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ASSISTED REPRODUCTION TECHNOLOGIES

Abnormal sperm concentration and motility as well as advanced paternal age compromise early embryonic development but not pregnancy outcomes: a retrospective study of 1266 ICSI cycles

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Abstract

Purpose To investigate the effect of sperm concentration, motility and advanced paternal age on reproductive outcomes.

Methods A retrospective analysis of 1266 intracytoplasmic sperm injection (ICSI) cycles between 2013 and 2017. The cohort was divided into four groups according to semen concentration based on the WHO criteria (2010): group A (conc. <1 M/ml), group B (1 ≤ conc. <5 M/ml), group C (5 ≤ conc. < 15 M/ml) and the control group D (conc. ≥15 M/ml). The primary outcome investigated was the blastulation rate. Secondary outcomes were fertilization rate, top quality blastocyst formation rate and ongoing pregnancy rate.

Results After adjustment for maternal age and number of oocytes recovered, a significant difference was observed between group A and group D on the rate of fertilized oocytes [66.7 (40.0–80.0) vs 75.0 (57.1–90.2), adjusted $p < 0.001$] and the blastocyst formation rate [50.0 (33.3–66.3) vs 55.6 (40.0–75.0), adjusted $p < 0.05$]. However, the male factor did not affect the top quality blastocyst formation rate nor the ongoing pregnancy rate. Considering the age of the male partner as confounding factor, at the increase of each year of age, a reduction of 0.3% on the fertilization rate was observed but no other outcome was impacted. A negative correlation was also observed between sperm motility and fertilization rate in the group with a motility <5%.

Conclusion Male factor infertility and advanced paternal age may compromise fertilization and blastulation rates but not top quality blastocyst formation rate or the establishment of pregnancy in ICSI cycles.

Keywords Abnormal sperm parameters · Paternal age · Male factor infertility · Blastulation rate

Introduction

Over the last two decades, the spread of the contraceptive pill, the extended period of education and various socio-economic factors have led a significant amount of couples in the developed world to delay parenthood [1]. The popularity and growing access to Assisted Reproduction Technology (ART) is also a consequence of the increased number of older candidate parents. One major burden to successful ART outcomes is, however, considered to be the conditions underlying male infertility. Several reports demonstrated the effect of male factor infertility, in terms of age and semen parameters, on the clinical pregnancy outcomes [2–5]. However, fewer studies have considered the consequence of semen abnormalities and advanced paternal age on embryologic outcomes, for example embryo developmental competence [6, 7]. This is particularly critical considering that recently, the high-paced advance of reproductive medicine has been driving the laboratory practice through introduction of improved culture mediums and optimization of embryo-transfer strategies. For example, transfer of the embryo later in development is nowadays preferred to early stage. Moreover, the controversial trend of a simultaneous transfer of multiple early stage

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embryos has been substituted by single blastocyst transfer. Considering the importance of embryonic developmental stage in the context of optimal uterine transfer, it is critical to elucidate factors that can jeopardize the competence of the embryo to develop.

In a recent retrospective study of 1219 couples undergoing intracytoplasmic sperm injection (ICSI) combined with preimplantation aneuploidy testing, male factor infertility had a detrimental effect on fertilization rate and the competence of embryo to blastulate (blastulation rate), but neither affected pregnancy rate or blastocyst quality [6]. Regarding the impact of male age the data are sparse and discordant. Ferreira et al. documented that paternal age could adversely impact implantation and pregnancy rates only in cases of oligozoospermia [8]. A larger retrospective study of 2627 ICSI cycles showed that high paternal age negatively influenced the number of high-quality embryos achieved, but had no effect on pregnancy outcomes [9]. Both studies assessed selectively only the ICSI cycles, with ICSI being the traditional treatment indication for severe male factor infertility. Controversially, ICSI has nowadays turned into a routine treatment strategy to avoid complete fertilization failure and circumvent all the potential effects deriving from poor spermatozoa motility and structure in their ability to cross the zona pellucida and fuse with the oocyte cytoplasm [10].

Herein, we scrutinized the plausible negative impact of male factor infertility, caused by semen abnormalities, and advanced paternal age on embryonic parameters in a retrospective analysis of the ICSI cycles in our center.

Materials and methods

Study population

This is a retrospective analysis of 1266 ICSI cycles in the San Raffaele Hospital between December 2013 and January 2017. The inclusion criteria were: (1) ICSI cycles with fresh mature oocytes between December 2013 and January 2017; (2) male factor or tubal factor infertility; (3) cycles on an agonist or antagonist protocol. Exclusion criteria were ICSI cycles using surgically retrieved or frozen sperm.

Male factor infertility was defined after a comprehensive diagnostic evaluation of all the female partners. According to the classic medical definition, infertility was defined as failing to conceive after at least 12 months of unprotected intercourse regardless of whether or not a pregnancy ultimately occurred (World Health Organization, 2010). Patients underwent at least two consecutive semen analyses, both showing below-standard values for normal semen parameters according to the World Health Organization (WHO) criteria (World Health Organization, 2010). Tubal factor infertility was defined as female infertility caused by diseases, obstructions, damage, scarring, congenital malformations or other factors which impede the descent of a fertilized or unfertilized ovum into the uterus through the Fallopian tubes.

The entire cohort was divided into four groups according to the sperm count, based on the WHO criteria (2010): group A (conc. <1 M/ml), group B (1 ≤ conc. <5 M/ml), group C (5 ≤ conc. < 15 M/ml) and group D (conc. ≥15 M/ml) (Table 1). The study protocol was approved by Ethical Review Board of San Raffaele Scientific Institute, Milano, Italy and a written informed consent was obtained from all participants.

Semen preparation

All male patients were required to undergo 2–7 days of abstinence before providing the semen samples for fertilization. After liquefaction of the semen (about 30 min at 37 °C), the sperm samples were analyzed for volume, concentration, and motility according to WHO criteria (2010) [11]. A hemocytometer (Bioanalytic GmbH, Germany) was used to estimate sperm concentration. Motility was scored manually, as percentages of fast forward progressive, slow forward progressive, non-progressive and immotile spermatozoa in 200 spermatozoa in at least five power fields per replicate.

The SEMinal QUAlity studies (SEMQUA) checklist was followed to improve accuracy and transparency of the study [12]. Both an internal and external quality control programme [European Society of Human Reproduction and Embryology (ESHRE)] has been established in the laboratory in order to control random and systematic errors and interlaboratory differences. All the personnel was trained based on the ESHRE Special Interest Group in Andrology Basic Semen Analysis Course.

Combinations of gradient centrifugation and swim-up were used for the preparation of ejaculated sperm. Semen samples were processed using a density gradient (Sperm gradient kit, Cook medical, Irleand) and Gamete Buffer medium (Gamete Buffer, Cook medical, Ireland). Sperm gradients of 80 and 40% were used for the experiment. All procedures were conducted under sterile conditions, and all media was warmed to 37 °C. Using a sterile syringe, 2 mL of the lower layer (80% Sperm gradient kit) was transferred into a conical centrifuge tube. Using a new sterile syringe, 2 mL of the upper layer (40% sperm gradient kit) was gently dispensed on top of the lower layer. A liquefied semen sample was then placed on top of the upper layer and the tube was centrifuged for 20 min at 1700 rpm. The upper and lower layers were carefully aspirated without disturbing the pellet. Using a transfer pipette, 3 mL of gamete buffer medium was added and the re-suspended pellet was centrifuged for 10 min at 1700 rpm. The supernatant was then removed and 0.5–1 mL of Fertilization medium (Sage In Vitro Fertilization, Inc. Trumbull, CT, USA) was gently dispensed on the top of the pellet. The tube was then

Table 1 Basal characteristics in the different paternal groups

Sperm concentration (M/ml)	Group A (< 1 M/ml)	Group B (1 - < 5 M/ml)	Group C (5 - < 15 M/ml)	Group D (≥ 15 M/ml)	*P*-value
Maternal age, years (mean ± SD)	35.7 ± 4.1	36.5 ± 3.9	36.9 ± 4.3	36.6 ± 4.2	< 0.05
Number of retrieved oocytes, mean ± SD	9.2 ± 6.0	9.0 ± 5.8	8.6 ± 5.4	9.3 ± 5.9	0.05
Mature oocytes, percentage [median (IQR)]	85.7 (74.4–94.7)	84.6 (68.1–100)	85.7 (76.4–95.8)	72.2 (50.0–87.5)	< 0.001
Paternal age, years (mean ± SD)	39.2 ± 5.2	39.2 ± 5.4	39.6 ± 5.2	39.8 ± 5.8	0.58
Sperm concentration, M/ml (mean ± SD)	0.4 ± 0.36	3.5 ± 1.3	10.5 ± 3.1	42.0 ± 21.4	< 0.001
Sperm motility, percentage [median (IQR)]	10.0 (5.0–20.0)	20.0 (10.0–30.0)	25.0 (20.0–30.0)	40.0 (25.0–50.0)	< 0.001

M/ml million per milliliter, SD standard deviation, IQR Interquartile Range

incubated at an angle of 45° for 30–60 min in the incubator at 37 °C and 5% CO_2.

Protocol for ovarian stimulation

Controlled ovarian stimulation was performed according to the standard clinical practice [13]. Either a GnRH agonist or GnRH antagonist daily protocol was used for pituitary down-regulation and ovarian stimulation was achieved by: a) recombinant FSH (rFSH) alone; or b) rFSH combined with recombinant LH (rLH); or c) highly purified human menopausal gonadotrophin alone (HP-hMG). Both initial dose and dose adjustments during treatment were chosen on a case-by-case basis according to patients' characteristics and response to gonadotrophins. Triggering of ovulation was performed with HP-human chorionic gonadotrophin (hCG) when one or more follicles had reached a diameter of ≥17–18 mm. Serial determinations of serum estrogen (E_2) and progesterone levels were performed during the treatment. The first (basal) determination was carried out before the beginning of gonadotrophin administration, while the last assessment of E_2 and progesterone levels was obtained on the day of hCG administration. Oocytes were retrieved after about 36 h after hCG administration.

ICSI and embryo culture

ICSI was performed in a standard way [14] by 9 biologists who worked in different daily shifts. Briefly, cumulus-corona-oocyte complexes were collected and washed in HTF medium with HEPES (Sage In Vitro Fertilization, Inc. Trumbull, CT, USA) supplemented with 5 g/l of human serum albumin (HSA) (Sage In Vitro Fertilization, Inc.). After 2–3 h incubation in HSA-supplemented-Fertilization medium (Sage In Vitro Fertilization, Inc.) under oil, denudation of the cumulus oophorus was performed by a brief exposure to 40 IU/ml hyaluronidase solution (Sage In Vitro Fertilization, Inc.) in HTF medium with HEPES. Then the cumulus was removed mechanically with the use of plastic pipettes (denuding pipettes; Cook) on a heated-stage stereomicroscope under the hood (IVF Tech); insemination of oocytes by means of ICSI was carried out immediately after denudation. ICSI was performed as previously described [14, 15]. Injected oocytes were group-cultured in microdrops of equilibrated Serum Substitute Supplement (SSS, Irvine, CA, USA)-supplemented Cleavage medium (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA) under oil.

At 16–18 h after ICSI, the oocytes were checked for fertilization and normal fertilization was assessed by the presence of two pronuclei. On day 3, embryos were evaluated according to the number of blastomeres, the percentage of fragmentation and the symmetry of the blastomeres. When, on day 3, at least 3 top quality embryos (i.e. ≥8 regular symmetrical blastomeres with no fragmentation on day 3) or 4 top/good quality embryos were present, embryo culture was extended to undergo day-5 blastocyst stage in SSS (Irvine, CA, USA)-supplemented Blastocyst medium (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA).

Extended embryo culture

Blastocyst evaluation was performed according to the Istanbul Consensus [16]. Briefly, on the morning of day 5–7 of development, blastocysts were given a rating based on: (i) the degree of expansion and hatching status (from 1 as early to 4 as hatched blastocyst); (ii) the inner cell mass scoring: "good" when prominent, easily discernible, with many cells that were compacted and tightly adhered together, "fair" easily discernible, with many cells that were loosely grouped together or "poor" when it was difficult to discern, with few cells, and (iii) the trophectoderm scoring: "good" when many cells were forming a cohesive epithelium, "fair" when few cells were forming a loose epithelium and "poor" when a small number of cells was present. According to the Consensus, an optimal embryo at this developmental stage is a fully expanded hatching blastocyst with a "good" inner cell mass and a "good" trophectoderm. Based on this, "top quality" blastocyst was defined as an expanded or hatched blastocyst with both an inner cell mass and multicellular trophectoderm scoring "good" or with only one of the two parameters scoring "fair"

and the other scoring "good". Day 7 blastocysts were excluded from the "top quality" classification. Blastocysts were never frozen before the expanded stage and only 4.5% of blastocyst were frozen on day 7. The number of embryos or blastocysts transferred in fresh cycles was established according to the American Society for Reproductive Medicine guidelines [17].

Aim of the study and outcome measures

The aim of the study was to investigate whether abnormal sperm concentration, motility and advanced paternal age might have a negative impact on reproductive outcomes, with particular attention to early embryonic development. The primary outcome of the study was the blastulation rate (number of blastocysts formed per fertilized oocyte cultured). Secondary outcomes were fertilization rate, top quality blastocyst formation rate per fertilized oocyte cultured and ongoing pregnancy rate. Ongoing pregnancy was defined as the presence of fetal heartbeat at 6 to 12 weeks of gestation.

Statistics

Data analysis was done with Statistical Package for Social Sciences (SPSS) version 17.0 (SPSS Incl., USA). The groups in question were included in the analysis as dummy variables where group D played the reference role. ANOVA test was used to analyze differences in basal characteristics of the different paternal groups. Information describing the female partner (age, number of oocytes retrieved and percentage of mature oocytes) was summarized in principal components in order to avoid errors related to the high degree of correlation of those variables (Supplementary Fig. 1). These, together with the motility of sperm (expressed with four different groups with motility respectively of <5%, 5 - < 10%, 10 - < 32% and ≥ 32%) and male age, have been used as confounding factors in the statistical analysis. Odds ratios for ongoing pregnancy rate were corrected also for day of the transfer and number of transferred embryos. Linear and logistic regression was used to analyze the continuous or dichotomous variables, respectively. All the reported p values were corrected for confounders, as previously described, and a value <0.05 was considered statistically significant. Results are expressed as mean ± standard deviation (SD) or median [interquartile range (IQR)] and odds ratio (OR) with 95% confidence interval (CI).

Results

A total of 1266 ICSI cycles were included in this study. Couples that performed multiple cycles were equally represented in each group, with a similar mean number of performed cycles ($p = 0.63$). The maternal age and percentage of mature oocytes were significantly different between the four groups, but not the number of retrieved and injected oocytes. Three of the parameters analyzed were significantly different in relation to the different sperm concentrations (Table 2). In group A, the fertilization rate was significantly lower than in group D [66.7 (40.0–80.0) vs 75.0 (57.1–90.2), adjusted $p < 0.001$]. The blastulation rate decreased significantly in group A compared to group D [50.0 (33.3–66.3) vs 55.6 (40.0–75.0), adjusted $p < 0.05$]. The rate of blastocyst in day V was significantly lower for groups B and C when compared with the control group D [50.0 (33.3–85.8) and 50.0 (31.2–84.0) vs 66.7 (40.0–100), adjusted $p < 0.05$ and $p < 0.01$, respectively]. There were no significant differences in ongoing pregnancy rate and top quality blastocyst formation rate per fertilized oocyte cultured among the groups. The age of the male partner, used as confounding factor in the linear regression analysis, negatively impacted the fertilization rate: at the increase of each year of age, a reduction of 0.3% on the fertilization rate (adjusted $p < 0.05$) was observed. The paternal age had no effect on any of the investigated clinical outcomes. A negative correlation was observed between sperm motility (used as confounding factor in the linear regression analysis) and fertilization rate, where the group with a motility <5% resulted significantly lower than the control group with a motility ≥32% [66.7 (45.5–83.3) vs 75.0 (60.0–90.0), adjusted $p < 0.001$]. A detailed representation of fertilization rate according to groups with different concentration and motility (n of groups = 16) is reported in Table 3. No effect on the other analyzed variables were detected for sperm motility.

Discussion

To date, little is known about the impact of abnormal sperm parameters on the developmental competence of the embryo. In the 1266 ICSI cycles of our study the association of embryological outcomes with semen concentration, motility and male age was scrutinized. In line with previous reports [6, 7], we confirmed a compromised fertilization and blastulation rate when spermatozoa of men diagnosed with severe oligozoospermia were utilized for ICSI. The negative impact of severe oligozoospermia on the blastulation rate might be associated with elevated DNA fragmentation of spermatozoa used to inject the oocyte [18, 19]. A growing body of literature suggests that sperm from infertile men contains more DNA damage than sperm from fertile men [20, 21]. Spermatozoa from oligozoospermic patients have reportedly reduced chromatin quality causally related to reduced preimplantation embryo development [22]. Although the oocyte ooplasm possesses a repair mechanism capable of repairing low scale DNA damage, rescue of severe DNA damage introduced by the spermatozoa with high level of DNA fragmentation may

Table 2 Cycle and embryologic outcomes by male factor

Sperm concentration (M/ml)	Group A (< 1 M/ml)	Group B (1 - < 5 M/ml)	Group C (5 - < 15 M/ml)	Group D (≥ 15 M/ml)
Number of cycles	260	216	265	525
Number of injected oocytes, mean ± SD	6.8 ± 4.4	6.5 ± 4.0	6.4 ± 4.1	6.9 ± 4.2
Fertilization rate, median (IQR)	66.7 (40.0–80.0)***	71.4 (50.0–90.1)	71.4 (55.5–90.0)	75.0 (57.1–90.2)
Rate of blastocyst formation, median (IQR)	50.0 (33.3–66.3)*	50.0 (36.4–70.0)	50.0 (37.6–71.4)	55.6 (40.0–75.0)
Rate of top quality blastocyst, median (IQR)	0.0 (0.0–33.3)	0.0 (0.0–30.0)	0.0 (0.0–20.0)	0.0 (0.0–25.0)
Rate of blastocyst in day V, median (IQR)	66.7 (33.3–100)	50.0 (33.3–85.8)*	50.0 (31.2–84.0)**	66.7 (40.0–100)
Number of transferred embryos, mean ± SD	1.7 ± 0.6	1.7 ± 0.6	1.7 ± 0.7	1.6 ± 0.7
Number of transfer day V (%)	86 (33.1%)*	78 (36.1%)	93 (35.1%)*	237 (45.1%)
Number of frozen embryos, mean ± SD	1.3 ± 2.0	1.6 ± 2.1	1.6 ± 2.1	1.9 ± 2.3
Number of transfer (%)	165 (63.4%)	141 (65.2%)	171 (64.5%)	351 (66.8%)
Ongoing Pregnancy, OR (<IC95%)	1.1 (0.7–1.8)	0.9 (0.5–1.5)	0.7 (0.4–1.1)	1

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (adjusted for confounders) vs group D. M/ml million per milliliter, SD standard deviation, OR odd ratio, IQR Interquartile Range

be ineffective, inevitably leading to an arrested embryo development [23]. Therefore, it is plausible that the spermatozoa in the group A of our study introduced an over-the-threshold damaged DNA into the oocyte upon fertilization and negatively impacted the rate of blastocyst formation. Our data highlight the reduced number of rapid developing blastocysts (less blastocysts observed on day V of development) in groups B and C compared to control group D, suggesting a possible effect of oocyte quality, which can be related to female idiopathic infertility paired with moderate male factor infertility. DNA damage may also explain the reduced fertilization rate in patients of group A compared to group D in our study. Consistent with this observation, DNA breaks in spermatozoa, which were more frequently observed in oligozoospermia compared to normozoospermia, correlated with a low fertilization rate in 100 ICSI cycles of a Danish IVF Center [24].

Table 3 Fertilization rate according to different sperm concentration and motility groups

Sperm concentration	Sperm motility			
	< 5%	5 - < 10%	10 - < 32%	≥ 32%
Group A (< 1 M/ml)	*n* = 108	*n* = 86	*n* = 57	n = 10
	55.6 * **	**66.7 ** **	**71.4**	**75.0**
Group B (1 - < 5 M/ml)	*n* = 50	n = 57	*n* = 87	*n* = 21
	**65.2 ** **	**71.4**	**77.0**	**75.0**
Group C (5 - < 15 M/ml)	*n* = 53	*n* = 38	*n* = 121	*n* = 52
	66.7	**66.7**	**75.0**	**73.9**
Group D (≥ 15 M/ml)	*n* = 140	*n* = 31	*n* = 122	*n* = 233 §
	**72.1 * **	**73.3**	**75.0**	**75.0**

Bold emphasis represents pergentage of fertilization

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (adjusted for confounders) vs reference group (§) with normal semen parameters, results are expressed as median value

Our finding is further reinforced by numerous reports where spermatozoa from semen with severely impaired parameters decrease fertilization rate in ICSI cycles of greater cohorts [2, 6, 7]. However, it should be noted that one smaller study (130 ICSI cycles), failed to find an association of abnormal semen parameters with fertilization rate [5].

Our data highlight the negative impact of motility on the fertilization rate, in line with a previous study that showed a significantly reduced fertilization rate in patients with less than 32% rapidly progressing spermatozoa [7]. This could be due to the limited availability of spermatozoa suitable for the treatment, which could lead to the selection of a suboptimal cell for ICSI. In fact, impaired motility has been proposed as a prognostic marker of low fertilization rate following ICSI [25, 26].

We have also shown that paternal age, which was used as confounding factor in the linear regression analysis, negatively affected the rate of fertilization: at the increase of each year of age we noted a reduction of 0.3% on the fertilization rate. Although the study from Chapuis et al. [7] has already reported a significant effect of male age in the fertilization rate, this result has not been corrected for female age and number of retrieved oocytes, thus making it impossible to determine whether the effect was due to the male or female age. Conversely, no impact could be demonstrated on the rate of top quality blastocysts formed and on pregnancy outcomes, in accordance with previous studies [27, 28]. The interpretation of the impact of paternal age is challenging due to the bias introduced by the maternal age; the two are inevitably correlated. There is some evidence supporting that paternal age has a detrimental effect on the number of high-quality embryos achieved [9, 29]. In contrast, other authors have argued that the parameters of the embryo quality are not influenced by the paternal age after correction for the female age or semen origin [27, 28]. Recently, evidence of a negative association between paternal age and live births was also presented [30].

We did not observe significant differences in the top quality blastocyst formation rate and in the clinical outcomes between the study groups. The ongoing pregnancy rate per ovum pick-up was similar among the groups. Despite the lower rate of blastulation, the implantation rate in Group A was unchanged for the evolving embryos. Consistent with our finding, a recent retrospective analysis showed that although the abnormal sperm parameters compromise embryo developmental competence, they do not negatively impact the rate of euploid blastocysts obtained or their implantation potential [6].

The impact of our results on clinical practice is uncertain. A blastocyst transfer policy could be considered for couples with mild male factor (group B and C), given the good blastulation rate comparable to the control group (group D). On the other hand, the embryo-transfer strategy in cases of severe oligozoospermia, could be fine-tuned to the progress of the cycle in order to reduce the rate of cycles without embryo transfer. In particular, a high number of oocytes retrieved (n. oocyte 8–10) as well as the high incidence of top quality day 3-embryos, could justify the choice of single transfer of a blastocyst. The lower blastulation rate associated with oligozoospermia is thought provoking in this context. Decision making in cases of low number of oocytes retrieved (n. oocyte 3–4), combined with the reduced blastulation rate is more straight-forward, pointing towards an early stage (cleaved) embryo transfer.

One flaw of our study is that we did not consider sperm morphology. This limitation is largely mitigated by the absence of strong evidence linking teratozoospermia with unfavorable IVF outcomes. Although some studies proposed that ICSI can improve the fertilization rate in cases where the sole diagnosis is teratozoospermia (assested by Kruger's strict criteria) [31], others argued that teratozoospermia did not influence embryonic outcomes such as blastulation or pregnancy outcome in their studies [32]. The fact that we did not consider the cumulative pregnancy rates, and the retrospective nature of the study are additional limitations of the study. Albeit at the expense of a retrospective nature, our study is strengthened by its large number of cycles.

In conclusion, we report abnormal sperm parameters compromised fertilization and blastulation rates but had no impact on the implantation potential of the obtained blastocysts. We also report for the first time a properly adjusted estimation of the impact of paternal age on fertilization rate. These results can stimulate development of novel hypotheses on the mechanisms by which oligozoospermic semen arrests embryonic development.

Compliance with ethical standars

Conflict of interest E.P. reports consultancies with MSD, Merck-Serono, Ferring, and IBSA Institut Biochimique SA; grants from MSD, Merck-Serono, Ferring, IBSA Institut Biochimique SA and FINOX; honoraria from MSD, Merck-Serono, and Ferring; and travel expenses paid by MSD, Merck-Serono, Ferring, and IBSA Institut Biochimique SA. All the other authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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