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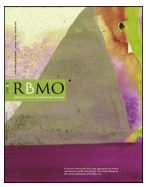

ARTICLE

Effect of ejaculatory abstinence period on fertilization and clinical outcomes in ICSI cycles: a retrospective analysis

BIOGRAPHY

Greta Chiara Cermisoni graduated from the University of Milan (Italy) with a degree in Medical and Pharmaceutical Biotechnology, focusing her Master's thesis on stem cell biology. Since 2017, she has been a Clinical Embryologist at the San Raffaele Scientific Institute in Milan, Italy. Her main research focuses are embryo development and preimplantation genetic testing.

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KEY MESSAGE

Longer ejaculatory abstinence periods decrease the rate of two-pronuclear zygotes and increase the rate of three-pronuclear zygotes in fresh intracytoplasmic sperm injection (ICSI) cycles without affecting blastulation and pregnancy rates. These findings underscore the critical role of optimizing ejaculation abstinence periods in ICSI cycles for infertile couples.

ABSTRACT

Research question: Does ejaculatory abstinence impact fertilization outcomes in intracytoplasmic sperm injection (ICSI) cycles in infertile couples?

Design: This single-centre retrospective observational study included 6919 ICSI cycles from 2013 to 2022. The primary outcome was the assessment of oocyte fertilization, measured in terms of the rate of formation of two-pronuclear (2PN), 3PN and 1PN zygotes. Secondary outcomes were blastulation, cumulative positive β-human chorionic gonadotrophin test and clinical pregnancy rates. Relationships between ejaculatory abstinence and fertilization outcomes, and ejaculatory abstinence and clinical outcomes were evaluated with multivariable analysis, including possible confounders.

Results: A positive association was observed between ejaculatory abstinence and semen sample volume ($P < 0.001$), sperm concentration ($P < 0.001$) and total motile sperm count ($P < 0.001$). No association was found between the 1PN zygote rate and ejaculatory abstinence ($P = 0.97$). Conversely, for each additional day of ejaculatory abstinence, the likelihood of obtaining 2PN zygotes from all inseminated oocytes decreased by 3% [adjusted odds ratio (aOR) 0.97, 95% CI 0.94–0.99], whilst the likelihood of obtaining 3PN zygotes from all inseminated oocytes increased significantly by 14% (aOR 1.14, 95% CI 1.07–1.22). No significant associations were found between ejaculatory abstinence and blastulation, cumulative pregnancy or miscarriage rates.

Conclusions: A longer ejaculatory abstinence period significantly decreases the rate of 2PN zygotes, and increases the rate of 3PN zygotes without directly affect blastulation and pregnancy rates.

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KEY WORDS
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Sperm

INTRODUCTION

Semen analysis represents a primary and crucial step in medically assisted reproduction (MAR). One of the main factors that can influence the quality of a biological sample of seminal fluid is the ejaculatory abstinence period. The latest edition of the World Health Organization (WHO) guidelines recommends ejaculatory abstinence for 2–7 days before seminal fluid examination (*WHO, 2021*). The European Society of Human Reproduction and Embryology (ESHRE) Special Interest Group of Andrology advises 3–4 days of ejaculatory abstinence prior to semen analysis (*Barratt et al., 2011*), based on WHO guidance and other research (*Bjornhdahl et al., 2010*; *Menkveld, 2007*).

A growing number of studies have focused on the impact of ejaculatory abstinence on semen quality (*Agarwal et al., 2016*; *Mayorga-Torres et al., 2015*; *Okada et al., 2020*) and MAR clinical outcomes (*Borges et al., 2019*; *Hanson et al., 2018*; *Li et al., 2020*; *Marshburn et al., 2010*; *Periyasamy et al., 2017*), but the effect of ejaculatory abstinence seems to remain contradictory and inconclusive for fertilization outcomes. Notwithstanding the controversial literature, recent meta-analyses have supported the positive effects of a short ejaculatory abstinence period (*Barbagallo et al., 2022*, *2023*). Short ejaculatory abstinence periods appear to be associated with improvements in semen parameters, such as viability and motility (*Agarwal et al., 2016*), and a decrease in sperm count and seminal volume (*Mayorga-Torres et al., 2015*). Moreover, an increase in oxidative stress has been observed in samples collected after 4 days of ejaculatory abstinence compared with 1 day, thus suggesting that prolonged storage of the spermatozoa in epididymal tracts may have a negative influence on both acrosome and mitochondrial activity, and nuclear DNA integrity (*Okada et al., 2020*). Considering the impact of ejaculatory abstinence on MAR clinical outcomes, a retrospective analysis showed higher pregnancy rates with ejaculatory abstinence for 0–2 days before intrauterine insemination compared with ejaculatory abstinence for >2 days (*Marshburn et al., 2010*). Consistently, the literature indicates that shorter ejaculatory abstinence periods are associated with higher fertilization, pregnancy and live birth rates in couples undergoing conventional IVF/intracytoplasmic sperm injection (ICSI) cycles. While the specific comparison intervals varied between studies, the consistent trend emphasizes the benefits of a shorter ejaculatory abstinence period (*Hanson et al., 2018*; *Li et al., 2020*; *Periyasamy et al., 2017*).

For MAR, optimal fertilization of the oocyte is confirmed 16–18 h after insemination by the presence of two polar bodies and two pronuclei (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*). Assessment of the fertilization status of zygotes after insemination is important to distinguish between zygotes with an abnormal number of pronuclei [i.e. mono-pronuclear (1PN) or tri-pronuclear (3PN) zygotes] and those with a normal display of two pronuclei (2PN). The incidence of 3PN zygotes has been estimated to be approximately 5–8% in IVF cycles and approximately 2–6% in ICSI cycles (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*; *Mutia et al., 2019*). Crucially, the prevalence of 3PN zygotes in all pregnancies has been estimated to be approximately 1–3%, and accounts for 15–18% of cytogenetic abnormalities among spontaneous miscarriages (*Li et al., 2015*). These data discourage the transfer or cryopreservation of embryos derived from 3PN zygotes to avoid a decrease in the chance of implantation or full-term pregnancy after embryo transfer (*Li et al., 2015*; *Mutia et al., 2019*). Over the years, various models have been postulated to understand the mechanism underlying the occurrence of supernumerary pronuclei zygotes; the presence of three haploid chromosome sets may be due to failure of extrusion of the second polar body (*Reichman et al., 2010*), fertilization of a diploid oocyte by a haploid spermatozoon (*Rosenbusch et al., 2008*), or fertilization of an oocyte by a diploid spermatozoon (*Rosen et al., 2006*).

The relationship between overall semen quality and ICSI outcomes has gained interest over time; thus, understanding and standardizing the optimal ejaculatory abstinence period could be helpful to improve further sperm selection and subsequent clinical outcomes of fertility treatments. Due to the complexities of the later stages of the IVF process, the authors chose to focus their research on the initial stages of fertilization. The choice of primary outcome enables straightforward and reliable assessment of the research findings. This decision was guided by the authors' intent to uncover subtle variations related to the ejaculatory abstinence period, which might be masked in more advanced outcomes. By analysing a total of 6919 ICSI cycles, this study emphasizes these particular outcomes to offer a more concise perspective on this complex area of research.

METHODS

Study design

This observational study was carried out at IRCCS San Raffaele Hospital, Milan, Italy between January 2013 and March 2022. All ICSI procedures (n = 7156) with non-donor and fresh gametes were included. Procedures involving cryopreserved gametes were excluded due to the clear impact of this condition on embryological outcomes (*Balaban et al., 2001*; *Loutradi et al., 2006*; *Mazzilli et al., 2017*; *Vernaeve et al., 2003*). Follow-up pregnancy data were collected up to July 2022.

The following cycles were excluded from the study: cycles with preimplantation genetic testing for structural chromosomal rearrangements (n = 157), due to the possible higher frequency of unbalanced gametes in carrier individuals (*Morin et al., 2017*); cycles with at least one 3PN zygote (n = 51), due to the rarity of the event and other possible causes that might come into play; and cycles with ejaculatory abstinence for <2 days or >7 days (n = 29), as couples are advised by gynaecological staff to observe sexual abstinence for 2–7 days, in accordance with WHO guidelines (FIGURE 1).

Semen samples and preparation

At the time of medical consultation, all male partners were instructed to observe 2–7 days of ejaculatory abstinence prior to treatments, in accordance with WHO guidelines (*WHO, 2010*). WHO laboratory guidelines and ISO 23162:*2021* were followed for assessment and processing of semen (*Björndahl et al., 2022*; *WHO, 2010*; see online supplementary material). The total motile sperm count (TMC) was also provided, calculated as: semen volume x semen concentration x (progressive motility/100). At the time of collection, the patient completed a form with details of: (i) time of collection; (ii) number of days of ejaculatory abstinence; (iii) any loss of ejaculate during collection; and (iv) location of semen collection (at home or in the

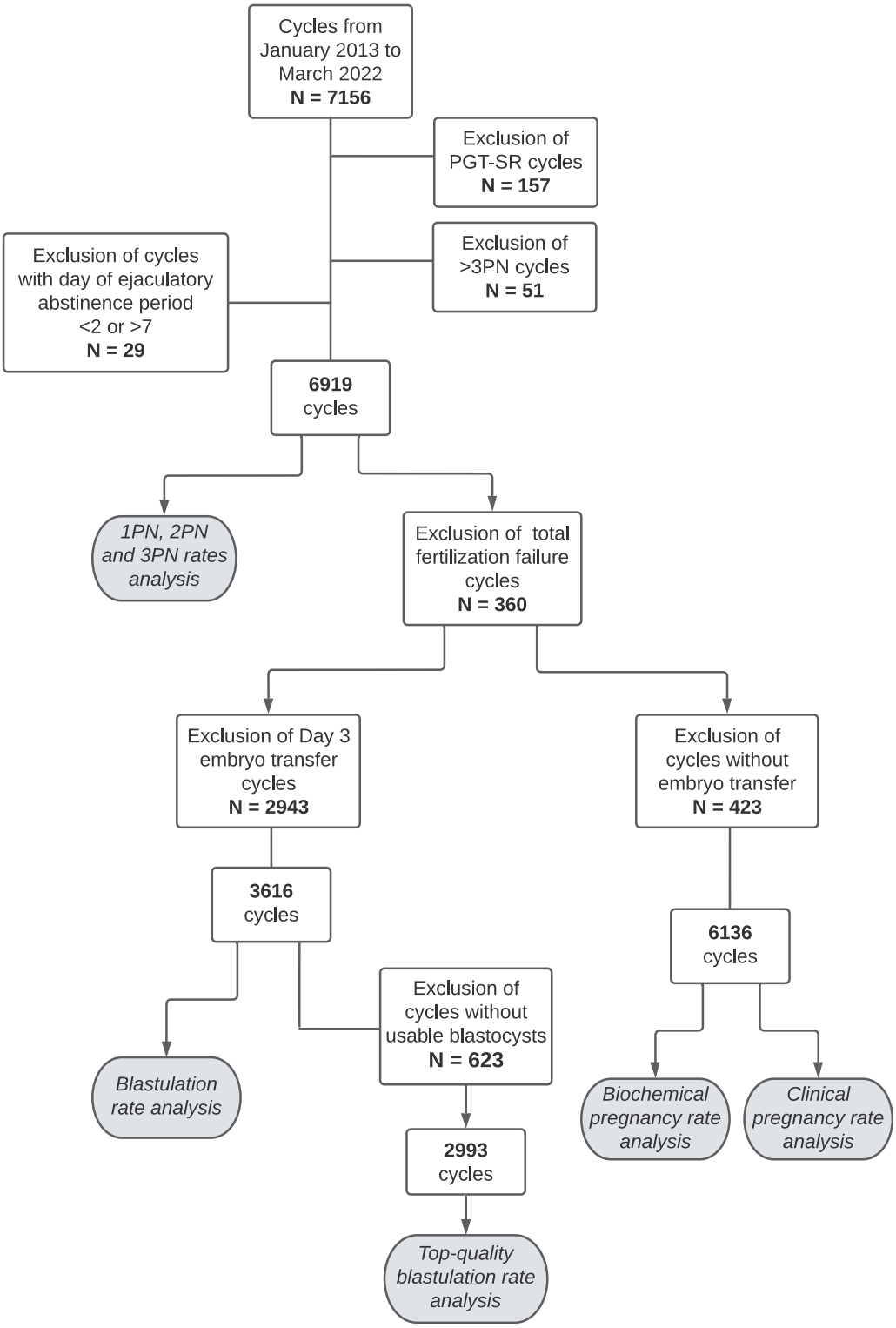

FIGURE 1 Flow chart of cycle inclusion of the analyses of different outcomes. PGT-SR, preimplantation genetic testing for structural chromosomal rearrangements; PN, pronuclear.

clinic). These parameters are relevant to the interpretation of analytical results. The laboratory operators participate in an external quality control programme for semen assessment (ESHRE EQA) Spermatozoa were prepared using 80/40 density gradient (Sydney IVF sperm gradient; Cook Medical, Bloomington, IA, USA) centrifugation. The obtained pellet was resuspended in 0.5 ml of sperm medium (Sydney IVF sperm medium; Cook Medical), and kept at 37°C in a controlled atmosphere (6% CO_2 and 5% O_2) in a standard incubator (Sanyo, Osaka, Japan) until use.

Assisted reproductive technology procedures

Recombinant gonadotrophins in antagonist or agonist protocols were used for ovarian stimulation, and ovulation triggering was performed with highly purified human chorionic gonadotrophin (HCG) or gonadotrophin-releasing hormone (GnRH) agonist, as described previously (*Papaleo et al., 2016*; *Vanni et al., 2017*). Oocyte collection was performed 36 h after triggering, and follicular fluids were screened for cumulus–oocyte complexes (COC). Once identified, COC were collected, rinsed and transferred into pre-equilibrated fertilization medium (Quinn's Advantage Medium; CooperSurgical Fertility, Ballerup, Denmark) supplemented with human serum albumin (HSA) (CooperSurgical Fertility) overlaid with mineral oil (CooperSurgical Fertility) in a four-well plate until insemination. Every step of embryo culture was performed at 37°C in a controlled atmosphere (6% CO_2 and 5% O_2) in a standard incubator (Sanyo). HEPES-buffered medium (Quinn's Advantage Medium with HEPES) supplemented with 5% HSA (sHEPES medium) was used for denuding and ICSI procedures.

COC denudation was performed 39 h post-HCG administration in a two-step procedure involving an initial period of enzyme exposure and a second mechanical step. Specifically, COC were exposed to hyaluronidase (Fujifilm Irvine Scientific, Irvine, CA, USA) in sHEPES medium (11.4 IU/ml) and pipetted repeatedly through a Pasteur pipette with an inner diameter of approximately 250 μm, with the total enzyme exposure time not exceeding 30 s. After the enzymatic-induced dispersion of cumulus cells, corona cells were removed gently through plastic denuding pipettes with decreasing inner diameters (170 μm then 140 μm; Cook Medical). Only mature metaphase II oocytes were considered for injection.

Injection of metaphase II oocytes was performed in an IVF/ICSI dish containing microdroplets of pre-warmed sHEPES and a drop of polyvinylpyrrolidone polymer (PVP; Irvine Scientific or Origio) covered with mineral oil. Giant oocytes were excluded from ICSI cycles (*ESHRE Special Interest Group in Embryology, 2016*). A few microlitres of sperm suspension were transferred into the PVP drop to allow sperm migration, facilitating sperm selection. A maximum of two oocytes were placed in each microdrop of sHEPES (10 μl). For each oocyte, a morphologically normal spermatozoon was selected, immobilised and finally aspirated with the micropipette. The metaphase II oocyte was placed with the first polar body in the 6 o'clock or 12 o'clock position; after rupturing the oolemma by gentle aspiration with the injection micropipette, the spermatozoon was slowly released into the ooplasm. At the end of the injection, the micropipette was removed carefully from inside the oocyte. The procedure was repeated for each oocyte.

Injected oocytes were rinsed and placed in continuous (Fujifilm Irvine Scientific) or sequential (Quinn's Advantage Sequential Media, CooperSurgical Fertility) pre-equilibrated culture medium supplemented with serum substitute supplement (SSS) (Fujifilm Irvine Scientific). The injected oocytes were cultured in groups of four, in separate 30-μl drops, at 37°C in a controlled atmosphere (6% CO_2 and 5% O_2) in a standard incubator (Sanyo) until the next day.

The presence of two equal-sized pronuclei was assessed 17 ± 1 h after insemination. Normally fertilized oocytes should have two polar bodies and two juxtaposed pronuclei. They should be approximately the same size, with two distinctly visible membranes (*ESHRE Special Interest Group in Embryology, 2016*). Oocytes with one or zero pronuclei were maintained in culture until day 3. Oocytes with three or more pronuclei were discarded. Zygotes were cultured individually in separate 30-μl drops up to blastocyst stage (days 5–7). All fertilized zygotes in the cohort were assessed for the purpose of fresh transfer or cryopreservation on day 3 and days 5–7, and a media change was performed. If conditions were suboptimal for fresh transfer due to any compromising factors affecting prognosis, a 'freeze-all' strategy was employed. Fresh embryo transfers were performed on day 3 or day 5. Blastocysts were cultured until day 5–7 and cryopreserved. Only expanded blastocysts (grade 3), with inner cell mass and trophectoderm graded as 1–3, were considered suitable for cryopreservation on days 5, 6 and 7. Cleavage-stage embryo and blastocyst grading were performed according to the Istanbul Consensus (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*). Based on this, a top-quality blastocyst was defined as an expanded blastocyst with at least one of inner cell mass and trophectoderm rated as very good/good quality. Expanded blastocysts were vitrified on day 5, 6 or 7 after artificial shrinkage by a single laser shot on the trophoblast cells. All viable embryos reaching the blastocyst stage up to 7 days after insemination were transferred or cryopreserved according to Italian Law 40/2004 (see web reference). Cleavage-stage embryos and expanded blastocysts were cryopreserved following the simplified embryo vitrification protocol by Irvine Scientific with slight modifications (https://www.irvinesci.com/) using Irvine (Vit Kit-Freeze; Irvine Scientific) or Kitazato solution (VT601 vitrification media; Kitazato, Shizuoka, Japan). The first equilibration was carried out in equilibration solution at room temperature for 10 min. Thereafter, the embryo was transferred in vitrification solution (3 × 30-μl drops) for 1 min before loading. Each device was used for one embryo. The vitrified–warmed cycles took place in either a natural or an artificial cycle, as described in detail elsewhere (*Alteri et al., 2022*). Cleavage-stage embryos and expanded blastocysts were warmed following the simplified embryo warming protocol by Irvine Scientific (https://www.irvinesci.com/) using Irvine (Vit Kit-Thaw; Irvine Scientific) or Kitazato solution (VT602 thawing media; Kitazato) (*Parmegiani et al., 2014*). After warming, both cleavage-stage embryos and blastocysts were placed in blastocyst medium (Quinn's Advantage Blastocyst Medium; CooperSurgical Fertility), supplemented with 20% SSS, and transferred as described elsewhere (*Alteri et al., 2022*).

Outcome measures

The primary outcome was to evaluate the effect of the ejaculatory abstinence period (2–7 days) on fertilization outcomes. Fertilization outcomes were the 1PN, 2PN and 3PN zygote rates, based on the total number of inseminated oocytes. Total fertilized zygotes included 2PN zygotes and cleavage-stage embryos on day 3 derived from non-pronucleated zygotes at the fertilization check on day 1, representing approximately 3.4% of the total fertilized zygotes. A recent study investigated the origins and clinical implications of a third pronucleus that differs in size from the other two pronuclei (*Capalbo et al., 2017*). However, based on the Istanbul consensus of 2011, this was considered abnormal (*Alpha Scientists in Reproductive Medicine*

and ESHRE Special Interest Group of Embryology, 2011). In light of this, these were included in this study, categorized as 3PN zygotes for the analysis.

As the analysis considered the total number of 1PN, 2PN and 3PN zygotes out of all inseminated oocytes, it was not affected by variables closely related to the individual cycle, such as the number of oocytes collected and inseminated, and enabled the authors to best visualize the variation in fertilization outcomes as a function of the ejaculatory abstinence period.

To evaluate associations between 1PN, 2PN and 3PN zygote rates and the ejaculatory abstinence period, the authors considered the rates per cycle, correcting for the number of oocytes collected, the number of mature oocytes and the number of inseminated oocytes. Using this approach, the coefficient estimates were not affected by the number of inseminated oocytes per cycle.

Moreover, blastulation and top-quality blastocyst rates were analysed as secondary outcomes to evaluate the effect of the ejaculatory abstinence period on embryo development after ICSI treatment. In particular, the blastulation rate was calculated as the mean percentage of blastocysts per number of fertilized zygotes per cycle. The blastulation rate was calculated by excluding cycles with total fertilization failure and cycles with embryo transfer on day 3 in order to correct for the bias of transferring the best embryo on day 3 which could have become a blastocyst. Subsequently, the top-quality blastocyst rate was investigated in cycles that obtained at least one blastocyst from fertilization and did not transfer embryos on day 3. This outcome was calculated as the mean percentage of top-quality blastocysts per total number of blastocysts per cycle.

Cumulative positive β-HCG test and clinical pregnancy rates were used as secondary outcomes to evaluate the effect of the ejaculatory abstinence period on post-transfer stages. A positive β-HCG test was defined as a serum β-HCG concentration ≥10 mIU/ml, measured ≥14 days after embryo transfer. Using transvaginal ultrasound performed >5 weeks after embryo transfer, clinical pregnancy was defined as the presence of an intrauterine gestational sac with visible fetal heart activity. The number of positive β-HCG tests and the number of clinical pregnancies obtained from both fresh and subsequent vitrified-warmed embryo transfer cycles were collected for each ICSI cycle. Only the first positive β-HCG test and clinical pregnancy per ICSI cycle were included in the results. Through this analysis, the effect of the ejaculatory abstinence period on cumulative positive β-HCG test and clinical pregnancy rates was evaluated, considering all embryo transfers originating from the same pool of fertilized oocytes from a single ICSI cycle. Next, through a stratified analysis by days of embryo transfer, associations between cumulative positive β-HCG test and clinical pregnancy rates and the ejaculatory abstinence period were examined in cycles with fresh embryo transfer on day 3 or day 5, and freeze-all cycles.

This work considered a comprehensive assessment of miscarriage types, including biochemical pregnancies, blighted ovum, and embryo without observed fetal heartbeat. Biochemical pregnancy was defined as a positive pregnancy test that resolved spontaneously, as evidenced by the absence of either an intrauterine or ectopic embryonic sac on ultrasound screening. Due to the constraints of the database, it was not possible to report these three events separately. Thus, they are presented collectively, conceptually representing those pregnancies where a positive β-HCG test was observed but no fetal heartbeat was ever detected. Miscarriages were reported using the same cumulative methodology as pregnancies, reflecting the percentage of ICSI cycles that showed at least one positive β-HCG test in all transfers but did not result in a detectable fetal heartbeat.

Covariates

Data were obtained from medical records stored in the database at the study centre. Data were collected on maternal and paternal age on the day of oocyte retrieval, maternal body mass index (BMI; kg/m^2), semen parameters (ejaculatory abstinence period, semen volume, sperm concentration, percentage of motile sperm, sperm motility), ovarian stimulation [days of stimulation, oestradiol level on the day of ovulation trigger, progesterone level on the day of ovulation trigger, total dose of recombinant FSH\human menopausal gonadotrophin (HMG)], oocyte retrieval procedure (number of oocytes retrieved, number of mature oocytes), ICSI treatment (number of inseminated oocytes, numbers of 1PN 2PN and 3PN zygotes), embryo transfer (number of embryos and blastocysts transferred or cryopreserved, day of embryo transfer) and pregnancies (positive β-HCG test, clinical pregnancies, miscarriages).

The discrete data of cumulative positive β-HCG tests and clinical pregnancies were converted to a dichotomous variable, with 0 indicating lack of pregnancies and 1 indicating the presence of at least one pregnancy from embryo transfers following the same ICSI treatment. Similarly, miscarriage data were analysed as a dichotomous variable, with 0 indicating lack of miscarriages and 1 indicating the presence of at least one miscarriage. The analysis was performed in embryo transfers derived from the same ICSI treatment in which at least one positive β-HCG test was achieved (cumulative positive β-HCG test = 1). The preimplantation genetic testing cycles were considered a two-category variable as follows: presence or absence of treatment. Infertility diagnoses were categorized as advanced maternal age (≥40 years), tubal factor, endocrine factor, endometriosis (endometriosis was laparoscopically diagnosed and endometriomas were diagnosed by transvaginal ultrasonography), at least two previous failed embryo transfer cycles (recurrent implantation failure and recurrent pregnancy loss), genetic factor (monogenic disorders), polycystic ovarian syndrome, poor ovarian response (according to the Bologna criteria; *Ferraretti et al., 2011*) and unexplained infertility. Male factor has been reported solely for descriptive purposes to better define the couple's infertility. The chosen threshold for severe oligozoospermia was <5 M/ml (*Practice Committee of the American Society for Reproductive Medicine, 2015*). Each infertility factor was considered as a dichotomous variable (presence or absence of the infertility factor); therefore, a patient could be characterized by more than one infertility factor.

Semen was collected on the same day as oocyte retrieval; as such, the cycles were categorized into four seasons based on the day of egg collection: winter (December–February), spring (March–May), summer (June–August) and autumn (September–November) (*De Giorgi et al., 2015*).

Statistical analysis

Outcomes were studied in relation to the ejaculatory abstinence period. Continuous

variables are presented as mean and SD, and were compared using Kruskal–Wallis test. Categorical variables are presented as absolute values and percentages (%), and were compared using Chi-squared test. Semen parameters are presented as median and interquartile range, and were compared using Kruskal–Wallis test. The ejaculatory abstinence period was categorized into four groups: 2 days, 3 days, 4 days, and 5–7 days. Cycles with ejaculatory abstinence periods of 5–7 days were grouped in a single category for descriptive purposes.

In the present study, certain variables had missing data: 3.0% for maternal BMI, 17.63% for length of stimulation, 23.4% for total dose of recombinant FSH/HMG, 1.7% for oestradiol concentration on the day of ovulation trigger, 1.8% for progesterone concentration on the day of ovulation trigger, and 1.0% for mature oocytes. For the analytical approach, univariate evaluations used the entirety of the available cohort corresponding to each variable. Conversely, multivariate methodologies were strictly applied to cycles encompassing comprehensive data across all variables under consideration. The precise number of cycles integrated within each distinct analysis is delineated in the respective analytical section.

Relationships between the ejaculatory abstinence period and fertilization outcomes (1PN, 2PN and 3PN zygote rates) were evaluated by multivariable analysis, including all possible confounders. Specifically, a Generalized Linear Mixed Model was used, specifying a quasi-binomial distribution and a logit link function. In this model, multiple cycles were taken into account by including the couple's identification code as a random effect. Additionally, the season in which the semen was collected was considered as a random effect. The ejaculatory abstinence period (in days) was incorporated as a continuous variable. The same model was used to evaluate potential relationships between the ejaculatory abstinence period and the blastulation rate and the top-quality blastocyst rate. Applying the formula *[exp(beta)-1]*100*, the percentage variation of odds as the ejaculatory abstinence period increased by one unit was estimated. Finally, relationships between the ejaculatory abstinence period and clinical outcomes (specifically, cumulative positive β-HCG test rate, cumulative clinical pregnancy rate and miscarriage rate) were evaluated using a multivariable logistic regression model with binomial distribution including all the potential confounders. Collinearity between covariates included in models was tested by calculating the variance inflation factor (VIF) of each variable, so that only variables with VIF <2.5 were retained in the final model (*Johnston et al., 2018*).

In all statistical models, infertility factors were included as covariates, except for advanced maternal age and poor ovarian response factors because these characteristics are already explained in the continuous variables 'maternal age' and 'number of mature metaphase II oocytes'. Odds ratios adjusted for confounders with corresponding 95% CI were calculated for each model. *P*-values ≤0.05 were considered to indicate significance. Data analysis was performed using RStudio Version 2022.07.1+554) (*RStudio Team, 2022*).

Ethical approval

Data collection followed the principles defined in the Declaration of Helsinki, and all patients signed a written informed consent agreeing to anonymous retrospective analysis and publication of their own anonymous information for research purposes (ID: BC-GINEOS, date of approval: 9 February 2012, San Raffaele Hospital Ethics Committee).

RESULTS

Patient selection process and baseline characteristics

In total, 6919 ICSI cycles were included in this analysis. TABLE 1 summarizes the baseline characteristics by ejaculatory abstinence period (2, 3, 4 or 5–7 days). Regarding semen parameters, significant differences in sample volume ($P < 0.001$) and sperm concentration ($P < 0.001$) were found to be associated with the ejaculatory abstinence period. The group with the longest ejaculatory abstinence period (5–7 days) had the highest semen volume and the highest sperm concentration. Additionally, a slight reduction in progressive motility was found to be associated with longer ejaculatory abstinence periods ($P = 0.043$). Finally, TMC was found to differ significantly between ejaculatory abstinence periods ($p<0.001$), with the highest median value in the group with 5–7 days of ejaculatory abstinence and the lowest value in the group with 2 days of ejaculatory abstinence. Supplementary Figure 1 shows the distribution of these semen parameters across ejaculatory abstinence groups.

The distribution of paternal age differed significantly between groups defined by ejaculatory abstinence period ($P = 0.002$); the group with 5–7 days of ejaculatory abstinence had the highest mean paternal age. Similarly, the group with 5–7 days of ejaculatory abstinence had the highest mean maternal age, although this difference was not significant for the continuous data. However, this same group showed an increase in infertility causes related to maternal age ($P = 0.023$). Finally, a significant association was found between embryo transfer method (day 3 or day 5 fresh embryo transfer or frozen embryo transfer) and ejaculatory abstinence period ($P = 0.045$).

Association between ejaculatory abstinence period and clinical outcomes of ICSI cycles

Primary outcome: fertilization rate

Adjusted odds ratios (aOR) with 95% CI for potential associations between the ejaculatory abstinence period and primary outcomes of ICSI cycles are summarized in TABLE 2. No association was found between the ejaculatory abstinence period and the 1PN zygote rate ($P = 0.969$). On the other hand, both the 2PN zygote rate ($P = 0.029$) and the 3PN zygote rate ($P < 0.001$) were significantly associated with the ejaculatory abstinence period. In fact, for each unit increase in days of ejaculatory abstinence, the likelihood of obtaining 2PN zygotes from all inseminated oocytes decreased by 3% (aOR 0.97, 95% CI 0.94–0.99). On the contrary, the longer the ejaculatory abstinence period, the greater the proportion of 3PN zygotes from the total number of inseminated oocytes. Thus, for every passing day of ejaculatory abstinence, the likelihood of obtaining 3PN zygotes from all inseminated oocytes increased significantly by 14% (aOR 1.14, 95% CI 1.07–1.22).

Supplementary Table 1 shows the full results of the univariate- and multivariable-adjusted models implemented to estimate the association between the ejaculatory abstinence period and the 1PN, 2PN and 3PN zygote rates.

Secondary outcomes: blastulation and pregnancy rates

The multivariable-adjusted models for blastulation rate ($n = 2280$ cycles after

TABLE 1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS ACCORDING TO EJACULATORY ABSTINENCE PERIOD

Characteristic	Population	Ejaculatory abstinence period (days)				*P*-value[1]
		2	3	4	5–7	
No. of cycles	6919	3152 (45.6)	2207 (31.9)	890 (12.9)	670 (9.7)	
Male characteristics, mean (SD)						
Paternal age (years)	40.2 (5.4)	40.2 (5.4)	40.1 (5.4)	40.4 (5.4)	41.0 (5.8)	**0.002**
Semen parameters, median (IQR)						
Total motile sperm count (million)	18.0 (4.5–42.0)	15.6 (4.0–37.5)	18.7 (4.5–45.0)	18.0 (4.5–43.6)	24.0 (5.6–60.0)	<**0.001**
Volume (ml)	2.5 (2.0–3.0)	2.0 (1.5–3.0)	2.5 (2.0–3.0)	2.5 (2.0–3.3)	3.0 (2.0–3.5)	<**0.001**
Sperm concentration (x 10^6/ml)	25.0 (10.0–46.0)	22.0 (10.0–42.0)	25.0 (10.0–50.0)	25.0 (10.0–45.0)	30.0 (10.0–60.0)	<**0.001**
Progressive motility (%)	35.0 (20.0–45.0)	35.0 (20.0–45.0)	35.0 (20.0–45.0)	30.0 (20.0–45.0)	30.0 (20.0–45.0)	**0.043**
Location of semen collection						0.297
Home	1054 (15.2)	457 (14.5)	360 (16.3)	135 (15.2)	102 (15.2)	
Clinic	5865 (84.8)	2695 (85.5)	1847 (83.7)	755 (84.8)	568 (84.8)	
Female characteristics, mean (SD)						
Maternal age (years)	37.9 (3.9)	37.8 (4.0)	37.8 (4.0)	37.8 (4.0)	38.1 (4.2)	0.154
Maternal BMI (kg/m^2)	22.1 (3.4)	22.2 (3.4)	22.1(3.4)	22.2 (3.4)	22.1 (3.5)	0.531
Length of stimulation (days)	10.0 (2.2)	9.9 (2.2)	10.1 (2.2)	10.1 (2.3)	9.8 (2.2)	**0.006**
Total dose of r-FSH/HMG (IU)	2690.6 (1365.9)	2666.6 (1343.6)	2726.9 (1407.9)	2762.0 (1368.8)	2590.4 (1318.2)	0.096
Oestradiol concentration on day of ovulation trigger (pg/ml)	1930.5 (1266.7)	1951.9 (1308.2)	1913.9 (1202.0)	1926.6 (1277.6)	1889.4 (1261.5)	0.584
Progesterone concentration on day of ovulation trigger (ng/ml)	0.9 (0.6)	0.9 (0.6)	0.8 (0.6)	0.9 (0.6)	0.8 (0.6)	0.077
Mature (metaphase II) oocytes	5.7 (4.5)	5.8 (4.7)	5.6 (4.3)	5.7 (4.5)	5.7 (4.6)	0.990
No. of oocytes retrieved	7.4 (5.6)	7.4 (5.7)	7.3 (5.4)	7.4 (5.6)	7.4 (5.7)	0.906
Female infertility factor[2], *n* (%)						
Maternal age	2408 (34.8)	1095 (34.7)	741 (33.6)	305 (34.3)	267 (39.9)	**0.023**
Tubal factor	441 (6.4)	205 (6.5)	140 (6.3)	60 (6.7)	36 (5.4)	0.701
Polycystic ovary syndrome	49 (0.7)	18 (0.6)	20 (0.9)	4 (0.4)	7 (1.0)	0.261
Endocrine factor	745 (10.8)	344 (10.9)	225 (10.2)	96 (10.8)	80(11.9)	0.619
Unexplained	1870 (27.0)	827 (26.2)	628 (28.5)	230 (25.8)	185 (27.6)	0.259
Endometriosis	592 (8.6)	277 (8.8)	180 (8.2)	77 (8.7)	58 (8.7)	0.876
At least two previous failed embryo transfer cycles	389 (5.6)	187 (5.9)	125 (5.7)	41 (4.6)	36 (5.4)	0.496
Poor ovarian response	2055 (29.7)	959 (30.4)	646 (29.3)	242 (27.2)	208 (31.0)	0.236
Couple infertility factor[2], *n* (%)						
Genetic factor	393 (5.7)	191 (6.0)	125 (5.7)	39 (4.4)	38 (5.7)	0.302
Male infertility factor[2], *n* (%)						
Severe oligozoospermia (<5 M/Ml)	1095 (15.8)	486 (15.4)	363 (16.4)	148 (16.6)	98 (14.6)	0.535
PGT cycles, *n* (%)						0.611
PGT-A, PGT-M	844 (12.2)	398 (12.6)	270 (12.2)	99 (11.1)	77 (11.5)	
No	6075 (87.8)	2754 (87.4)	1937 (87.8)	791 (88.9)	593 (88.5)	
Season at oocyte retrieval, *n* (%)						0.076
Winter	1811 (26.2)	870 (27.6)	563 (25.5)	219 (24.6)	159 (23.7)	
Spring	1926 (27.8)	815 (25.9)	656 (29.7)	259 (29.1)	196 (29.3)	
Summer	1268 (18.3)	584 (18.5)	388 (17.6)	173 (19.4)	123 (18.4)	
Autumn	1914 (27.7)	883 (28.0)	600 (27.2)	239 (26.9)	192 (28.7)	

(*continued on next page*)

TABLE 1 (*Continued*)

Characteristic	Population	Ejaculatory abstinence period (days)				*P*-value[1]
		2	3	4	5–7	
Embryo transfer, *n* (%)						**0.045**
No. of cycles with embryo transfer	6136	2800	1931	800	605	
No. of cycles with day 3 embryo transfer	2943 (48.0)	1350 (48.2)	900 (46.6)	386 (48.3)	307 (50.7)	
No. of cycles with day 5 embryo transfer	984 (16.0)	459 (16.4)	318 (16.5)	138 (17.3)	69 (11.4)	
No. of freeze all-cycles	2209 (36.0)	991 (35.4)	713 (36.9)	276 (34.5)	229 (37.9)	
Mean no. of embryos transferred on day 3	1.7	1.7	1.7	1.7	1.7	0.891
Mean no. of blastocysts transferred on day 5	1.2	1.2	1.2	1.2	1.2	0.884

Bold text indicates significance.

[1] Chi-squared test used to evaluate associations between ejaculatory abstinence period and category of embryo transfer (day 3, day 5, freeze-all).

Kruskal–Wallis test used to evaluate associations between continuous variables and ejaculatory abstinence period.

[2] Infertility factors were considered as dichotomous variables to represent patients with multiple causes of infertility.

IQR, interquartile range; BMI, body mass index; r-FSH, recombinant follicle-stimulating hormone; HMG, human menopausal gonadotrophin; PGT, preimplantation genetic testing; PGT-A, preimplantation genetic testing for aneuploidy; PGT-M, preimplantation genetic testing for monogenic/single gene disorders.

excluding those without complete data for multivariable analysis), top-quality blastulation rate (n = 1911 cycles included), cumulative positive β-HCG test rate (n = 3386 cycles included), cumulative clinical pregnancy rate (n = 3386 cycles included) and miscarriage rate (n = 2676 cycles included) did not find a significant association between the ejaculatory abstinence period and the examined rates (TABLE 3). The results of the multivariable-adjusted models with confounding factors are shown in Supplementary Table 2 (blastulation rate), Supplementary Table 3 (pregnancy) and Supplementary Table 4 (miscarriage).

DISCUSSION

Main findings

To date, there are no clear and evidence-based recommendations regarding the ejaculatory abstinence period prior to ICSI treatment of infertile couples. To answer this question, the results of 6919 ICSI cycles were analysed to assess the potential impact of the ejaculatory abstinence period. The findings showed that the ejaculatory abstinence period strongly influences oocyte fertilization in terms of 2PN and 3PN zygote rates, thus suggesting its impact not only on seminal parameters but also in terms of sperm functionality. Conversely, ejaculatory abstinence was not found to be associated with any later effects regarding embryological or clinical outcomes.

TABLE 2 MAIN CYCLE OUTCOMES ACCORDING TO EJACULATORY ABSTINENCE PERIOD

Main outcome	Population	Ejaculatory abstinence period (days)				Adjusted odds ratio (95% CI)	Adjusted *P*-value
		2	3	4	5–7		
Cycles (*n*)	6919	3152	2207	890	670		
Oocytes retrieved (*n*)	51,399	23,511	16,250	6670	4968		
Inseminated oocytes (*n*)	39,847	18,234	12,648	5142	3823		
1PN zygotes (*n*)	1204	543	411	146	104		
2PN zygotes (*n*)	30,220	13,870	9652	3821	2877		
3PN zygotes (*n*)	931	390	291	140	110		
1PN zygote rate	3.0%	3.0%	3.2%	2.8%	2.7%	1.00 (0.94–1.06)	0.969
2PN zygote rate	75.8%	76.1%	76.3%	74.3%	75.3%	0.97 (0.94–0.99)	**0.029**
3PN zygote rate	2.3%	2.1%	2.3%	2.7%	2.9%	1.14 (1.07–1.22)	**<0.001**

Bold text indicates significance.

One-pronuclear (1PN), 2PN and 3PN zygote rates are per total inseminated oocytes. The effect estimation of ejaculatory abstinence period on fertilization outcomes was obtained from Generalized Linear Mixed Model odds adjusted for maternal age, paternal age, maternal body mass index, total motile sperm count, location of semen collection, length of stimulation, number of oocytes retrieved, mature (metaphase II) oocytes, total dose of recombinant FSH/human menopausal gonadotrophin, oestrogen concentration on day of ovulation trigger, progesterone level on day of ovulation trigger, and infertility factors. The couple's identification code and the season of semen collection were incorporated into the model as random effects.

TABLE 3 SECONDARY OUTCOMES ACCORDING TO EJACULATORY ABSTINENCE PERIOD

Secondary outcome	Population	Ejaculatory abstinence period (days)				Adjusted odds ratio (95% CI)	Adjusted *P*-value
		2	3	4	5–7		
Outcomes before embryo transfer[1]							
Blastocyst culture cycles	3616	1650	1175	456	335		
No. of blastocysts	10,351	4818	3277	1313	943		
Blastulation rate, mean[2]	43.3%	43.9%	42.5%	44.1%	41.4%	0.97 (0.94–1.00)	0.168
Top-quality blastulation rate, mean[3]	13.4 %	12.5 %	14.3%	14.0%	14.4%	1.03 (0.97–1.10)	0.223
Outcomes after embryo transfer							
No. of cycles with embryo transfer[4]	6136	2800	1931	800	605		
No. of cumulative positive β-HCG[5]	2676	1225	824	373	254		
Cumulative positive β-HCG rate							
All cycles	43.6%	43.8%	42.7%	46.6%	42.0%	0.98 (0.93–1.04)	0.698
Cycles with embryo transfer on day 3	33.2%	32.3%	32.7%	36.3%	34.5%	1.03 (0.95–1.12)	0.441
Cycles with embryo transfer on day 5	63.4%	63.8%	63.2%	64.5%	59.4%	1.06 (0.89–1.26)	0.470
Freeze-all cycles	48.7%	50.0%	46.1%	52.2%	46.7%	0.93 (0.84–1.02)	0.118
No. of miscarriages[6]	920	413	275	140	92		
Miscarriage rate	34.4%	33.7%	33.4%	37.5%	36.2%	1.02 (0.94–1.11)	0.609
No. of cumulative clinical pregnancies[7]	1944	896	605	264	179		
Cumulative clinical pregnancy rate							
All cycles	31.7%	32.0%	31.3%	33.0%	29.6%	0.98 (0.92–1.04)	0.596
Cycles with embryo transfer on day 3	21.4%	21.6%	21.1%	22.7%	19.8%	0.95 (0.86–1.05)	0.358
Cycles with embryo transfer on day 5	48.9%	49.2%	48.7%	49.2%	47.8%	1.14 (0.97–1.35)	0.108
Freeze-all cycles	37.6%	38.1%	36.4%	39.1%	37.1%	0.97 (0.88–1.06)	0.553

[1] Outcomes exclude cycles with day 3 embryo transfer/embryo freezing.
[2] Number of blastocysts/number of two-pronuclear zygotes per cycle.
[3] Number of top-quality blastocysts/number of available blastocysts per cycle.
[4] Each cycle refers to an oocyte retrieval that subsequently led to at least one embryo transfer, whether from a fresh embryo or a frozen embryo in a later cycle.
[5] Number of cycles where at least one subsequent embryo transfer resulted in a positive β-HCG test.
[6] Number of cycles with at least one biochemical pregnancy, blighted ovum or embryo without observed fetal heartbeat calculated in embryo transfers derived from the same intracytoplasmic sperm injection treatment in which at least one positive β-HCG test was achieved.
[7] Number of cycles where at least one subsequent embryo transfer resulted in a clinical pregnancy.
Results for outcomes before embryo transfer were obtained from Generalized Linear Mixed Models and logistic regression adjusted for maternal age, paternal age, maternal body mass index, total motile sperm count, length of stimulation, mature (metaphase II) oocytes, total dose of recombinant FSH/human menopausal gonadotrophin, oestradiol concentration on day of ovulation trigger, progesterone concentration on day of ovulation trigger, and infertility factors. For outcomes after embryo transfer, the analysis was further adjusted for preimplantation genetic testing and day of embryo transfer. The couple's identification code and the season of semen collection were incorporated into the model as random effects.
HCG, human chorionic gonadotrophin.

The influence of ejaculatory abstinence on ICSI outcomes has been evaluated extensively in previous studies, but the results remain inconclusive. This is probably due to confounding variables, different clinical outcomes, different patient groups, and the heterogeneity of ejaculatory abstinence periods and sperm quality. This study investigated the effect of the ejaculatory abstinence period in a large population on early embryological and clinical outcomes using a Generalized Linear Mixed Model with a quasi-binomial distribution, which is the best way to analyse proportions with a large variance, such as the study outcomes. Statistical models were corrected for all known confounding factors. Additionally, the effect of collinearity between covariates was minimized by removing those with a high VIF. Use of these methods enabled the authors to increase the accuracy of the model estimates.

A highly significant increase in semen volume, sperm concentration and TMC was observed as the ejaculatory abstinence period increased. In contrast, a prolonged ejaculatory abstinence period resulted in a slight, but significant, reduction in progressive motility. It should be underscored that these observations were derived from a purely descriptive analysis, devoid of statistical correction. These findings support the results of three recent systematic reviews which reported that the longer the ejaculatory abstinence period, the higher the seminal volume, sperm concentration and total sperm count (*Ayad et al., 2017*; *Hanson et al., 2018*; *Sokol et al., 2021*). Nonetheless, results related to sperm motility remain inconclusive.

In addition, analysis of the associations between ejaculatory abstinence and embryological results of ICSI cycles showed that spermatozoa derived from semen following a longer ejaculatory abstinence period are less likely to achieve successful fertilization, with a consequent reduction in usable zygotes. Specifically, the likelihood of obtaining 2PN zygotes from all inseminated oocytes was reduced significantly by 3% for each additional day of ejaculatory abstinence. Conversely, the likelihood of obtaining 3PN zygotes from all inseminated oocytes increased significantly by 14% for each day of ejaculatory abstinence. No differences were observed in blastulation rate per cycle and top-quality blastocyst rate per cycle.

In light of these findings, the effect sizes related to 2PN and 3PN zygotes may initially seem subtle. However, it is crucial to underscore that each additional day of ejaculatory abstinence augments this effect. Thus, the cumulative impact of prolonged ejaculatory abstinence can influence fertilization outcomes considerably. Notably, the adjustment of ejaculatory abstinence represents a strategic, cost-neutral intervention, poised to optimize the zygote pool and enhance the yield of viable zygotes without any adverse effects.

Few studies have focused on the impact of the ejaculatory abstinence period on fertilization, and to the authors' knowledge, no studies have examined the 3PN zygote rate. Previous studies included a heterogeneous mix of retrospective studies which used different ejaculatory abstinence periods and different study populations. Nevertheless, some of the studies observed no association between the ejaculatory abstinence period and the fertilization rate (*Azizi et al., 2022*), even when considering a single oocyte in infertile couples undergoing ICSI cycles *Gutpa et al. (2021)* observed no difference when considering a single oocyte in infertile couples undergoing ICSI cycles. In contrast, *Borges et al. (2019)* observed a negative impact of the ejaculation abstinence period on the fertilization rate per cycle in ICSI cycles, which agrees with the present findings. *Borges et al. (2019)* reported that 4 days of ejaculatory abstinence was the threshold for observing a negative effect on sperm DNA and ICSI results. Unfortunately, they did not report the number of abnormal fertilization phenomena in terms of 3PN zygotes.

The presence of three (or more) pronuclei is indicative of abnormal fertilization. A 3PN zygote can be produced as the result of the entry of two spermatozoa during conventional IVF, the entry of diploid spermatozoa (*Rosenbusch et al., 2008*), a diploid maternal chromosome set (*Rosenbusch et al., 2008*), or failure to extrude the second polar body (*Reichman et al., 2010*). The present study evaluated the contribution of the oocyte by considering confounders such as ovarian stimulation, maternal BMI and maternal age on the day of oocyte retrieval. However, the contribution of spermatozoa should not be underestimated. Prolonged ejaculatory abstinence may be associated with impaired sperm DNA because, in the absence of ejaculation, cells accumulate in the epididymis and are subjected to a potentially detrimental seminal microenvironment for a prolonged time, mainly due to reactive oxygen and nitrogen species (*Agarwal et al., 2003*; *Pons et al., 2013*; *Sukprasert et al., 2013*). It is known that high levels of reactive oxygen species may have a negative effect on capacitation, the acrosome reaction, and the motility of hyperactivated human spermatozoa (*de Lamirande et al., 1993*; *Zini et al., 1995*), thus compromising their fertilizing competence (*du Plessis et al., 2010*). Furthermore, during ICSI, where natural sperm selection is circumvented, functionally suboptimal spermatozoa (with fragmented DNA) may be injected into the oocyte causing early paternal effects (*Barroso et al., 2009*), such as impaired formation of 2PN zygotes at fertilization.

Furthermore, the statistical analyses revealed an interesting result regarding the relationship between TMC, ejaculation abstinence period and 3PN zygote rate. In the descriptive analysis (**TABLE 1**), the TMC values for the four groups (2, 3, 4 and 5–7 days of ejaculatory abstinence) differed, with the group with the longest ejaculatory abstinence period having the highest median TMC value. The subsequent univariate analysis showed a significant relationship between TMC and the 3PN zygote rate, and between the ejaculatory abstinence period and the 3PN zygote rate (Supplementary Table 1). The multivariable model results showed that the relationship between the ejaculatory abstinence period and the 3PN zygote rate remained significant regardless of TMC and other covariates. No association was found between TMC and the 3PN zygote rate (Supplementary Table 1). These results show that TMC differs with the ejaculatory abstinence period, but it is not a mediator of the effect of the ejaculatory abstinence period on the 3PN zygote rate. It is postulated that a longer ejaculatory abstinence period affects other seminal characteristics that ultimately contribute to a higher 3PN zygote rate.

Strengths and limitations

To the best of the authors' knowledge, this is the first study to analyse fertilization in ICSI cycles with a focus on abnormal fertilization patterns (observation of 3PN zygotes). A further strength of this analysis is that it deals with a large population over a long time frame, and with a wide range of semen quality. Moreover, statistical models characterized by a quasi-binomial distribution were used to determine proportions more accurately as response variables. All statistical models were corrected for potential confounders and collinearity between variables. Finally, by reporting the cumulative clinical pregnancy rate, this study seeks to provide a more comprehensive assessment of treatment efficacy across multiple cycles (*Maheshwari et al., 2015*).

This study also has some limitations that deserve attention. The retrospective design of the study is the main limitation of this analysis. Although no particular indication was given to the patients regarding the number of days to abstain from ejaculation, it is not possible to exclude the presence of hidden biases for which it was not possible to adjust the analysis. In addition, it is also important to note that oocyte fertilization patterns were assessed with static observations without any time-lapse imaging technology. This may reflect some biases in terms of incorrect observation of pronuclei, inability to estimate the number of polar bodies reliably, and the novel morphokinetic markers of fertilization phenomenon (*Bartolacci et al., 2021*). Nonetheless, it is important to note that at the study centre, the observed number and rate of 2PN zygotes, failed fertilization rate and oocyte degeneration (data not shown) were consistent with the key performance indicator values indicated in the Vienna consensus (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*). This indicates the reliability and accuracy of the observational methodology at the IVF centre.

CONCLUSIONS

Over the years since the introduction of ICSI, the idea has emerged that spermatozoa abnormalities may contribute to functional or chromosomal abnormalities that have not yet been studied. To the authors' knowledge, this is the first study to investigate the contribution of spermatozoa in abnormal oocyte fertilization. Although recent articles have shown that a proportion of blastocysts that develop from 3PN zygotes are euploid (*Kemper et al., 2023*; *Mutia et al., 2019*), abnormal pronuclear development is one of the first morphological parameters used for embryo selection in MAR clinics worldwide. Thus, limiting the formation of 3PN zygotes could optimize the number of usable embryos obtained from the same oocyte cohort.

These findings support the concept that current recommendations regarding ejaculatory abstinence may need to be modified in infertile couples undergoing MAR cycles. In particular, shorter ejaculatory abstinence periods are associated with a higher percentage of usable zygotes, and thus the efficiency of an ICSI cycle can be increased without additional cost.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

G.C.C., A.A. and L.P. conceptualized the study. G.C.C., S.M. and D.M. acquired the data. G.C.C. drafted the manuscript. D. M., N.S. and A.N. performed the statistical analyses. L.P. oversaw the statistical analyses. All authors interpreted the results. All authors critically revised the manuscript for important intellectual content. L.P. had full access to the data in the study, and took full responsibility for the integrity of the data and the accuracy of the data analyses. All authors read and approved the final paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.103401.

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