Article

Paternal contribution to aneuploidy in preimplantation embryos

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Abstract

The association between sperm indices and the chromosomal status of preimplantation embryos was assessed in 230 couples with a female partner younger than 36 years undergoing 295 cycles of preimplantation diagnosis for aneuploidy: 105 cycles had normozoospermic samples, 134 cycles presented with oligoasthenoteratozoospermia (OAT), while the remaining cycles had spermatozoa retrieved from the seminal tract due to obstructive (29 cycles) or non-obstructive azoospermia (NOA, 27 cycles). One blastomere was biopsied from day-3 embryos and analysed for chromosomes XY, 13, 15, 16, 17, 18, 21, and 22. From the testing of 1549 embryos, the proportion of chromosomally abnormal embryos was significantly lower in normozoospermic patients (55%) than in OAT (62%, $P < 0.025$) and NOA patients (69%, $P < 0.005$). Complex abnormalities were the most frequent defect in NOA (68%), which also demonstrated the highest incidence of gonosomal aneuploidy (12%). From the re-analysis of all blastomeres in 493 non-transferred embryos, 95% of NOA embryos were chaotic mosaics. In conclusion, a severe male infertility condition could contribute to the generation of chromosomal abnormalities in the resulting embryos. This might occur especially in NOA patients in which the high incidence of chromosomal abnormalities is mainly due to mosaicism and gonosomal aneuploidy.

Keywords: *aneuploidy, ICSI, male infertility, preimplantation genetic diagnosis, sperm quality*

Introduction

An inverse relationship between sperm indices and the prevalence of karyotype abnormalities in infertile men has been reported, with numerical and structural chromosomal abnormalities (gonosomal aneuploidy, balanced translocations) appearing to be more frequent in men with pathological spermatozoa compared with the general population (Van Assche *et al.*, 1996; Yoshida *et al.*, 1996). In agreement with these reports, two findings have focused the attention on whether the paternal contribution to aneuploidy could be especially relevant in cases of severe male factor infertility: (i) the increased incidence of de-novo chromosomal abnormalities in the children born after intracytoplasmic sperm injection (ICSI) with a notable rise in sex chromosome aneuploidy (1.6% versus 0.45% according to Liebaers *et al.*, 1995; Bonduelle *et al.*, 2002); and (ii) a higher aneuploidy rate in spermatozoa from patients with severe oligoasthenoteratospemia (OAT) or azoospermia compared to normozoospermic men (Bernardini *et al.*, 2000; Levron *et al.*, 2001; Calogero *et al.*, 2003, Gianaroli *et al.*, 2005a).

Meiotic disorders are reported to occur frequently in infertile males, and increase proportionally with the severity of OAT condition and/or FSH concentrations (Egozcue *et al.*, 2000). As these patients produce spermatozoa with autosomal and sex chromosome disomies and diploid spermatozoa, their contribution to recurrent abortion or failed implantation could depend on the production of embryos with trisomies, monosomies and triploidy of male origin (Egozcue *et al.*, 2000; Bernardini *et al.*, 2004, 2005). This condition could be originated by either meiotic mutations or by a compromised testicular environment affecting chromosome segregation during meiotic divisions (Egozcue *et al.*, 2000). The effects

seem to be related to the severity of OAT and are especially relevant in testicular and epididymal sperm cells both in non-obstructive azoospermia (NOA) (Lange *et al.*, 1997; Egozcue *et al.*, 2000; Gianaroli *et al.*, 2005a) and obstructive azoospermia (Mroz *et al.*, 1999; McVicar *et al.*, 2005).

In light of these considerations, it is possible that the well-known maternal preponderance to the generation of euploid fetuses in natural conceptions (Koehler *et al.*, 1996) could be subverted in cases of severe male factor infertility, including azoospermia, in which ICSI totally bypasses any mechanism of natural sperm selection.

As the chromosomal defects in gametes can be transmitted to the resulting embryos, the information derived by preimplantation genetic diagnosis for aneuploidy, currently known as preimplantation genetic screening (PGS), is especially relevant in selected categories of patients. Accordingly, embryos derived from azoospermic patients could have a high incidence of chromosomal abnormalities even when the female age is below 36 years (Gianaroli *et al.*, 2000; Silber *et al.*, 2003; Platteau *et al.*, 2004; Gianaroli *et al.*, 2005b).

The aim of this study was to evaluate the paternal contribution to embryo aneuploidy in selected groups of patients characterized with a poor prognosis of pregnancy having undergone at least one previous IVF failure. The effect of sperm indices on the chromosomal constitution of preimplantation embryos was assessed in couples in whom female partners were younger than 36 years.

Materials and methods

Patients

A total of 230 patients underwent 295 cycles of PGS. Only patients younger than 36 years were included in the study resulting in a mean maternal age of 32.3 ± 2.2 years. Indications for PGS were: three or more previous IVF failures (135 cycles, mean maternal age 32.7 ± 2.1 years); two IVF failures in combination with one or more spontaneous abortions (104 cycles, mean maternal age 32.2 ± 2.1 years); and azoospermic patients with at least one previous IVF failure, who had spermatozoa microsurgically retrieved due to obstructive azoospermia (29 cycles, mean maternal age 31.0 ± 2.5 years) or non-obstructive azoospermia (NOA, 27 cycles, mean maternal age 32.1 ± 2.4 years).

Induction of multiple follicular growth was performed by administering exogenous gonadotrophins after a long desensitization protocol with long-acting gonadotrophin-releasing hormone analogues (Ferraretti *et al.*, 1996, 2004). Oocytes were collected transvaginally under ultrasound guidance at 34–36 h after human chorionic gonadotrophin (HCG) administration, and cultured in human tubal fluid supplemented with 5% human serum albumin (HSA) in a 5% CO_2 humidified gas atmosphere at 37°C.

Oocytes were inseminated by conventional IVF or ICSI depending on sperm indices and couples' reproductive histories. Patients were arbitrarily divided into the following subgroups depending on the characteristics of sperm indices as defined by the criteria described by the World Health Organization (2000): normozoospermic (105 cycles), oligoasthenoteratozoospermic (134 cycles, of which 76 were severe OAT with $<0.5 \times 10^6$ total motile count), and azoospermic patients due to obstructive azoospermia (29 cycles) or NOA (27 cycles). The conditions of obstructive azoospermia and NOA was based on a clinical examination, ultrasound and hormonal concentrations. The diagnosis was also confirmed during surgery.

The study was discussed and approved by the local institutional review board.

Assessment of fertilization, embryo biopsy and fluorescence in-situ hybridization (FISH)

At 16 h post-insemination, oocytes were scored for the presence of pronuclei and polar bodies (Gianaroli *et al.*, 2003a). Regularly fertilized oocytes were cultured individually and scored at 40, 62, 88 and 112 h post-insemination. Number and morphology of nuclei and blastomeres and the percentage and type of fragmentation were recorded (Magli *et al.*, 2007). For the biopsy procedure, developing embryos with at least four cells and no more than 40% fragmentation were manipulated individually in HEPES-buffered medium overlaid with pre-equilibrated mineral oil at 62–64 h post-insemination. A nucleated blastomere was gently aspirated by using a polished glass needle, which was introduced into the perivitelline space after opening a breach of approximately 20 µm in the zona pellucida with acidic Tyrode's solution (Gianaroli *et al.*, 2003b). After blastomere biopsy, embryos were meticulously rinsed and transferred to blastocyst growing medium.

Each blastomere was incubated in hypotonic solution and, after removal of all the cytoplasm, the nucleus was fixed on a glass slide with methanol:acetic acid. For the multicolour FISH analysis, specifically labelled probes were used in a two-round protocol for the simultaneous detection of chromosomes XY, 13, 15, 16, 18, 21, and 22 (Magli *et al.*, 2001; Munné *et al.*, 2003; Gianaroli *et al.*, 2005c). The scoring criteria for FISH signals were previously described (Munné *et al.*, 1998; Magli *et al.*, 2001). Briefly, euploidy, haploidy and polyploidy were defined by the presence of two sets, one set and three or more sets respectively for the tested chromosomes; monosomy and trisomy were defined by the presence of an abnormal number of copies for one or two chromosomes, whereas the presence of three or more chromosomes with an abnormal number of copies defined the embryo as complex abnormal.

Embryo transfer and clinical outcome

Only chromosomally normal embryos were transferred by replacement into the uterus, mainly on day 4 (Gianaroli *et al.*, 1999b). Clinical pregnancies were confirmed by the presence of a gestational sac with fetal heartbeat. The implantation rate represented the ratio between the number of gestational sacs with fetal heart beat divided by the total number of embryos transferred.

Embryo spreading and blastomere analysis

A total of 493 embryos were disaggregated and all their blastomeres were fixed and analysed. They had been diagnosed as non-transferable after PGS and were reanalysed in order to estimate the overall rate of mosaicism and to verify the efficiency of the technique (Gianaroli *et al.*, 2001). For the reanalysis, the nuclei were fixed and hybridized with the same probes used for PGS following the same protocol described above.

Mosaic embryos were classified as previously reported, chaotic mosaics being those in which all cells had a different chromosome count (Munné *et al.*, 2002).

Statistical analysis

Data were analysed by Student's *t*-test and chi-squared analysis applying the Yates' correction, 2 × 2 contingency tables.

Results

A total of 1565 day-3 embryos were biopsied and the FISH analysis was informative for 1549 (99%), 618 were diagnosed as chromosomally normal (40%) whereas the remaining 931 had an abnormal chromosomal complement (60%). The analysis of the detected abnormalities revealed that 39% (n = 366) were due to monosomy and trisomy, 11% (n = 101) to haploidy and polyploidy, and 50% (n = 464) to complex abnormalities.

No euploid embryos resulted in 65 cycles (22% of oocyte retrievals), for which embryo transfer was cancelled. In the remaining 230 cycles, a mean of 1.9 ± 0.7 FISH normal embryos were transferred generating 82 clinical pregnancies (36% per transferred cycle and 28% per oocyte retrieval) with an implantation rate of 25.2% (108/428). One ectopic pregnancy occurred, while the incidence of spontaneous abortions was 8.5% (n = 7) accounting for a take-home-baby rate per patient of 32%. Of the 82 pregnancies, 59 were singletons (72%), 21 were twins, one was a triplet and one was a quadruplet.

Normozoospermic and OAT samples were equally distributed in the two categories of indication for PGS. In the category of three of more previous IVF failures, 42% (56/135) of samples were normozoospermic and the remaining 58% (79/135) were OAT; similarly, in patients with two IVF failures in combination with one or more spontaneous abortions, 47% (49/104) were normozoospermic and 53% (55/104) were OAT.

When evaluated according to sperm indices, the four subgroups were homogeneous in terms of maternal age, number of retrieved oocytes, administered units of FSH and concentration of oestradiol on the day of HCG. The resulting clinical pregnancy rate per oocyte retrieval and the take-home-baby rate per patient were slightly lower in NOA patients (19% for both parameters versus 30% and 36% respectively in normozoospermic), although the difference was not statistically significant (**Table 1**).

The chromosomal constitution of the embryos was evaluated in the different sub-groups. As represented in **Table 2**, the incidence of chromosomal abnormalities was significantly lower in normozoospermic patients (55%), when compared with OAT (62%, $P < 0.025$) and NOA patients (69%, $P < 0.005$). In cases of obstructive azoospermia, the proportion of chromosomally abnormal embryos (63%) was comparable to that of OAT patients.

Regarding the type of chromosomal defects, monosomy and trisomy were significantly lower in NOA (25%) when compared with normozoospermic (45%, $P < 0.05$), OAT (37%, $P < 0.05$) and obstructive azoospermia patients (45%, $P < 0.01$). **Figure 1** shows the contribution of gonosomal aneuploidy to total abnormalities. There was a significant increase in the proportion of gonosomal aneuploidy along with the severity of the male factor condition, ranging from 2.4% in normozoospermic patients (n = 8/328) to 12% in NOA (n = 11/92; $P < 0.001$). Interestingly, if the category of OAT was divided into moderate OAT (≥0.5 × 10^6 total motile count) and severe OAT (<0.5 × 10^6 total motile count), the proportion of gonosomal aneuploid embryos was 3.0% (n = 6/202) and 6.1% (n = 14/229) respectively, the last figure being significantly higher when compared with normozoospermic patients ($P < 0.05$).

Embryos carrying complex abnormalities, were more common in NOA (68%) patients versus all the other sub-groups (**Table 2**). The proportion of top-quality embryos, intended as those with seven or eight cells and no fragmentation, was inversely proportional to the typology of sperm samples (from 40% in normozoospermic to 16% in NOA patients, $P < 0.001$).

When looking at the type of anomalies within each sperm category, complex abnormalities were the prevailing defect in embryos from OAT (49% versus 37% monosomy and trisomy, $P < 0.001$) and NOA patients (68% versus 25% monosomy and trisomy, $P < 0.001$). In the group of OAT, this difference was detected both in moderate [49% (100/202) versus 39% (79/202), $P < 0.05$] and in severe OAT [49% (111/229) vs, 36% (81/229), $P < 0.01$].

In order to evaluate the rate of mosaicism, 493 non-transferred embryos had all their blastomeres disaggregated and re-analysed with the same FISH probes used for PGS on day 3. As represented in **Table 3**, the overall error rate of the technique was 6% as the PGS result on a single cell was confirmed in 464 embryos. Most of the misdiagnoses were due to mosaicism which was detected in 293 (59%) of the re-analysed embryos (21 embryos diagnosed as normal, aneuploid or haploid/polyploidy on single cell analysis, and 272 embryos diagnosed as complex abnormal on day 3). Accordingly, the incidence of clinically relevant errors was 2%: two embryos diagnosed as normal were mosaics, while four embryos that were not transferred being diagnosed as monosomic and four as trisomic, after re-analysis appeared to be chromosomally normal.

As shown in **Figure 2**, the proportion of chaotic mosaics was significantly higher in NOA patients (95%) compared with the other sub-groups (51% in normozoospermic, 59% in OAT, 62% in obstructive azoospermia, $P < 0.001$).

Table 1. Clinical outcome according to sperm parameters.

	Normozoospermia	*Oligoasthenoter-atozoospermia*	*Obstructive azoospermia*	*Non-obstructive azoospermia*
No. cycles at oocyte retrieval/patients	105/81	134/104	29/23	27/21
Mean age in years (± SD)	32.5 ± 2.2	32.5 ± 2.2	31.0 ± 2.5	32.1 ± 2.4
Mean no. of retrieved oocytes (± SD)	10 ± 4.5	9.4 ± 4.1	10.7 ± 3.4	10.6 ± 4.8
No. of transfer cycles	84	110	20	16
No. of clinical pregnancies	31	37	9	5
Percentage per transfer	37	34	45	31
Percentage per cycle at oocyte retrieval	30	25	31	19
No. of spontaneous abortions	2	2 + 1 ectopic	2	1
Implantation rate (%)	44/166 (26.5)	48/204 (23.5)	9/32 (28.1)	7/26 (26.9)
Take-home-baby rate/patient (%)	29/81 (36)	34/104 (33)	7/23 (30)	4/21 (19)

Table 2. Chromosomal abnormalities in IVF and intracytoplasmic sperm injection embryos according to sperm parameters.

	Normozoospermia	*Oligoasthenoter-atozoospermia*	*Obstructive azoospermia*	*Non-obstructive azoospermia*
Embryos diagnosed	594	695	127	133
FISH abnormal (%)	328 (55)^{a,b}	431 (62)^{a}	80 (63)	92 (69)^{b}
Monosomies and trisomies (%)	147 (45)^{c}	160 (37)^{d}	36 (45)^{e}	23 (25)^{c,d,e}
Haploidy and polyploidy (%)	30 (9)	60 (14)	5 (6)	6 (7)
Complex abnormalities (%)	151 (46)^{f}	211 (49)^{g}	39 (49)^{h}	63 (68)^{f,g,h}
No. day-3 embryos with 7–8 regular cells, no fragmentation (%)	237 (40)^{i}	243 (35)^{j}	37 (29)^{k}	21 (16)^{i,j,k}

Values with same superscript letter are significantly different: $^{ahk}P < 0.025$; $^{bg}P < 0.005$; $^{cd}P < 0.05$; $^{e}P < 0.01$; $^{fij}P < 0.001$.
FISH = fluorescence in-situ hybridization.

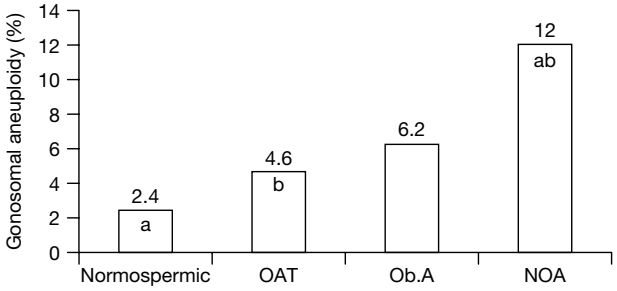

Figure 1. Proportion of embryos carrying gonosomal aneuploidy in the different sperm categories. OAT = oligoasthenoteratozoospermia; Ob.A = obstructive azoospermia; NOA = non-obstructive azoospermia. Values with the same superscript letter are significantly different ($^{a}P < 0.001$; $^{b}P < 0.025$).

Table 3. Re-analysis of non-transferred embryos with the same probes used for preimplantation genetic screening (PGS).

PGS result	*No. of embryos analysed*	*Confirmed*	*Not confirmed*	
			Normal	*Mosaics*
Normal	47	45	–	2
Monosomy	82	69	4	9
Trisomy	74	64	4	6
Haploid/polyploid	18	14	–	4
Complex abnormalities	272	272	–	–
Total (%)	493	464 (94)	8 (2)	21 (4)

Total mosaics = 293 (21 + 272).

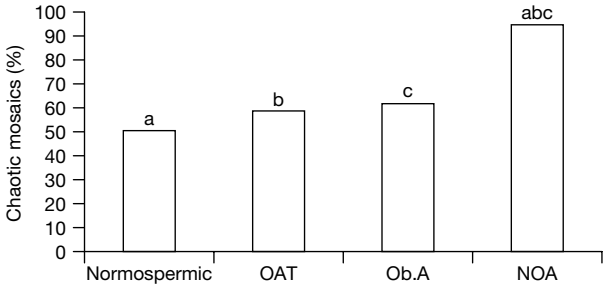

Figure 2. Proportion of embryos carrying chaotic mosaics in the different sperm categories. OAT=oligoasthenoteratozoospermia; Ob.A = obstructive azoospermia; NOA = non-obstructive azoospermia. Values with the same superscript letter are significantly different ($^{a,b,c}P < 0.001$).

Discussion

The clinical relevance of assessing the chromosomal status in preimplantation embryos has become manifest in the treatment of patients with a poor prognosis of term pregnancy. According to previous studies, the selection for transfer of euploid embryos is associated with increased implantation rates and reduced incidence of spontaneous abortions (Gianaroli *et al.*, 1997, 1999a, 2005a; Munné *et al.*, 2003, 2006a). The clinical advantage related to this approach is especially evident when a comparison is made with the patients' reproductive history, resulting in a significantly improved take-home baby rate after PGS (Gianaroli *et al.*, 2005c, Verlinsky *et al.*, 2005). On the other hand, three important studies have been published claiming that no advantage is associated with PGS compared to the controls (Staessen *et al.*, 2004, 2007; Mastenbroek *et al.*, 2007). Although these studies were randomized control trials, they have been severely criticized due to several biases in their approach that made their conclusion of unclear relevance (Cohen and Grifo, 2007; Kuliev and Verlinsky, 2007; Munné *et al.*, 2007b; Harper *et al.*, 2008).

The data derived from PGS have confirmed that maternal age has an unquestionable link with embryo aneuploidy and the risk of trisomic pregnancies (Màrquez *et al.*, 2000; Kuliev *et al.*, 2005; Munné *et al.*, 2005). Besides, it has been demonstrated that the incidence of aneuploidy is much higher in preimplantation embryos, up to a mean value of 67% in couples with a poor prognosis of pregnancy (Gianaroli *et al.*, 2005c) than in live-borns (0.3%, Warburton *et al.*, 1986). This demonstrates that a very strong selection mechanism against aneuploidy exists throughout embryo development, determining a progressive loss of abnormal embryos at specific stages. It is not known to what extent the results obtained from IVF patients are representative of the general population, but some studies on oocytes from unstimulated cycles seem to confirm an overall aneuploidy rate of more than 20% (Hassold and Hunt, 2001).

The study of preimplantation embryos in young patients has demonstrated that the rate of chromosomal abnormalities can be similar to that detected in older patients (Munné *et al.*, 2006b). This is probably due to the fact that young patients resorted to PGS for a poor prognosis indication, mainly repeated IVF failures and/or recurrent miscarriages. The chromosomal status of their preimplantation embryos suggests that they could carry a condition of infertility that is more severe compared with what characterizes standard IVF patients. Interestingly, the type of abnormalities are significantly different from those detected in the group of patients with an age factor, post-zygotic abnormalities being the most frequent reason for chromosomal alterations (Munné *et al.*, 2002; Gianaroli *et al.*, 2003b; Mantzouratou *et al.*, 2007). It can be concluded that there might be factors either inherent to the IVF procedure (Munné *et al.*, 2006b), or to the genetic background of each couple that predispose them to an increased risk of generating chromosomally abnormal embryos.

As the alterations detected in embryos may come from either parent, one possibility is that the fertilizing spermatozoa could have an important role in determining the chromosomal status of the conceptus. This could be due the generation of aneuploid gametes during spermatogenesis, or to a defective organization of the mitotic plates at the first cleavage divisions that, in human embryos, are organized by the sperm centrosome (Sathananthan *et al.*, 1991). According to the data from the present study, the proportion of chromosomally abnormal embryos increased with the severity of the male factor condition, with the highest incidence in those generated by NOA patients (**Table 2**). The analysis of the type of defects confirmed that both mechanisms contribute to the total abnormalities, but in a different way depending on the type of sperm characteristics. Basically, embryos from normozoospermic samples have the same type of abnormality distribution as obstructive azoospermia, monosomy and trisomy being as frequent as complex abnormalities, implying that a mechanical azoospermia is not necessarily associated with a pathological sperm sample. Conversely, complex abnormalities were by far the prevailing defect in embryos generated by NOA patients, as well as, although to a minor extent, in those derived from OAT patients. This points to post-zygotic errors as the predominant cause of abnormal development that could be dependent either on a defective sperm centrosome or on a molecular mechanism that is genetically inherited (Kay *et al.*, 2006). The presence of defects in the sperm centrosome from pathological spermatozoa has been reported from ultrastructural studies (Sathananthan *et al.*, 1991). These observations have supported the hypothesis that sperm centrosomal dysfunction could cause aberrant embryonic development based on centriolar defects in spermatozoa with impaired motility (**Table 2**). Accordingly, the occurrence of mosaicism in preimplantation embryos is one of the major consequences (Palermo *et al.*, 1994; Silber *et al.*, 2003). This condition was evaluated in the present study by analysing the chromosomal status of 493 non-transferred embryos that had all the blastomeres analysed with the same probes that were used for PGS. The incidence of chaotic mosaicism was significantly higher in embryos from NOA patients compared with all the other sperm categories, confirming the hypothesis that testicular spermatozoa might have difficulties in properly organizing the sperm aster within the oocyte for bringing pronuclei into apposition and the following chromosome congression on the first mitotic spindle, whose poles are generated by the replication of the male centrosome.

Several studies on spermatozoa have reported a higher frequency of aneuploidy in cases of severe OAT or azoospermia due to testicular failure compared with the normal population (Egozcue *et al.*, 2000; Gianaroli *et al.*, 2005a). In these

men, there could be a higher frequency of pairing disruption resulting in meiotic arrest and in the development of embryos with numerical chromosome abnormalities or mosaicism. Generally, with the exception of NOA, the increase in sperm aneuploidy is modest but big enough to lead to the increase in sex chromosome anomalies that has been reported in ICSI children (Bonduelle *et al*., 2002). Accordingly, the data from embryos generated by severe male factor patients showed a notable variation of gonosomes, reaching the highest level in NOA (**Figure 1**). The study of preimplantation embryos demonstrates that in young patients (<36 years), post-meiotic abnormalities such as mosaicism, haploidy and polyploidy are the most frequent type of chromosome abnormalities (Munné *et al*., 2007a). However, a male effect is evident with an increase in these anomalies that is proportional to the severity of the male factor condition (**Table 2**). This suggests that sperm integrity, including an optimal centriole function, are essential for a normal distribution of chromosomes to sister cells.

In conclusion, the data from this study propose that a severe male infertility condition could contribute to the generation of chromosomal abnormalities in the resulting embryos. This might occur especially in NOA patients in which the high incidence of chromosomal abnormalities is mainly due to mosaicism and gonosomal aneuploidy. Nevertheless, as the data reported here were originated from couples with at least one repeated IVF failure, they cannot be extended to the general IVF/ICSI population.

Although mitotic defects are often associated with embryo dimorphism like fragmentation, unevenness and multinucleation (Magli *et al*., 2007; Munné *et al*., 2007a), development to blastocyst can still occur (Magli *et al*., 2000) and PGS has been reported to be beneficial especially in cases of single embryo transfer performed at the blastocyst stage (Donoso *et al*., 2006). Provided that conclusive studies could finally demonstrate if benefits derive from the application of PGS on preimplantation embryos, this strategy could be the prevailing approach in the treatment of patients with a severe male factor of infertility in order to reduce the risk of transferring chromosomally abnormal embryos (Donoso *et al*., 2006).

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