozoa accumulate in the epididymis and are subjected to a harmful seminal microenvironment, mainly reactive oxygen and nitrogen species (ROS and RNS), for a prolonged time. Additionally, a shorter EA was associated with higher seminal total antioxidant capacity, which would enhance sperm protection from oxidative damage (Marshburn *et al.*, 2014). Agarwal *et al.* (2016) observed that the incidence of spermatozoa exhibiting fragmented DNA was significantly lower after EA periods of up to 2 days. On the other hand, a progressive increase was noted when EA was compared among short, recommended and long EA groups. Interestingly, the incidence of ROS-positive semen samples was similar among all EA periods, suggesting that prolonged EA periods do not increase ROS levels. This finding corroborates the fact that sperm antioxidant capacity may be reduced during prolonged storage in the epididymis (Marshburn *et al.*, 2014). Indeed, studies show that immature spermatozoa, which produce high levels of ROS, can induce DNA damage in mature spermatozoa (Ollero *et al.*, 2001). An interesting finding from previous studies is that the degree of sperm DNA fragmentation in ejaculated spermatozoa is generally higher than that in testicular spermatozoa (Steele *et al.*, 1999). This was corroborated by Greco *et al.* (2005), who showed higher pregnancy rates in patients with DNA fragmentation levels in semen >15%, using testicular spermatozoa compared to ejaculated spermatozoa.

Few studies evaluated the influence of EA on intrauterine insemination (IUI), conventional in vitro fertilization (IVF) and ICSI outcomes, and suggested that shorter EA periods result in improved pregnancy rates; however, cut-off values for EA length are inconsistent. One study observed higher pregnancy rates post-IUI using sperm samples which had been collected after an EA of ≤3 days (Jurema *et al.*, 2005), while another study found higher pregnancy rates with an EA of ≤2 days before IUI (Marshburn *et al.*, 2010). Sugiyam *et al.* (2008) found higher fertilization rates post-IVF when semen samples were collected after 30–60 min of abstinence. Another study observed that recurrent ejaculation every 24 h for 4 days with a final abstinence of 12 h results in a significant higher pregnancy rate post-ICSI (Sanchez-Martin *et al.*, 2013). Colturato *et al.* (2007) observed lower pregnancy rates after ≥ 5 days of EA, which is in agreement with our findings, despite the abstinence intervals in that study were different from ours. A recent study showed that an EA of > 7 days adversely affects live-birth rate, clinical pregnancy rate and implantation rate, compared with the recommended period of 2–7 days. Moreover, an EA of 2–4 days was associated with higher live-birth rates compared with an EA of >7 days (Periyasamy *et al.*, 2017). Another study found no significant differences in fertilization and clinical pregnancy rates post-ICSI between groups of 2–4 and 5–7 day of EA (Lee *et al.*, 2015). Notwithstanding the fact that pregnancy outcomes depend on many factors, shorter EA lengths seem to be associated with higher pregnancy rates following ART. It is well known that sperm cells are particularly vulnerable to oxidative damage, and this has been directly correlated with reduced fertilization rates (du Plessis *et al.*, 2010). Additionally, as ICSI bypasses natural sperm selection, it is possible that spermatozoa with fragmented DNA are injected into the oocyte, leading to early and late paternal effects (Barroso *et al.*, 2009) such as impaired fertilization and blastocyst formation, respectively.

This study established a cut-off point for EA length at 4 days, through a discriminant analysis. The EA ≤ 4 days group showed lower semen volume, sperm concentration, total sperm count and TMSC compared to EA > 4 days group. On the other hand, the EA ≤ 4 days group showed lower SDF index and higher rates of fertilization, high-quality embryos on day 3, blastocyst formation, implantation and pregnancy compared to EA > 4 days group. It is possible that the ICSI outcomes are positively influenced by even shorter EA lengths. In the present study, we split patients with EA lengths below the cut-off point into four groups and found a significant higher implantation rate and a trend towards higher pregnancy rate in patients with one day of EA, despite significant lower semen volume and total sperm count, compared to those with 2, 3 and 4 days of EA. This value is considerably lower than the maximum interval outlined by the WHO and is more comparable to the interval suggested by the ESHRE. Nevertheless, it is important to emphasize that the patients with one day of EA were under-represented in the study population (6.7%, 31/463); therefore, statistical significance for pregnancy rate could possibly be obtained with a larger sample size.

Historically, it has been suggested that optimal EA should be based on copulatory regularity (Macleod & Gold, 1952). Moreover, couples trying to conceive naturally are encouraged to have intercourse every other day during the fertile period (Agarwal *et al.*, 2016). In general, the aforementioned studies suggest that, while lengthy EA is detrimental, short EA does not endanger sperm quality. Recently, Agarwal *et al.* (2016) suggested that reduced EA length may also be recommended for sperm banking.

The strengths of our study are (i) the number of analysed subjects, mainly because the majority of previous studies have included only a limited casuistic; (ii) the adjustment of statistical analyses for potential confounders; (iii) the establishment of cut-off values for the EA length over which sperm DNA integrity and ICSI outcomes are negatively affected. The main limitation of this study is the facts that DNA fragmentation assessment was performed by SCD and may not be equivalent to the SCSA/TUNEL DNA fragmentation reported by others.

CONCLUSIONS

EA periods of >4 days have a detrimental effect on sperm DNA and ICSI outcomes. Our results suggest that one day of EA significantly improves implantation rate and tends to increase pregnancy rate, compared to 2, 3 and 4 days of EA. The study findings are useful for couples planning for ART treatment, in which shortening of EA length could be used as a strategy to optimize sperm quality, fertility preservation and pregnancy outcomes, by keeping epididymal stasis at a minimum. Documentation that even shorter EA lengths would lessen these potentially harmful influences will require further study in larger populations.

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