

Negative influence of paternal age on clinical intracytoplasmic sperm injection cycle outcomes in oligozoospermic patients

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Objective: To evaluate the effect of male age on clinical outcomes of intracytoplasmic sperm injection (ICSI) cycles, according to sperm concentration.

Design: Retrospective, observational study.

Setting: Assisted reproduction center.

Patient(s): The study included 1,024 couples undergoing ICSI cycles with fresh spermatozoa.

Intervention(s): The influence of paternal age on ICSI outcomes of oligozoospermic and normozoospermic patients was evaluated.

Main Outcome Measure(s): Rates of high-quality embryos, pregnancy, implantation, and miscarriage were evaluated through linear logistic regression analyses.

Result(s): When the sperm concentration was abnormal, paternal age influenced implantation (regression coefficient value = -0.7009) and pregnancy rates (odds ratio = 0.95 , 95% confidence interval $0.91-0.99$). However, in normozoospermic patients, no influence of paternal age was observed on implantation (regression coefficient value = 0.0566) or pregnancy rates (odds ratio = 1.00 , 95% confidence interval $0.97-1.03$).

Conclusion(s): For couples in which the men are oligozoospermic, the implantation rate could be impaired by increased paternal age. In these couples, the chance of pregnancy decreased 5% for each year of paternal age. When men are normozoospermic, this effect is not observed. (Fertil Steril® 2010;93:1870-4. ©2010 by American Society for Reproductive Medicine.)

Key Words: Paternal age, ICSI, implantation, pregnancy rate

With increased life expectancy in developed countries, many couples are delaying childbearing. However, the risk of reproductive difficulties is clearly increased for couples who delay childbearing until after the age of 35 years. As a result, the relationship between maternal age and a progressive decline in fertility has been widely discussed. It has been reported that female fertility starts to markedly decline at approximately age 35 years, when there is a sharp decrease in the follicle reserves (1, 2). Moreover, other complications have been described in older women: spontaneous abortion, pregnancy complications, congenital abnormalities, and perinatal mortality (3).

Received August 12, 2008; revised November 11, 2008; accepted December 10, 2008; published May 5, 2009.

R.C. has nothing to disclose. D.B. has nothing to disclose. T.B. has nothing to disclose. F.P. has nothing to disclose. A.I. has nothing to disclose. E.B. has nothing to disclose.

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Meanwhile, the effects of advancing paternal age on assisted reproductive technology outcomes are controversial. Male reproductive function does not cease abruptly as in women, but some factors can become fundamentally changed with age (4). Decline in semen parameters, such as volume, concentration, motility, and morphology, has been observed in men of increasing age (5-8). Morphologic changes in the testes could induce a qualitative and quantitative decrease in spermatogenesis (7, 8) due to a decline in blood T concentration with advancing age (9).

At present there is controversy regarding the influence of paternal age on clinical outcomes after IVF. Some investigators have associated a decline in pregnancy rates with advancing paternal age in couples in which the woman is younger (10-12). On the other hand, Spandorfer *et al.* (13) suggest that the pregnancy rate is not affected by male age.

The aim of this study was to evaluate the effect of male age on clinical outcomes of intracytoplasmic sperm injection (ICSI) cycles, according to sperm concentration.

MATERIALS AND METHODS

Experimental Design

This case-control study analyzed 1,024 couples undergoing ICSI cycles using fresh spermatozoa from January 2002 to December 2007. To exclude poor-responding patients, who would influence the results, cycles in which fewer than four metaphase II oocytes were recovered were not included (14, 15).

Written, informed consent was obtained from all patients, who agreed to share the outcomes of their cycles for research purposes, and the study was approved by the local institutional review board.

The influence of paternal age on sperm concentration, sperm motility, and rates of high-quality embryos, implantation, pregnancy, and miscarriage was evaluated in two different groups of patients: normozoospermics patients (sperm concentration $\geq 20 \times 10^6/\text{mL}$) ($n = 690$) and oligozoospermics patients (sperm concentration $< 20 \times 10^6/\text{mL}$) ($n = 334$). Oligozoospermics patients were previously diagnosed through seminal analyses, according to World Health Organization criterion (16).

High-quality embryo rate was defined by the number of high-quality embryos divided by the total number of normally fertilized oocytes. The implantation rate was defined as the total number of gestational sacs divided by the total number of embryos transferred. Clinical pregnancy was defined as the presence of a gestational sac visualized by ultrasound 4–6 weeks after ET.

Seminal Processing

After a 3–5-day ejaculatory abstinence, semen samples were collected by masturbation into a sterile container. After liquefaction for 30 minutes at room temperature, all semen samples were analyzed, and volume, concentration, linear progressive motility, agglutination, presence of round cells, and morphology were recorded before semen preparation. These analyses followed World Health Organization and Kruger's strict criteria.

Sperm samples were prepared by discontinuous density gradient centrifugation or swim-up. For discontinuous density gradient centrifugation, the bottom fraction was aspirated and washed twice at $300 \times g$ for 8 minutes. For swim-up, sperm samples were diluted 1:1 with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered medium (Irvine Scientific, Santa Ana, CA), centrifuged at $300 \times g$ for 8 minutes, and incubated at 37°C for 1 hour, allowing spermatozoa to move from the resulting pellet to the overlaid culture medium.

Controlled Ovarian Stimulation

Controlled ovarian stimulation was achieved by a long pituitary down-regulation using a GnRH agonist, followed by ovarian stimulation with recombinant FSH. The follicular dynamic was assessed by ultrasound, starting on day 4 of gonad-

otropin administration, and when adequate follicular growth and serum E_2 levels were observed, urinary or recombinant hCG was administered to trigger ovulation. Thirty-six hours after urinary or recombinant hCG administration, oocytes were collected by transvaginal ultrasound ovum pick-up.

Preparation of Oocytes

After retrieval, oocytes were incubated in culture medium covered with mineral oil for 3 hours at 37°C and 6% CO_2 . Cumulus cells were removed by a 30-second exposure to HEPES-buffered medium containing 80 IU/mL hyaluronidase, after which coronal cells were carefully removed using finely drawn glass Pasteur pipettes.

Denuded oocytes were then assessed for nuclear status. Oocytes showing the release of the first polar body were considered mature and were used for ICSI (13).

Assessment of Fertilization, Embryo Quality Evaluation, and ET

Fertilization was assessed 18 hours after ICSI, and normal fertilization was considered when two clearly distinct pronuclei were present.

Embryo quality was evaluated under an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan), and the following parameters were recorded: number of blastomeres, fragmentation percentage, variation in blastomere symmetry, presence of multinucleation, and defects in the zona pellucida and cytoplasm.

Embryo transfer was performed on the third day of development. High-quality embryos were defined as those showing four cells on the second day or six to eight cells on the third day of development, less than 15% fragmentation, symmetric blastomeres, absence of multinucleation, colorless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity, and absence of zona pellucida dysmorphism.

For each couple, from one to four embryos were transferred, depending on the embryo quality and the female partner's age.

Statistical Analysis

Multiple linear regression was performed to study the influence of paternal age on high-quality embryos and implantation rates, and the results were expressed as the regression coefficient (RC) value and *P* value. To assess the influence of paternal age on pregnancy and miscarriage rates, a multiple binary logistic regression model was applied, and the results were expressed as odds ratios (OR), 95% confidence interval (CI), and *P* value.

All regression analyses were adjusted for maternal age, numbers of oocytes retrieved, sperm concentration, and fertilization rate, because these would be considered potential confounders of the association between paternal age and ICSI outcomes.

Results were considered significant at the 5% critical level ($P \leq .05$). Data analysis was carried out with the Minitab (version 14) statistical program (State College, PA).

RESULTS

The general characteristics of the cycles are described in Table 1.

No correlation between sperm concentration and paternal age was observed for either normozoospermic ($P=.148$; Pearson $r = 0.055$) or oligozoospermic patients ($P=.382$; Pearson $r = 0.048$). Sperm motility was also not correlated with paternal age for normozoospermic ($P=.135$; Pearson $r = -0.057$) or oligozoospermic patients ($P=.304$; Pearson $r = -0.056$).

To evaluate whether paternal age influenced the high-quality embryo rate, multiple linear regression was performed and adjusted as mentioned in Materials and Methods. No influence of paternal age was observed on the high-quality embryo rate for either group (oligozoospermic: $P=.442$, $RC = 0.1822$; normozoospermic: $P=.368$, $RC = -0.1725$; Fig. 1). On the other hand, paternal age had a negative influence on implantation rate only in couples in which the man had an abnormal sperm concentration ($P=.008$, $RC = -0.7009$; Fig. 2) but not for normozoospermic patients ($P=.752$, $RC = 0.0566$; Fig. 2).

To study the influence of paternal age on pregnancy and miscarriage rates, a multiple binary logistic regression was conducted and adjusted as mentioned in Materials and Methods. Paternal age influenced the pregnancy rate when the patient was oligozoospermic ($P=.017$, $OR = 0.95$ [95% CI 0.91–0.99]) but not when the sperm concentration was normal ($P=.878$, $OR = 1.00$ [95% CI 0.97–1.03]). These results suggest that when the sperm concentration is abnormal, the chance of pregnancy decreased 5% for each year of paternal age.

On the other hand, independent of sperm concentration, paternal age did not influence miscarriage outcomes (oligozoospermic patients: $P=.128$, $OR = 0.92$ [95% CI 0.82–1.03]; normozoospermic patients: $P=.916$, $OR = 1.00$ [95% CI 0.94–1.06]).

DISCUSSION

The literature has demonstrated an effect of advancing paternal age on pregnancy (10, 11), miscarriage (3, 8), failure to conceive (17), recurrent pregnancy loss (18), and live birth rates (12). However, little is known about the effect of advancing paternal age on implantation rates. The present study evaluated the effect of paternal age on ICSI outcomes in normozoospermic and oligozoospermic patients. Our results show that paternal age negatively influences the implantation and pregnancy rates only in couples in which the man's sperm concentration was $<20 \times 10^6/\text{mL}$.

The decrease in implantation and pregnancy rates in couples in which the man has oligozoospermia can be due to several factors, including sperm quality and genetic alterations. Many studies have demonstrated that sperm quality is severely affected by increasing age (6, 19, 20). Aboulghar *et al.* (5) showed that patients aged <50 years had sperm concentration, motility, and morphologic quality that was significantly increased when compared with patients with aged ≥ 50 years. These effects were also observed in male volunteers. The mean sperm total count and motility in these volunteers decreased according to age (20, 21).

The reason there is no effect of paternal age on clinical outcomes in couples in which the men are normozoospermic is unclear; however, it could be argued that the negative age-related effects would be magnified by male-factor infertility. Spermatogenesis is the sequence of cytologic events that result in the formation of mature spermatozoa from precursor cells. Three events constitute spermatogenesis: stem cell renewal by mitosis, reduction of chromosomal number by meiosis, and the transformation of a conventional cell into the spermatozoon (22, 23). Our findings might suggest that in patients in whom spermatogenesis is already compromised, important sperm-related factors, such as centrosomic factors and oocyte activation factor, may be affected by age.

Indeed, in mammalian oocytes, an intracellular calcium rise is the signal responsible for the resumption of meiosis and the beginning of embryo development, thus playing an important role during fertilization and embryo development

TABLE 1

Characteristics of ICSI cycles from oligozoospermic and normozoospermic patients.

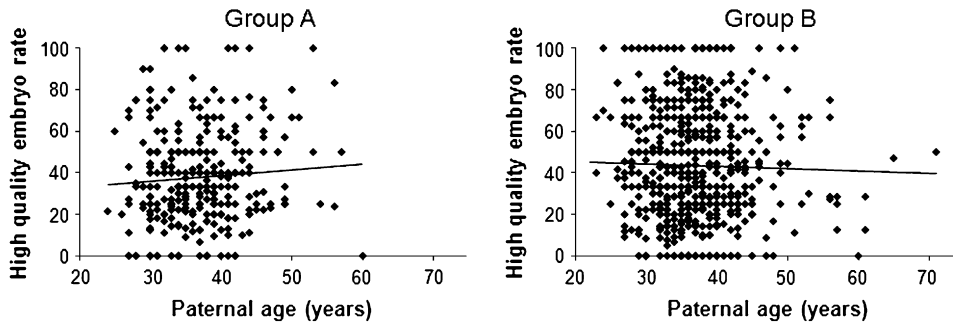
Parameter	Oligozoospermic (n = 334)	Normozoospermic (n = 690)
Paternal age (y)	36.85 ± 6.28	37.18 ± 6.12
Sperm concentration ($10^6/\text{mL}$)	7.03 ± 5.90	70.62 ± 46.95
Sperm motility (%)	41.07 ± 19.85	61.54 ± 13.02
Maternal age (y)	32.80 ± 4.86	34.23 ± 4.48
No. of retrieved oocytes	16.15 ± 8.30	14.29 ± 8.91
No. of metaphase II oocytes	12.09 ± 6.62	10.58 ± 6.63

Note: Oligozoospermic patients: sperm concentration $<20 \times 10^6/\text{mL}$. Normozoospermic patients: sperm concentration $\geq 20 \times 10^6/\text{mL}$. Values are mean ± SD.

Ferreira. Paternal age and ICSI outcomes. *Fertil Steril* 2010.

FIGURE 1

Influence of paternal age on high-quality embryo rate. Multiple linear regression was performed and was adjusted for maternal age, numbers of oocytes retrieved, sperm concentration, and fertilization rate. Group A (oligozoospermic patients): $P=.442$, $RC = 0.1822$ (high-quality embryo rate regression equation: in high-quality embryo rate = $42.3 + 0.182$ paternal age $- 0.214$ maternal age $- 0.802$ number of oocyte retrieved $- 0.347$ sperm concentration $+ 0.155$ fertilization rate); group B (normozoospermic patients): $P=.368$, $RC = -0.1725$ (high-quality embryo rate regression equation: in high-quality embryo rate = $59.9 - 0.172$ paternal age $- 0.115$ maternal age $- 0.569$ number of oocyte retrieved $+ 0.0029$ sperm concentration $+ 0.0225$ fertilization rate).



Ferreira. Paternal age and ICSI outcomes. *Fertil Steril* 2010.

(24). It has been suggested that sperm provide a soluble factor that is released into the egg upon sperm-egg fusion (25), which is expressed in the spermatid stage (26); however, whether its release and activation may be affected by paternal age has yet to be determined.

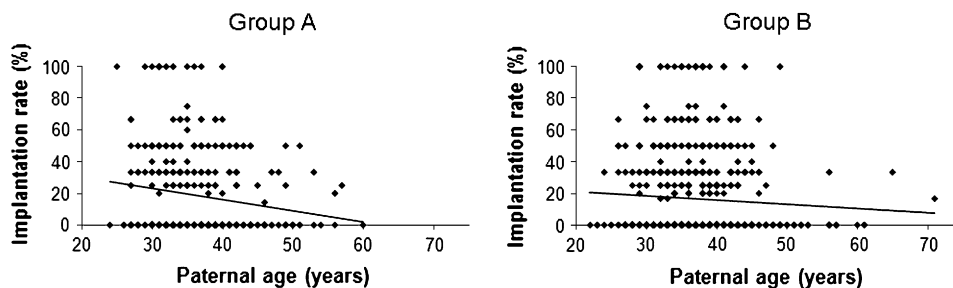
The identification of DNA fragmentation is an important tool to predict IVF outcomes. The DNA fragmentation could be related to semen parameters and paternal age. Flow cytometry data showed a negative correlation between normal morphology, motility, concentration, or total count of spermatozoa and percentage of DNA fragmentation (27). Moskovtsev *et al.* (19) demonstrated significantly reduced

sperm motility and increased DNA fragmentation in patients aged ≥ 45 years. Furthermore, patients with a high percentage of DNA fragmentation had a reduction in potential for *in vivo* fertility (28). Tesarik *et al.* (29) suggested that DNA fragmentation on spermatozoa has a late adverse effect on embryo development, without any apparent damage to morphology at the moment of transference.

Male genetic alterations could be mediated by age-related increases in germ cell mutations, impairment of DNA repair mechanisms, and apoptotic processes (30). Some mutations of single-base substitutions were exclusively paternal in origin. Because of countless divisions of stem cells, older men

FIGURE 2

Influence of paternal age on implantation rate. Multiple linear regression was performed and was adjusted for maternal age, numbers of oocytes retrieved, sperm concentration, and fertilization rate. Group A (oligozoospermic patients): $P=.008$, $RC = -0.7009$ (implantation rate regression equation: in implantation rate = $48.2 - 0.701$ paternal age $- 0.177$ maternal age $- 0.220$ number of oocyte retrieved $- 0.092$ sperm concentration $+ 0.082$ fertilization rate); group B (normozoospermic patients): $P=.752$, $RC = 0.0566$ (implantation rate regression equation: in implantation rate = $32.1 + 0.057$ paternal age $- 0.758$ maternal age $+ 0.426$ number of oocyte retrieved $- 0.024$ sperm concentration $+ 0.0542$ fertilization rate).



Ferreira. Paternal age and ICSI outcomes. *Fertil Steril* 2010.

may be at major risk for errors in DNA transcription (8). Increased paternal age was also found to increase the frequency of numeric and structural aberrations, which were significantly greater in chromosomes of older sperm donors (4, 31, 32). Furthermore, an increase in the risk of birth defects was also associated with advancing paternal age (33). These genetic alterations may be associated with impairment in implantation and consequently impairment in pregnancy (10, 11).

Our finding suggests that, in oligozoospermic patients, the chance of pregnancy decreases 5% for each year of increased paternal age. This evidence corroborates the findings of Mathieu *et al.* (10) and Ford *et al.* (11), who showed a decreased likelihood of pregnancy associated with advancing paternal age.

Some studies have reported an association between the risk of miscarriage and advanced male age (3, 8, 13, 34). We did not find any relationship between paternal age and miscarriage. Differences in study design and statistical analyses could explain those differences.

In conclusion, in couples in which the man is oligozoospermic, implantation rates and pregnancy outcomes could be impaired with increasing paternal age; however, the same is not observed in couples in which the man is normozoospermic.

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