Human Reproduction, Vol.35, No.1, pp. 32–43, 2020
Advance Access Publication on January 9, 2020 doi:10.1093/humrep/dez234

human reproduction

ORIGINAL ARTICLE *Embryology*

The effect of ICSI-related procedural timings and operators on the outcome

Roberta Maggiulli[1,§], Danilo Cimadomo[1,2,§], Gemma Fabozzi[1,2], Letizia Papini[2], Lisa Dovere[1], Filippo Maria Ubaldi[1,2], and Laura Rienzi[1,2,*]

[1]Clinica Valle Giulia, G.EN.E.R.A. Centers for Reproductive Medicine, Rome, Italy [2]G.EN.E.R.A. Umbria, G.EN.E.R.A. Centers for Reproductive Medicine, Umbertide, Italy

*Correspondence address. E-mail: rienzi@generaroma.it

Submitted on April 8, 2019; resubmitted on September 11, 2019; editorial decision on October 1, 2019

STUDY QUESTION: Do the ICSI-related procedural timings and operators affect the outcomes of an ART cycle?

SUMMARY ANSWER: The ICSI-related timings and operators do not associate with the mean blastulation rate per cohort of inseminated oocytes and the cumulative delivery rate per concluded cycle, except for a mild association between the times from induction of ovulation to oocyte denudation and the former outcome.

WHAT IS KNOWN ALREADY: In ART, specific timings, protocols and conditions must be complied with to preserve gamete developmental and reproductive competence during the required manipulations. ICSI represents a groundbreaking advancement that has been widely implemented. Nevertheless, the studies that examined the putative impact of ICSI-related procedural timings were mainly conducted in old-fashioned settings or in good prognosis patients. No report addressed issues like operators' skills and experience and uncertainties exist dealing with the effect of cumulus cells in the pre-incubation period *in vitro* before ICSI. However, all this information is crucial to efficiently plan the daily routine of an IVF lab, fill the existing gaps of knowledge and define proper key performance indicators.

STUDY DESIGN, SIZE, DURATION: Observational study conducted at a private IVF clinic (January 2016 to January 2018). We included all consecutive ICSI procedures ($n = 1084$ infertile couples undergoing 1444 cycles with or without preimplantation genetic testing (PGT); mean ± SD maternal age: 38.1 ± 4.0 years) with fresh autologous oocytes ($n = 7999$ oocytes, 5.5 ± 3.2 per treatment) inseminated with fresh non-donor ejaculated sperm. All operators and critical procedural timings (induction of ovulation to oocyte denudation, denudation and ICSI) were automatically recorded through an electronic witnessing system. The primary outcome measure was the cumulative delivery rate among both non-PGT and PGT-concluded cycles (i.e. delivery achieved or no supernumerary cryopreserved blastocyst available). The secondary outcome measure was the mean blastulation rate per cohort of inseminated oocytes. All confounders were registered and included in generalized linear models and multivariate logistic regression analyses.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Fourteen and 12 operators were involved in denudation and ICSI procedures, respectively. Denudation was performed after 4.1 ± 1.2 h (2–7) of pre-incubation *in vitro* after oocyte retrieval, and ICSI was started immediately after. Beyond procedural timings and operators, all the putative confounders (patients' and cycles' characteristics) on the primary and/or secondary outcomes were systematically registered and included in the statistical analyses.

MAIN RESULTS AND THE ROLE OF CHANCE: The mean time from induction of ovulation to oocyte denudation was 39.3 ± 1.3 h. The mean procedural timings for denudation and ICSI were 8.1 ± 3.8 and 12.6 ± 6.4 min; both these variables were significantly dependent on the number of inseminated oocytes and the operators' skills and experience. The overall mean blastulation rate per cohort of inseminated oocytes was 34.0 ± 27.9%. This outcome was significantly associated with the time from induction of ovulation to oocyte denudation (mean blastulation rate stable in the time interval 38–42 h, but significantly higher for timings <38 h), maternal age (the mean blastulation rate drops especially beyond the age of 40 years) and categorized sperm concentration (highest mean blastulation rate for sperm concentrations ≥15 mil/ml and lowest for cryptozoospermic patients) through a generalized linear model that showed an adjusted $r^2 = 0.053$ ($P < 0.01$). No association was found for denudation and ICSI timings and operators. Lastly, when adjusted for maternal age and number of inseminated oocytes, both ICSI-related procedural timings and operators did not associate with the cumulative delivery rate among both non-PGT- or PGT-concluded cycles.

LIMITATIONS, REASONS FOR CAUTION: This is a single private IVF center study. Its reproducibility should be assessed in different laboratory conditions, with different protocols and in the hands of different operators. Moreover, specific studies are warranted to address

§These authors contributed equally

the beneficial/detrimental effect of the other putative confounders under investigation (e.g. kind of ovulation trigger, culture media, incubator, etc.).

WIDER IMPLICATIONS OF THE FINDINGS: Proactive communication between the embryologists and the clinicians might contribute to a reasoned and more efficient organization of the daily workload and increase the mean blastulation rate, especially when poor prognosis couples (advanced maternal age, reduced sperm count and/or ovarian reserve) are treated.

STUDY FUNDING/COMPETING INTEREST(S): No funding. The authors declare no conflict of interest related to the present study.

Key words: oocyte metaphase II rate / oocyte denudation / ICSI timings / live birth / blastocyst / electronic witnessing system / IVF quality control / induction of ovulation

Introduction

ICSI represents one of the main breakthroughs in ART. It provided the embryologists worldwide with a tool to bypass the mechanical barriers irrespective of semen source and quality and inject a selected sperm directly into the cytoplasm of an oocyte (Palermo *et al*., 1992; Palermo *et al*., 1995). In a review published in 2017, Palermo and colleagues celebrated the 25th anniversary of ICSI. In that manuscript, they underlined the data collected from the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) that outlined a prevalence of ICSI ranging between 60 and 98% of the ART treatments performed across 60 different countries (Palermo *et al*., 2017). These numbers demand attention, especially considering the limited evidence published to outline the ideal workflow of this technique, which undoubtedly stands out as a game-changer in the history of ART.

Gaps in knowledge include the definition of the optimal timing between induction of ovulation (IO) and insemination, as well as of oocyte denudation (Den) and ICSI itself; similarly, the putative impact of less experienced operators or different *in vitro* pre-incubation strategies on ICSI-related outcomes needs to be assessed. Lastly, only a few reports have addressed the putative sensitivity of oocytes retrieved from poor prognosis women (e.g. advanced maternal age, AMA) to *in vitro* manipulations.

Oocyte competence requires both nuclear and cytoplasmatic maturations (Rienzi *et al*., 2012; Coticchio *et al*., 2015), which should be achieved during the pre-incubation time before insemination, and published results dealing with this topic are controversial. Specifically, both the duration of pre-incubation *in vitro* and the whether or not surrounding cumulus cells are removed have both resulted in conflicting results when compared with outcomes such as oocyte metaphase II (MII) rate, fertilization, cleavage and pregnancy rates mainly after Day 2/3 embryo transfer (ET) (Rienzi *et al*., 1998; Van de Velde *et al*., 1998; Yanagida *et al*., 1998; Andrews *et al*., 2001; Hassan, 2001; Jacobs *et al*., 2001; Ho *et al*., 2003; Dozortsev *et al*., 2004; Isiklar *et al*., 2004; Falcone *et al*., 2008; Patrat *et al*., 2012; Garor *et al*., 2015; Barcena *et al*., 2016; Pujol *et al*., 2018; Mizuno *et al*., 2019).

The largest dataset focused on ICSI-related timings has been published by Barcena and colleagues (~4000 ICSI cycles). These authors observed no impact on live birth rates (LBR) imputable to pre-incubation timings ranging between 1 h and 25 min up to 17 h and 13 min in the absence of surrounding cumulus cells. However, Barcena's study was conducted in an ideal population of fertile young egg donors (mean age ~27 years, ranging 18–35). In other terms, it did not outline whether the oocytes suffering from the issues of *in vivo* aging (Cimadomo *et al*., 2018) might be more sensitive to the *in vitro* stresses involved by ICSI and its related manipulations (Wale and Gardner, 2016). Therefore, the same group published a second study in 2018 which instead included 1468 ICSI cycles from 1322 AMA women undergoing ART with their own eggs (mean age ~38 years, ranging 21–46) (Pujol *et al*., 2018). Similarly, in this case, no significant impact on LBR was reported when denuded oocytes were submitted to extended pre-incubation timings between oocyte retrieval (OR) and ICSI, except for a negative trend if such interval was longer than 10 h.

In conclusion then, Barcena's and Pujol's studies together provide a comprehensive overview of the effect of prolonged denuded oocyte pre-incubation timings before ICSI on the clinical outcomes of an ART cycle (mostly with a Day 2/3 ET strategy). Still, some information are missing: (i) the putative beneficial/detrimental effect of the surrounding cumulus cells during the pre-incubation period OR-to-ICSI, (ii) the putative impact of ICSI-related procedural timings on the mean blastulation rate (m-BR) per cohort of inseminated oocytes and on the cumulative delivery rate (CDR) per cycle (as defined by (Zegers-Hochschild *et al*., 2017, 2017)), as well as (iii) the effect of the operators involved in each procedure. To this end, we designed this study in which all the ICSI timings were accounted (i.e. IO-to-Den, Den and ICSI) together with the operators involved, automatically and systematically registered via an electronic witnessing system (EWS) (Rienzi *et al*., 2015). The primary outcome measure was defined as the CDR per cycle. The main secondary outcome measure was instead the m-BR per cohort of inseminated oocytes. Both these outcomes were addressed in an unselected population of infertile women undergoing ICSI, with or without PGT, with own fresh oocytes and fresh non-donor ejaculated sperm at our private IVF center.

Materials and Methods

Study design

This is an observational study conducted at a private Italian IVF clinic between January 2016 and January 2018. All elective ICSI procedures ($n = 1444$ from 1084 patients) during both non-PGT ($n = 452$) and PGT ($n = 992$) cycles performed with fresh own oocytes ($n = 7999$) inseminated with fresh non-donor ejaculated sperm were included. Only procedures entailing a single round of insemination were included (i.e. maximum 12 oocytes). All operators, number of ICSI procedures per working day and critical procedural timings (i.e. IO-to-Den time in hours, Den and ICSI procedures time in minutes) were recorded automatically through an EWS (RI Witness^{TM}, cooper surgical, USA). Fourteen and 12 operators were involved in Den and ICSI proce-

dures, respectively. The operators had minimum of 1 and maximum of 14 years of experience. A sub-analysis was also performed by categorizing their experience as ≤3 years (i.e. threshold to apply for the 'clinical embryologist' certificate at ESHRE), 4–6 years (i.e. threshold to apply for the 'senior embryologist' certificate at ESHRE) and > 6 years.

The primary outcome measure was the effect of the ICSI procedural timings on the CDR among concluded cycles. A cycle was considered concluded when at least one delivery was achieved or no supernumerary cryopreserved transferable blastocysts were still available (Zegers-Hochschild *et al*., 2017, 2017). The secondary outcome measure was the m-BR per cohort of inseminated oocytes. All viable embryos reaching the blastocyst stage up to Day 7 post-insemination were either transferred or cryopreserved according to the Italian Law 40/2004. All confounders were prospectively registered in a relational database (Fertilab Manager, Italy) and used as corrective measures in generalized linear models and multivariate logistic regression analyses. All patients signed an informed consent to the anonymous retrospective analysis and publication of the data related to their cycles. Institutional Review Board (IRB) approval was obtained from the clinic.

ART procedures

Ovarian stimulation was conducted with recombinant-gonadotrophins in antagonist or agonist protocols, as previously described (Ubaldi *et al*., 2015; Vaiarelli *et al*., 2018). When three follicles of ≥18 mm diameter were recorded by ultrasound, induction of oocyte final maturation was triggered with the administration of 10 000 IU of hCG (Gonasi, IBSA, Switzerland) or a single subcutaneous bolus of GnRH agonist (50 IU buserelin; Sanofi-Aventis, Canada). The latter was adopted in case of risk of ovarian hyperstimulation syndrome and in all cases when a freeze-all strategy was planned (i.e. hyper-response, inadequate endometrium or PGT). The time of IO was confirmed by the patient to the embryologist at the time of OR and noted in the relational database (mean ± SD, range; 35.2 ± 0.4, 34–37 h). Immediately after egg collection, follicular fluids were screened for cumulus-oocyte complexes (COCs). Once identified, COCs were rinsed, transferred into pre-equilibrated IVF medium (continuous: CSCM, Irvine Scientific, or sequential: Quinn's Advantage Fertilization, Origio) in a sterile tube and incubated at 37°C in controlled atmosphere (6%CO_2 and 5%O_2, either it was single step or sequential culture system) until Den. The time between OR and Den was automatically registered from the EWS and then added to the time between IO and OR to calculate the hours IO-to-Den. Oocyte stripping was performed immediately before the insemination in a HEPES-buffered medium (Modified HTF Medium, Irvine Scientific, USA or Quinn's Advantage Medium with HEPES, Origio, USA) supplemented with human serum albumin (5 mg/ml, Irvine Scientific or Quinn's Advantage,) and containing 20 IU/ml of bovine-derived hyaluronidase (Irvine Scientific or Quinn's Advantage). COCs (up to 12 per procedure) were first exposed to hyaluronidase to disperse the cumulus cells and then pipetted repeatedly through a Pasteur pipette with an inner diameter of ∼250 µm for up to 30–40 s. Once initial cell dissociation was observed, further Den was carried out removing the corona cells through denuding pipettes with decreasing inner diameters (170 to 140 µm). After Den, the oocytes were thoroughly washed and examined under an inverted microscope to assess integrity and stage of maturation. Only MII oocytes were considered for injection. The times of Den and of ICSI were automatically registered from the EWS. ICSI was performed in a Petri culture dish containing six pre-warmed HEPES-buffered medium microdroplets supplemented with human serum albumin (HSA) and one drop of PVP (polyvinylpyrrolidone solution with HSA—7%, Irvine Scientific or Origio) covered by mineral oil (Oil for Tissue Culture, Irvine Scientific or Origio). Sperm samples were analyzed the day of each ICSI cycle and processed according to the WHO guidelines as previously described (Mazzilli *et al*., 2017). After processing, few microliters of sperm suspension (2–5 µl) were transferred to the left side of the PVP droplet in order to allow for sperm migration and facilitate sperm selection, and up to two oocytes were allocated in each drop. At the inverted microscope (magnification ×400), a normal spermatozoon was selected and immobilized by slicing on the sperm tail using the injection needle. When the first polar body could be visualized at 6 o'clock, the oocyte was gently secured by the holding pipette and intracytoplasmic sperm injection was performed. The oolemma breakage was conducted by applying a gentle suction by the injection pipette; then, the spermatozoon was slowly released into the ooplasm. The injection pipette was withdrawn gently and the oocyte released from the holding pipette. The procedure was repeated for each oocyte. Injected oocytes were then rinsed and placed in pre-equilibrated culture medium (continuous: CSCM, Irvine Scientific, or sequential: Quinn's Advantage Cleavage-Blastocyst, Origio; only the latter culture strategy entailed a media change-over in Day 3 post-insemination) in a controlled atmosphere (6%CO_2 and 5%O_2). The presence of two equally sized pronuclei was assessed 17 ± 1 h after insemination. Embryos were cultured in separate 30 µl drops up to the blastocyst stage (Days 5–7) in an atmosphere containing 6%CO_2 and 5%O_2 in either a standard incubator (MINC, Cook Medical, USA) or time-lapse undisturbed system (EmbryoScope, Vitrolife, Sweden). PGT was conducted according to the indications after thorough genetic and gynecological counseling. If required, trophectoderm biopsy was performed according to (Capalbo *et al*., 2014), a method that does not entail zona pellucida opening at the cleavage stage. Of note, both trophectoderm biopsy–related timings and operators were already shown previously not to impact the clinical outcomes at our center (Capalbo *et al*., 2016; Maggiulli *et al*., 2019). Vitrification and warming procedures were conducted as previously detailed in (Cobo *et al*., 2012; Cimadomo *et al*., 2018). ETs were conducted as described in (Ubaldi *et al*., 2015; Vaiarelli *et al*., 2018). Specifically, in case of freeze-all, ETs were performed in an artificial (estradiol valerate and progesterone) or natural cycle. All pregnancies were followed up to delivery or miscarriage.

Statistics

Categorical variables are presented as percentages with 95% CI. Chi-squared or Fisher's exact tests were used to asses statistically significant differences. Continuous variables are presented as mean ± SD and range. Shapiro–Wilk tests were conducted to investigate whether the data followed a normal (Gaussian) distribution. Kruskal–Wallis or Mann–Whitney *U* tests were performed to assess statistically significant differences. The oocyte MII rate (MII oocytes per COCs retrieved) and the m-BR (blastocysts obtained per MII oocytes inseminated) across IO-to-Den timing were shown through locally estimated scatterplot

smoothing (LOESS) regression with a fit to 50% of the points and an Epanechnikov weight function. The same approach was used also to plot the m-BR across increasing maternal age. The association between the variables under investigation and the m-BR per cohort of oocytes was assessed through generalized linear models adjusted for confounders. The main confounders tested were maternal/paternal age and karyotype, categorized sperm concentration (≥15, 6–14 and ≤5 mil/ml or cryptozoospermia defined according to the WHO guidelines and (Barratt *et al.*, 2017)), main cause of infertility, protocol of stimulation and trigger of ovulation, culture media, incubator, number of ICSI procedures performed during the same working day, and sequential number of ICSI cycle undertaken by each patient during the study period. Generalized linear models were adopted also to investigate the association between the procedural timings and the operators involved. These data were corrected also for number of oocytes processed and operators' clinical experience in years. The associations with the CDR among concluded cycles were assessed through multivariate logistic regression analyses. This last analysis was conducted for non-PGT and PGT cycles separately due to the significantly different population of patients undergoing these two clinical strategies (Supplementary Table SI). In case of non-PGT cycles, beyond the same confounders previously listed, also the number of oocytes collected, the strategy for the first ET (fresh or freeze-all) and the protocol for endometrial preparation were accounted among confounders. The software SPSS (IBM, USA) was used for statistics.

Results

Tables I and II summarize the main patient and cycle and procedural and embryological data. In detail, 1444 ICSI cycles were performed during the study period from 1084 patients (up to 5 ICSI cycles per patient), on average 3.6 ± 2.2 (1–14) procedures per day. The mean maternal and paternal ages were 38.1 ± 4.0 years (21–45) and 40.8 ± 5.7 years (26–69), respectively. Supplementary Table SII summarizes the mean maternal age and sperm concentration across patients undergoing 1, 2, 3, 4 or 5 ICSI cycles during the study period. Most of the cycles involved PGT ($n = 992/1444$, 68.7%) (main data summarized in Supplementary Table SI). An antagonist protocol was adopted for almost all the ovarian stimulations ($n = 1374/1444$, 95.2%), and the hCG or GnRH agonist was almost equally used to trigger ovulation ($n = 661/1444$, 45.8% and $n = 783/1444$, 54.2% respectively).

The oocyte MII rate (on average 73.4 ± 20.5% per cohort of COCs retrieved) did not show any association with the IO-to-Den timing (Supplementary Figure S1), also when corrected for maternal age and number of COCs retrieved in a generalized linear model. Overall, 7999 MII oocytes were inseminated, 5.5 ± 3.2 (1–12) per procedure. The mean hours IO-to-Den were 39.4 ± 1.3 h (36.5–42), the mean time of Den was 8.1 ± 3.8 min (2–20) and the mean time of ICSI was 12.6 ± 6.3 min (2–36). Among the inseminated oocytes, 5774 were fertilized, on average 4.0 ± 2.6 (0–12) per cohort, resulting in a mean fertilization rate of 71.0 ± 27.4% (0–100%). The blastocysts obtained were 2818, 1.9 ± 1.8 (0–9) per cohort of inseminated oocytes. The m-BR was 34.0 ± 27.9% (0–100%).

Table I Patient and cycle data. All data are *n* (%) unless stated otherwise.

Patients, *n*	**1084**
Maternal age year, mean ± SD (min–max)	38.1 ± 4.0 (21–45)
Paternal age year, mean ± SD (min–max)	40.8 ± 5.7 (26–69)
Parental karyotype:	
Normal	1064 (98.2)
Abnormal	20 (1.8)
Sperm concentration 10^6/ml:	
≥15	1037 (71.8)
6–14	224, (5.5)
≤5	129 (8.9)
Cryptozoospermia	54 (3.7)
Main cause of female infertility:	
Idiopathic	900 (83.0)
Tubal factor	83 (7.7)
Endometriosis	70 (6.5)
Endocrine-ovulatory	31 (2.9)
ICSI cycles performed during the study period:	
One	824 (76.0)
Two	189 (17.4)
Three	46 (4.2)
Four	21 (1.9)
Five	4 (0.4)
Cycle, *n*	**1444**
ICSI procedures per working day, mean ± SD (min–max)	3.6 ± 2.2 (1–14)
Preimplantation genetic testing (PGT):	
No	452 (31.3)
Yes	992 (68.7)
Protocol of ovarian stimulation:	
Antagonist	1374 (95.2)
Agonist	70 (4.8)
Trigger of ovulation:	
hCG	661 (45.8)
Agonist	783 (54.2)
Culture media:	
Continuous	1165 (80.7)
Sequential	279 (19.3)
Incubator:	
Standard	1024 (71.0)
Undisturbed	420 (29.0)

The time of Den and of ICSI are different across different operators but do not associate with the m-BR per cohort of inseminated oocytes

The time of Den was significantly variable across the 14 operators involved (Kruskal–Wallis test, $P < 0.01$). Figure 1a and b shows the box plots of this timing across each operator and the related mean values. The fastest operator denuded on average 5.4 ± 3.2 (1–12) oocytes across 174 procedures in 6.6 ± 3.0 min (3–20), while the slowest denuded 6.0 ± 3.3 (1–12) oocytes across 24 procedures in

Table II Main timings and embryological outcomes per ICSI cycle. Data are mean ± SD (min–max) unless stated otherwise. IO, induction of ovulation; Den, oocyte denudation; MII, metaphase II.

ICSI cycles, *n*	**1444**
Hours between IO and Den	39.4 ± 1.3 (36.5–42)
Duration of Den min	8.1 ± 3.8 (2–20)
Duration of ICSI min	12.6 ± 6.3 (2–36)
MII oocytes, *n*	**7999**
MII oocytes inseminated per cycle	5.5 ± 3.2 (1–12)
Fertilized oocytes, *n*	**5774**
Fertilized oocytes per cohort of inseminated oocytes	4.0 ± 2.6 (0–12)
Fertilization rate per cohort of inseminated oocytes, %	71.0 ± 27.4 (0–100)
Blastocysts, *n*	**2818**
Blastocysts per cohort of inseminated oocytes	1.9 ± 1.8 (0–9)
Blastulation rate per cohort of inseminated oocytes, %	34.0 ± 27.9 (0–100)

13.9 ± 5.1 min (6–20). Figure 1c and d shows the mean time of Den according to the operators' experience clustered in three groups (≤3, 4–6 and > 6 years). Also, in this case, a significant difference was reported (Kruskal–Wallis test, $P < 0.01$). The statistically significant association between the Den timing and the Den operator (partial eta-squared 0.147, $P < 0.01$) was confirmed in a generalized linear model (r-squared = 0.45) adjusted for the number of oocytes processed (partial eta-squared 0.380, $P < 0.01$). Nevertheless, such variability had no effect on the m-BR: neither the time of Den ($P = 0.5$) nor the Den operator ($P = 0.8$) associated with this outcome.

The time of ICSI was also significantly variable across the 12 operators involved (Kruskal–Wallis test, $P < 0.01$). Figure 2a and b shows the box plots of this timing across each operator and the related means. The fastest operator inseminated on average 5.5 ± 3.2 (1–12) oocytes across 197 procedures in 10.4 ± 5.4 min (2–36), while the slowest inseminated 5.4 ± 2.7 (2–11) oocytes across 23 procedures in 18.7 ± 8.3 min (9–36). Figure 2c and d shows the mean time of ICSI according to the operators' experience clustered in three groups (≤3, 4–6 and > 6 years). Also, in this case, a significant difference was reported (Kruskal–Wallis test, $P < 0.01$). The statistically significant association between the ICSI timing and the ICSI operator (partial eta-squared 0.254, $P < 0.01$) was confirmed in a generalized linear model (r-squared = 0.74) adjusted for the number of oocytes processed (partial eta-squared 0.713, $P < 0.01$). Nevertheless, also in this case, such variability had no effect on the m-BR: neither the time of ICSI ($P = 0.1$), nor the ICSI operator ($P = 0.7$) associated with this outcome.

Time IO-to-Den, maternal age and sperm concentration are significantly associated with the m-BR per cohort of inseminated oocytes

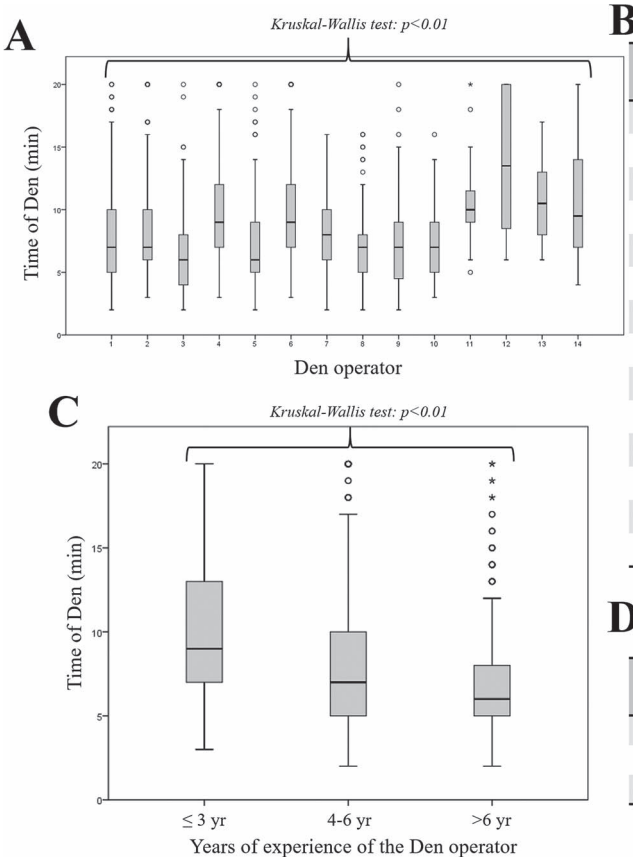
A significant association was found between the m-BR per cohort of inseminated oocytes and the hours from IO-to-Den. Specifically, Fig. 3a shows the LOESS curve that outlines a constant decrease in the m-BR between 36 and 38 h and a plateau between 38 and 42 h. Specifically, the m-BR was 44.6 ± 27.5% when Den was started <37 h from the IO ($n = 62$ cycles) then gradually decreased to 39.8 ± 27.2% for the range 37–37.5 h ($n = 59$ cycles) to reach 36.9 ± 28.4% for the range 37.5–38 h ($n = 102$ cycles). Lastly, a stable 33.0 ± 27.8% m-BR per cohort of inseminated oocytes was reported when Den was started >38 h from the IO ($n = 1221$ cycles).

The strongest association was reported between m-BR and maternal age at OR (Fig. 3b). In detail, this outcome was stable for women aged <35 years ($n = 234$ cycles, 41.2 ± 26.5%) then started decreasing in the range 35–40 years ($n = 541$ cycles, 37.8 ± 27.7%), until it dropped in women >40 years ($n = 669$ cycles, 28.6 ± 27.6%). Also, the categorized sperm concentration showed an association with the m-BR (Fig. 3c) that resulted in a gradual decrease from 35.6 ± 28.6% with a sperm concentration ≥ 15 mil/ml to 26.0 ± 26.8% for cryptozoospermic patients (Kruskal–Wallis test: $P < 0.01$). The generalized linear model (adjusted r-squared = 0.053) investigating the effect of all putative confounders on the m-BR per cohort of inseminated oocytes confirmed a significant association only for the time from IO-to-Den (partial eta-squared = 0.005, power = 76%, $P < 0.01$), maternal age (partial eta-squared = 0.04, power = 99%, $P < 0.01$) and sperm concentration (partial eta-squared = 0.017, power = 99%, $P < 0.01$).

Of note, no association was reported between the m-BR per cohort of inseminated oocytes and the sequential number of ICSI cycle performed by each specific couple ($P = 0.12$), even if adjusted for the time IO-to-Den, maternal age and sperm concentration ($P = 0.61$). Supplementary Figure S2A shows the overall m-BR for first, second, third, fourth and fifth ICSI cycles, while Supplementary Figure S2 B–F shows the m-BR in each sequential ICSI cycle achieved by the population of patients undergoing just 1, 2, 3, 4 or 5 treatments during the study period. In both cases, no significant difference was found.

No association was found between ICSI-related timings and operators with the CDR among concluded treatments

The ICSI-related timings were tested for their association with the CDR among concluded cycles (i.e. $n = 1345/1444$, 93.1% of the started cycles). Overall, 332 cycles resulted in at least one live birth ($n = 332/1345$, 24.7%). These data were addressed separately for non-PGT and PGT cycles since the populations of patients and cycle strategies were heterogenous in the two groups (Supplementary Table I). The former resulted in a 17.7% (95% CI 14.2–21.8) CDR per concluded cycle ($n = 72/407$; 45 cycles still open), while the latter resulted in a 27.7% (95% CI 24.9–30.7) CDR per concluded cycle ($n = 260/938$; 54 cycles still open). Table III presents the ICSI-related timings across the concluded non-PGT and PGT cycles resulting in no delivery versus at least one live birth. No difference was reported for the IO-to-Den timing, while significant differences were reported for the time of Den and of ICSI (Mann–Whitney U test, $P < 0.01$). However, both these associations were imputable to the higher number of oocytes retrieved and therefore processed, during the cycles resulting in at least one live birth (Mann–Whitney U test, $P < 0.01$), rather than to the procedural timings themselves. In fact, when the Den and ICSI timings were assessed in a multivariate logistic regression analysis adjusted for maternal age at OR and number of MII



B

Op.	Experience (yr)	N of procedures	N of oocytes mean±SD, min-max	Time (min) mean±SD, min-max
1	4	235	5.6±3.3, 1-12	7.7±3.9, 2-20
2	6	201	5.6±3.3, 1-12	8.0±3.5, 3-20
3	12	174	5.4±3.2, 1-12	6.6±3.0, 3-20
4	2	157	5.7±3.0, 1-12	9.6±3.8, 3-20
5	14	136	5.3±3.4, 1-12	7.1±3.6, 2-20
6	6	121	5.9±3.2, 1-12	9.5±3.9, 3-20
7	6	114	5.3±2.9, 1-12	7.9±3.0, 2-16
8	6	111	4.9±2.9, 1-12	7.2±2.9, 2-16
9	11	60	5.2±3.3, 1-12	7.7±4.0, 2-20
10	4	51	5.5±3.0, 1-12	7.4±2.8, 3-16
11	2	24	6.2±3.3, 1-12	10.5±3.5, 5-20
12	1	24	6.0±3.3, 1-12	13.9±5.1, 6-20
13	2	18	6.1±2.8, 2-12	10.7±3.4, 6-17
14	2	18	6.6±3.4, 1-12	10.5±5.1, 4-10

D

Experience (range and N of op.)	N of procedures	N of oocytes mean±SD, min-max	Time (min) mean±SD, min-max
≤ 3 yr (5 op.)	241	5.9±3.0, 1-12	10.3±4.2, 3-20
4-6 yr (6 op.)	833	5.5±3.2, 1-12	8.0±3.6, 2-20
> 6 yr (3 op.)	370	5.4±3.3, 1-12	7.0±3.4, 2-20

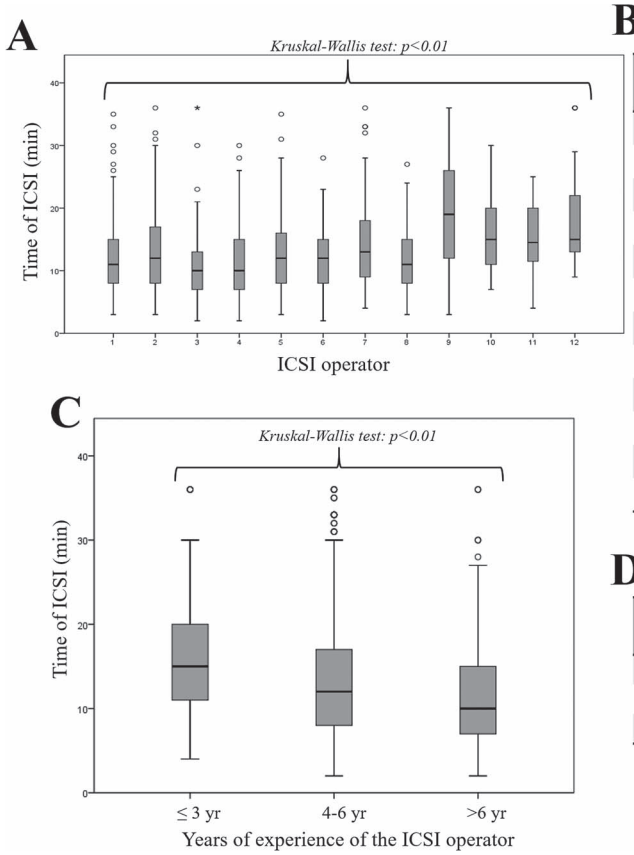
Figure 1 Variability in the time of oocyte denudation (Den) among different operators. (**A**) Box plots showing the time of Den in minutes versus the operators listed according to the number of procedures performed. (**B**) Years of clinical experience, number of procedures, mean number of denuded oocytes per procedure and mean time of Den according to operator. (**C**) Time of Den according to years of operators' clinical experience clustered in three groups (≤3, 4–6 and > 6 years). (**D**) Number of procedures, mean number of denuded oocytes/procedure and mean time of Den according to operators' clinical experience clustered in three groups (≤3, 4–6 and > 6 years).

oocytes collected (i.e. the only confounders found significant from the univariate analyses), no association was found (odds ratio in non-PGT cycles: 0.93 and 1.0 for Den and ICSI timing, respectively; odds ratio in PGT cycles: 0.99 and 1.0 for Den and ICSI timings, respectively). No association was reported also for the Den and ICSI operators involved in each procedure (Fig. 4a and b; Fig. 5a and b).

Discussion

This study was designed to fill the main research gaps in our knowledge regarding the putative effect of ICSI-related timings and operators on the key embryological and clinical outcomes in a modern ART unit. To this end, we focused on m-BR per cohort of inseminated oocytes and the CDR per concluded treatment in ICSI cycles with or without PGT. The study was conducted in infertile and mostly-AMA women, which represent an unselected population of patients at our Italian IVF unit. All procedures performed in a single round with up to 12 fresh oocytes, and ejaculated non-donor fresh sperms were included to ensure a homogenous population of gametes. The only procedures excluded were the ones involving azoospermic male partners, due to the clear impact of this condition on the fertilization and blastulation rates (Palermo *et al.*, 1999; Balaban *et al.*, 2001; Vernaeve *et al.*, 2003; Loutradi *et al.*, 2006; Mazzilli *et al.*, 2017). The EWS was adopted as a tool to automatically, systematically and objectively register all the procedural timings and operators. The only timing that was retrieved from our relational database was the hour and date of IO as confirmed by the patient to the embryologists according to what planned with the clinician.

The pre-incubation time between OR and ICSI ranks among the main issues addressed by several previous papers, whose authors were concerned of both the achievement of oocyte cytoplasmic maturity and the prevention of oocyte *in vitro* aging. These papers came to contrasting indications but most importantly highlighted the wide variability in the time between IO and OR across different laboratories, which ranges between 34 and 38 h (Rienzi *et al.*, 1998; Van de Velde *et al.*, 1998; Yanagida *et al.*, 1998; Andrews *et al.*, 2001; Hassan, 2001; Jacobs *et al.*, 2001; Ho *et al.*, 2003; Isiklar *et al.*, 2004; Dozortsev *et al.*, 2004; Falcone *et al.*, 2008; Patrat *et al.*, 2012; Garor *et al.*, 2015; Barcena *et al.*, 2016; Mizuno *et al.*, 2019). In other terms, a consensus on this topic is clearly missing also because of different *modus operandi* regarding how OR procedures are scheduled. In this



B

Op.	Experience (yr)	N of procedures	N of oocytes mean±SD, min-max	Time (min) mean±SD, min-max
1	4	271	5.7±3.3, 1-12	11.9±5.8, 3-35
2	6	245	5.5±3.3, 1-12	13.3±6.4, 3-36
3	12	197	5.5±3.2, 1-12	10.4±5.4, 2-36
4	14	160	5.6±3.4, 1-12	11.7±5.8, 2-30
5	6	143	4.9±2.6, 1-12	12.6±6.0, 3-35
6	6	120	5.5±3.1, 1-12	11.9±4.8, 2-28
7	6	112	5.8±3.2, 1-12	14.0±7.0, 4-36
8	11	65	5.6±3.3, 1-12	11.7±5.5, 3-27
9	4	57	5.6±2.9, 1-12	18.7±8.3, 3-36
10	2	27	5.8±3.1, 1-11	16.0±5.9, 7-30
11	2	24	6.3±3.0, 1-12	15.2±6.0, 4-25
12	2	23	5.4±2.7, 2-11	18.7±8.3, 9-36

D

Experience (range and N of op.)	N of procedures	N of oocytes mean±SD, min-max	Time (min) mean±SD, min-max
≤ 3 yr (3 op.)	74	5.8±2.9, 1-12	16.7±7.0, 4-36
4-6 yr (6 op.)	948	5.5±3.2, 1-12	13.0±6.4, 2-36
> 6 yr (3 op.)	422	5.4±3.3, 1-12	11.1±5.6, 2-36

Figure 2 Variability in the time of ICSI among different operators. (**A**) Box plots showing the timing of ICSI in minutes versus the operators ordered according to the number of procedures performed. (**B**) Years of clinical experience, number of procedures, mean number of inseminated oocytes/procedure and mean time of ICSI according to operator. (**C**) Time of ICSI according to years of operators' clinical experience clustered in three groups (≤3, 4–6 and > 6 years). (**D**) Number of procedures, mean number of inseminated oocytes per procedure and mean time of ICSI according to operators' clinical experience clustered in three groups (≤3, 4–6 and > 6 years).

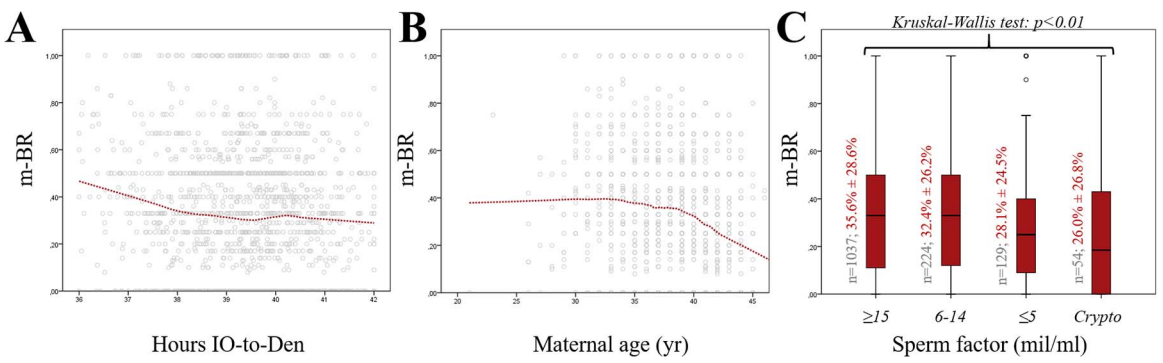


Figure 3 Mean blastulation rate (m-BR) per cohort of inseminated oocytes according to hours between induction of ovulation and oocyte denudation (IO-to-Den) (A), maternal age at oocyte retrieval (B) and categorized sperm concentration (C). The gray spots in panels A and B identify the outcome achieved in each specific cycle, while the dotted red lines represent the locally estimated scatterplot smoothing (LOESS) curves. For sperm concentration, the data are summarized through box plots and average ± SD values.

Table III Absence of an association between the ICSI-related procedural timings (adjusted for maternal age and number of oocytes inseminated) with the cumulative delivery rate (CDR) per concluded non-PGT and PGT cycle. Data are mean ± SD (min–max). A cycle was considered concluded when at least one live birth was achieved or no blastocysts were left available for transfer. PGT, preimplantation genetic testing.

	Non-PGT cycles		*Association with the CDR*	PGT cycles		*Association with the CDR*
	No live birth achieved *n* = 335	**≥1 live birth achieved *n* = 72**	***Multivariate odds ratio, 95% CI; p-value***	**No live birth achieved *n* = 678**	**≥1 live birth achieved *n* = 260**	***Multivariate odds ratio, 95% CI; p-value***
Maternal age year	39.0 ± 4.2 (21–45)	33.2 ± 2.7 (26–40)	*0.76, 0.70–0.82, <0.01*	39.7 ± 3.3 (26–45)	37.3 ± 3.4 (28–44)	*0.84, 0.81–0.88, <0.01*
MII oocytes	3.5 ± 2.7 (1–12)	7.2 ± 3.2 (1–12)	*1.29, 1.17–1.42, <0.01*	5.4 ± 2.9 (1–12)	7.4 ± 2.9 (1–12)	*1.21, 1.15–1.28, <0.01*
Time IO-to-Den hour	39.3 ± 1.3 (36–42)	39.0 ± 1.3 (36–41)	*0.89, 0.70–1.13, 0.35*	39.4 ± 1.2 (36–42)	39.4 ± 1.3 (36–42)	*0.96, 0.85–1.09, 0.53*
Time of Den minutes	6.7 ± 3.5 (2–20)	9.3 ± 4.4 (3–20)	*0.93, 0.84–1.04, 0.21*	8.0 ± 3.4 (2–20)	9.4 ± 4.2 (3–20)	*0.99, 0.94–1.04, 0.64*
Time of ICSI minutes	9.4 ± 5.7 (2–36)	15.9 ± 6.8 (4–33)	*1.0, 0.92–1.08, 0.99*	12.4 ± 5.8 (2–36)	15.6 ± 6.1 (4–36)	*1.0, 0.97–1.04, 0.77*

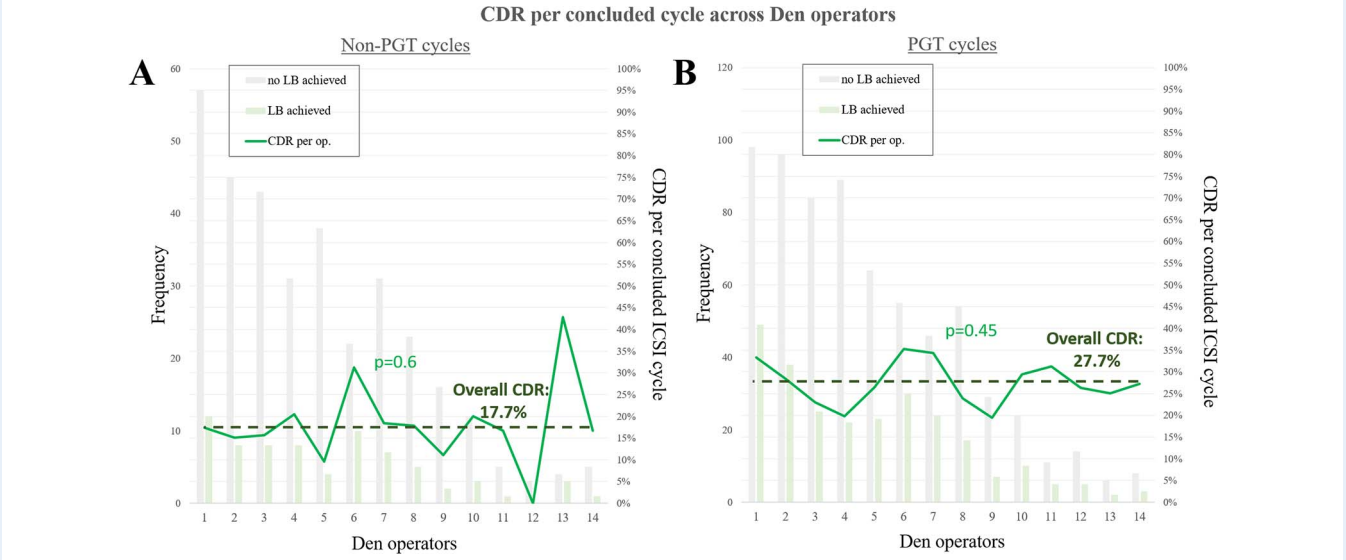


Figure 4 Cumulative delivery rate (CDR) per concluded cycle across different oocyte denudation (Den) operators (op.). Non-PGT **(A)** and PGT **(B)** cycles. Light gray = number of concluded cycles with no live birth (LB) achieved; light green = number of concluded cycles with at least one LB achieved; continuous light green line = CDR per Den operator; dotted dark green line = overall CDR per concluded cycle. Chi-squared tests were conducted for statistics.

study, the ICSI procedures were performed 4.1 ± 1.2 h (2–7) after the OR, but this time interval was not evaluated by itself: it was instead added to the time between IO and OR to produce an inclusive and more standardized parameter, named IO-to-Den time.

A crucial aspect of our workflow was pre-incubating the oocytes still enclosed in their cumulus until ICSI, which is another practice still awaiting for a general consensus (Rubino *et al.*, 2016). Our rationale was to prevent (i) the potential impact of the absence of cumulus cells

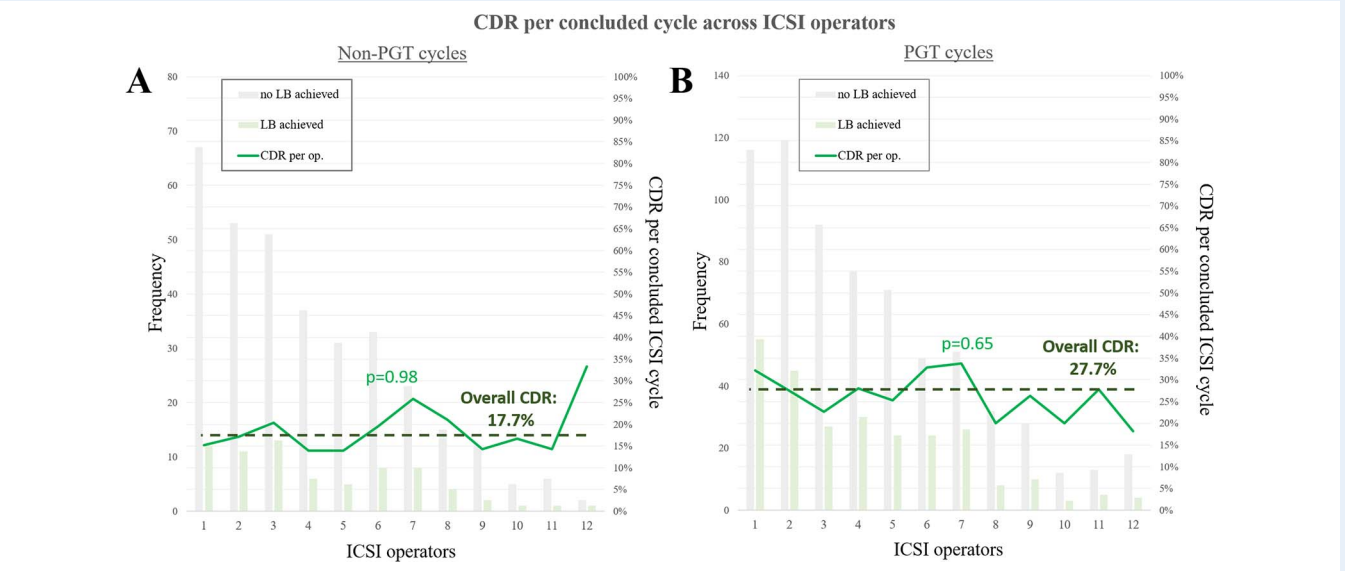


Figure 5 CDR per concluded cycle across different oocyte ICSI operators (op.). Non-PGT (**A**) and PGT (**B**) cycles. Light gray = number of concluded cycles with no live birth (LB) achieved; light green = number concluded cycles with at least one LB achieved; continuous light green line = CDR per ICSI operator; dotted dark green line = overall CDR per concluded cycle. Chi-squared test was conducted for statistics. PGT, preimplantation genetic testing; LB, live birth.

on both embryonic metabolism and oxidative stress (McKenzie *et al.*, 2004; Fatehi *et al.*, 2005), and (ii) the exposure of the eggs to Hepes-buffered media twice in a few hours (Phillips *et al.*, 2000; Will *et al.*, 2011). In this regard, the group of Barcena and Pujol recently published two important studies claiming that long pre-incubation timings before ICSI do not affect the reproductive potential of the oocytes obtained from both young fertile women (Barcena *et al.*, 2016) and AMA infertile ones (Pujol *et al.*, 2018). Yet, a negative trend was shown in the latter population when the pre-incubation timing was extended beyond 10 h (Pujol *et al.*, 2018). Of note, in both Barcena's and Pujol's studies, the oocytes were denuded on average ~1 h after OR and ~4 h before ICSI. In this study, we reported that IO-to-Den timings up to 42 h (of which 4.1 ± 1.2 h from 2 to 7 h *in vitro*) in presence of the surrounding cumulus cells do not impact the CDR per concluded treatment (with or without PGT), thereby in part confirming Barcena's and Pujol's data with a different oocyte pre-incubation strategy *in vitro*. An association was instead found with the m-BR per cohort of inseminated oocytes, namely an outcome that is missing from several previous studies (mostly based on a Day 2/3 ET strategy). In this study, we reported the highest m-BR for IO-to-Den timings <38 h (~37–45%) and then a plateau up to 42 h (~33%), a trend which is not imputable to the *in vitro* maturation of immature oocytes along extended pre-incubation timings. In fact, the oocyte MII rate was very stable (~74%) across the whole IO-to-Den time interval. Therefore, future investigations of the oocyte MII rate and m-BR are warmly suggested from centers that pre-incubate the oocytes before ICSI in the absence of their surrounding cumulus cells.

Maternal age represented the only putative confounder significantly associated with both embryological and clinical outcomes, while sperm concentration was confirmed only for its association with the former (Mazzilli *et al.*, 2017). Standing these evidences, we suggest scheduling the daily workload so that the ICSI procedures of poor prognosis couples (i.e. AMA, reduced sperm count and/or ovarian reserve) are performed within an IO-to-Den time interval < 38 h. Such expedient might in fact elicit a slight, yet significant, beneficial effect on the related outcomes. In other terms, those busy IVF units where ORs are planned across the whole working day might benefit from proactive communication between the embryologists and the clinicians aimed at a reasoned and more efficient organization of the daily workload.

The use of the EWS, like in Barcena's and Pujol's studies, provided us with the unprecedented possibility of investigating ICSI-related timings across the operators involved in the Den and ICSI procedures. Similarly, the Den and ICSI 'operator' variables were tested for their association with both the m-BR per cohort of inseminated oocytes and the CDR per concluded cycle. Interestingly, both the Den and ICSI timings were highly variable across the operators, mainly as a consequence of a shorter/longer clinical experience in years, even when the model was adjusted for the number of oocytes processed. However, this did not translate in an impact on the embryological and clinical outcomes under investigation. Indeed, neither the Den nor the ICSI timings associated with the m-BR per cohort of inseminated oocytes and the CDR per concluded treatment. These results are reassuring towards less experienced operators or operators that have just completed their training for the ICSI procedure. Clearly, the maximum timings were 20 and 36 min for Den and ICSI, respectively, to inject no more than 12 fresh oocytes with ejaculated fresh sperm in standard conditions (Alpha and ESHRE, 2011; Rubino *et al.*, 2016; ESHRE and Alpha, 2017). Therefore, future studies should be conducted in different conditions to confirm the reproducibility of these evidences.

Our study represents the first comprehensive investigation of the ICSI-related timings and operators with respect to the embryological

outcomes up to the blastocyst stage and the CDR across concluded non-PGT and PGT cycles. Future studies are warranted to investigate the reproducibility of these results in a different setting and in the hands of other operators. Of note, different operators in our clinical workflow performed the different procedures required along each cycle (Den, ICSI, biopsy, vitrification, warming, transfer). This might represent a bias when investigating the outcomes and their correlation with the operator involved in each procedure. Nevertheless, in several studies previously published from our group we already defined the absence of an impact due to different biopsy, vitrification and warming operators (Capalbo *et al*., 2016; Cimadomo *et al*., 2018; Maggiulli *et al*., 2019). Therefore, further investigation is suggested mainly to address whether the embryo transfer operator might affect the clinical outcomes after IVF.

This study involves a sufficient amount of cycles ($n = 1444$ with 7999 MII oocytes from 1084 patients) to achieve an adequate statistical power to outline the main variables significantly affecting the m-BR per cohort of inseminated oocytes and the CDR per concluded cycle. Nevertheless, further investigations focused on all the other putative confounders (here found not significant) are warranted. For instance, a sequential culture strategy and an undisturbed incubation were respectively adopted only in ~20 and ~30% of the cycles included in this study; therefore, future studies conducted only with a sequential culture media and/or only with an undisturbed incubation strategy are suggested. Similarly, the putative association of the main outcomes defined in this study with the different strategies of ovarian stimulation and trigger of ovulation, or with the strategies for endometrial preparation and ET, as well as with repeated ICSI cycles undertaken from the same patient, requires deeper investigation.

Lastly, this study outlines also how the EWS, via an automatic and systematic registration of each procedure-related timings and operators, represents a valuable tool to define key performance indicators (KPIs) internal to each IVF unit. Such KPIs might then be prospectively adopted to constantly monitor on operators' performance, as well as shared with the international scientific community in reports like in Barcena's and Pujol's.

Conclusion

Neither ICSI-related procedural timings nor operators affected the CDR per concluded cycle, even when mostly-AMA women undergo the insemination of fresh own oocytes pre-incubated in presence of their surrounding cumulus cells (for 4 h on average, from 2 to 7 h from oocyte retrieval), culture to the blastocyst stage and untested/euploid ET.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

DC, GF, RM and LR designed the study. DC analyzed the data. DC and GF drafted the manuscript. All authors contributed to the interpretation and discussion of the results.

Funding

None.

Conflict of interest

The authors declare no conflict of interest related to the present study.

References

Alpha SIRM, ESHRE SIGE. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011a;**22**:632–646.

Alpha SRM, ESHRE SIGE. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Hum Reprod* 2011b;**26**:1270–1283.

Andrews MM, Fishel SB, Rowe PH, Berry JA, Lisi F, Rinaldi L. Analysis of intracytoplasmic sperm injection procedures related to delayed insemination and ejaculated, epididymal and testicular spermatozoa. *Reprod Biomed Online* 2001;**2**:89–97.

Balaban B, Urman B, Isiklar A, Alatas C, Mercan R, Aksoy S, Nuhoglu A. Blastocyst transfer following intracytoplasmic injection of ejaculated, epididymal or testicular spermatozoa. *Hum Reprod* 2001;**16**:125–129.

Barcena P, Rodriguez M, Obradors A, Vernaeve V, Vassena R. Should we worry about the clock? Relationship between time to ICSI and reproductive outcomes in cycles with fresh and vitrified oocytes. *Hum Reprod* 2016;**31**:1182–1191.

Barratt CLR, Bjorndahl L, De Jonge CJ, Lamb DJ, Osorio Martini F, McLachlan R, Oates RD, van der Poel S, St John B, Sigman M *et al.* The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum Reprod Update* 2017;**23**:660–680.

Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, Nagy ZP, Ubaldi FM. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. *Hum Reprod* 2014;**29**:1173–1181.

Capalbo A, Ubaldi FM, Cimadomo D, Maggiulli R, Patassini C, Dusi L, Sanges F, Buffo L, Venturella R, Rienzi L. Consistent and reproducible outcomes of blastocyst biopsy and aneuploidy screening across different biopsy practitioners: a multicentre study involving 2586 embryo biopsies. *Hum Reprod* 2016;**31**:199–208.

Cimadomo D, Capalbo A, Levi-Setti PE, Soscia D, Orlando G, Albani E, Parini V, Stoppa M, Dovere L, Tacconi L *et al.* Associations of blastocyst features, trophectoderm biopsy and other laboratory practice with post-warming behavior and implantation. *Hum Reprod* 2018a.

Cimadomo D, Fabozzi G, Vaiarelli A, Ubaldi N, Ubaldi FM, Rienzi L. Impact of maternal age on oocyte and embryo competence. *Front Endocrinol (Lausanne)* 2018b;**9**.

Cobo A, de los MJ, Castello D, Gamiz P, Campos P, Remohi J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: evaluation of 3,150 warming cycles. *Fertil Steril* 2012;**98**:1138, e1131–1146.

Coticchio G, Dal Canto M, Mignini Renzini M, Guglielmo MC, Brambillasca F, Turchi D, Novara PV, Fadini R. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. *Hum Reprod Update* 2015;**21**:427–454.

Dozortsev D, Nagy P, Abdelmassih S, Oliveira F, Brasil A, Abdelmassih V, Diamond M, Abdelmassih R. The optimal time for intracytoplasmic sperm injection in the human is from 37 to 41 hours after administration of human chorionic gonadotropin. *Fertil Steril* 2004;**82**:1492–1496.

SIGoE ESHRE, SiRM A. The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reprod Biomed Online* 2017;**35**:494–510.

Falcone P, Gambera L, Pisoni M, Lofiego V, De V, Mencaglia L, Piomboni P. Correlation between oocyte preincubation time and pregnancy rate after intracytoplasmic sperm injection. *Gynecol Endocrinol* 2008;**24**:295–299.

Fatehi AN, Roelen BA, Colenbrander B, Schoevers EJ, Gadella BM, Beverst MM, van den R. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. *Zygote* 2005;**13**:177–185.

Garor R, Shufaro Y, Kotler N, Shefer D, Krasilnikov N, Ben-Haroush A, Pinkas H, Fisch B, Sapir O. Prolonging oocyte in vitro culture and handling time does not compensate for a shorter interval from human chorionic gonadotropin administration to oocyte pickup. *Fertil Steril* 2015;**103**:72–75.

Hassan HA. Cumulus cell contribution to cytoplasmic maturation and oocyte developmental competence in vitro. *J Assist Reprod Genet* 2001;**18**:539–543.

Ho JY, Chen MJ, Yi YC, Guu HF, Ho ES. The effect of preincubation period of oocytes on nuclear maturity, fertilization rate, embryo quality, and pregnancy outcome in IVF and ICSI. *J Assist Reprod Genet* 2003;**20**:358–364.

Isiklar A, Mercan R, Balaban B, Alatas C, Aksoy S, Urman B. Impact of oocyte pre-incubation time on fertilization, embryo quality and pregnancy rate after intracytoplasmic sperm injection. *Reprod Biomed Online* 2004;**8**:682–686.

Jacobs M, Stolwijk AM, Wetzels AM. The effect of insemination/injection time on the results of IVF and ICSI. *Hum Reprod* 2001;**16**:1708–1713.

Loutradi KE, Tarlatzis BC, Goulis DG, Zepiridis L, Pagou T, Chatziioannou E, Grimbizis GF, Papadimas I, Bontis I. The effects of sperm quality on embryo development after intracytoplasmic sperm injection. *J Assist Reprod Genet* 2006;**23**:69–74.

Maggiulli R, Giancani A, Cimadomo D, Ubaldi FM, Rienzi L. Human blastocyst biopsy and vitrification. *J Vis Exp* 2019.

Mazzilli R, Cimadomo D, Vaiarelli A, Capalbo A, Dovere L, Alviggi E, Dusi L, Foresta C, Lombardo F, Lenzi A *et al*. Effect of the male factor on the clinical outcome of intracytoplasmic sperm injection combined with preimplantation aneuploidy testing: observational longitudinal cohort study of 1,219 consecutive cycles. *Fertil Steril* 2017.

McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 2004;**19**:2869–2874.

Mizuno S, Ishikawa Y, Matsumoto H, Sato M, Ida M, Fukuda A, Morimoto Y. The timing of cumulus cell removal for intracytoplasmic sperm injection influences the capability of embryonic development. *Reprod Med Biol* 2019;**18**:111–117.

Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:17–18.

Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Development and implementation of intracytoplasmic sperm injection (ICSI). *Reprod Fertil Dev* 1995;**7**:211–217 discussion 217-218.

Palermo GD, O'Neill CL, Chow S, Cheung S, Parrella A, Pereira N, Rosenwaks Z. Intracytoplasmic sperm injection: state of the art in humans. *Reproduction* 2017;**154**:F93–F110.

Palermo GD, Schlegel PN, Hariprashad JJ, Ergun B, Mielnik A, Zaninovic N, Veeck LL, Rosenwaks Z. Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. *Hum Reprod* 1999;**14**:741–748.

Patrat C, Kaffel A, Delaroche L, Guibert J, Jouannet P, Epelboin S, De Ziegler D, Wolf JP, Fauque P. Optimal timing for oocyte denudation and intracytoplasmic sperm injection. *Obstet Gynecol Int* 2012;**2012**:403531.

Phillips KP, Leveille MC, Claman P, Baltz JM. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;**15**:896–904.

Pujol A, Garcia D, Obradors A, Rodriguez A, Vassena R. Is there a relation between the time to ICSI and the reproductive outcomes? *Hum Reprod* 2018;**33**:797–806.

Rienzi L, Balaban B, Ebner T, Mandelbaum J. The oocyte. *Hum Reprod* 2012;**27**:i2–i21.

Rienzi L, Bariani F, Dalla Zorza M, Romano S, Scarica C, Maggiulli R, Nanni Costa A, Ubaldi FM. Failure mode and effects analysis of witnessing protocols for ensuring traceability during IVF. *Reprod Biomed Online* 2015;**31**:516–522.

Rienzi L, Ubaldi F, Anniballo R, Cerulo G, Greco E. Preincubation of human oocytes may improve fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**:1014–1019.

Rubino P, Vigano P, Luddi A, Piomboni P. The ICSI procedure from past to future: a systematic review of the more controversial aspects. *Hum Reprod Update* 2016;**22**:194–227.

Ubaldi FM, Capalbo A, Colamaria S, Ferrero S, Maggiulli R, Vajta G, Sapienza F, Cimadomo D, Giuliani M, Gravotta E *et al*. Reduction of multiple pregnancies in the advanced maternal age population after implementation of an elective single embryo transfer policy coupled with enhanced embryo selection: pre- and post-intervention study. *Hum Reprod* 2015;**30**:2097–2106.

Vaiarelli A, Cimadomo D, Patrizio P, Venturella R, Orlando G, Soscia D, Giancani A, Capalbo A, Rienzi L, Ubaldi FM. Biochemical pregnancy loss after frozen embryo transfer seems independent of embryo developmental stage and chromosomal status. *Reprod Biomed Online* 2018;**37**:349–357.

Van de H, De A, Joris H, Nagy ZP, Van AC. Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**:3160–3164.

Vernaeve V, Tournaye H, Osmanagaoglu K, Verheyen G, Van A, Devroey P. Intracytoplasmic sperm injection with testicular spermatozoa is less successful in men with nonobstructive azoospermia than in men with obstructive azoospermia. *Fertil Steril* 2003;**79**: 529–533.

Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016;**22**: 2–22.

Will MA, Clark NA, Swain JE. Biological pH buffers in IVF: help or hindrance to success. *J Assist Reprod Genet* 2011;**28**: 711–724.

Yanagida K, Yazawa H, Katayose H, Suzuki K, Hoshi K, Sato A. Influence of oocyte preincubation time on fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**: 2223–2226.

Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de J, Sokol R, Rienzi L, Sunde A, Schmidt L, Cooke ID *et al*. The International Glossary on Infertility and Fertility Care, 2017. *Hum Reprod* 2017a;**32**:1786–1801.

Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de J, Sokol R, Rienzi L, Sunde A, Schmidt L, Cooke ID *et al*. The international glossary on infertility and fertility care, 2017. *Fertil Steril* 2017b;**108**:393–406.