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GENETICS

Contributing factors for the incidence of aneuploidy in older patients undergoing intracytoplasmic sperm injection cycles

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Abstract
Purpose To evaluate different factors that might affect the incidence of embryo aneuploidy in intracytoplasmic sperm injection cycles (ICSI).
Methods One hundred and ninety ICSI cycles in conjunction with preimplantation genetic screening (PGS) were included. The influence of the following variables on the aneuploidy incidence was evaluated: (i) maternal and (ii) paternal ages, (iii) dose of FSH administered, (iv) dose of FSH per number of retrieved matured oocytes (FSH/MII), (v) serum 17β-oestradiol levels on the ovulation trigger day, (vi) aspirated follicles and (vii) retrieved oocytes.
Results A total of 440 embryos were successfully biopsied, of which 240 were considered euploid and 200 were considered aneuploid. The paternal age (Slope: 0.2, p=0.372), total dose of FSH (Slope: 0.2, p=0.218), FSH/MII (Slope: 0.1, p=0.296) and 17β-oestradiol levels (Slope: 0.2, p= 0.378) were not correlated with the presence of aneuploidy. However, the maternal age (Slope: 1.7, p<0.01), aspirated follicles (Slope: 1.9, p<0.01) and retrieved oocytes (Slope: 2.6, p<0.01) were negatively correlated with the incidence of aneuploidy.
Conclusions Even in older patients, lower oocyte yields may represent a more appropriate response to ovarian stimulation, allowing the most competent follicles and oocytes to develop and thereby reducing the occurrence of embryo aneuploidy.

Keywords Aneuploidy · Ovarian stimulation · FSH · PGS · FISH

Capsule Lower oocyte yields reduces the occurrence of embryo aneuploidy.

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Introduction

The main challenge for successful in vitro fertilization (IVF) is the production of viable embryos with high implantation potential. Although high-quality embryos may be available for transfer, only a small proportion of patients undergoing IVF will ever achieve a pregnancy. The main cause for the relatively low efficiency of human reproduction appears to be the high incidence of chromosomal abnormalities in oocytes and embryos [11, 28].

The introduction of fluorescence in situ hybridization (FISH) labelling of interphase nuclei allows for the investigation of oocytes and preimplantation embryos for aneuploidies of specific chromosomes. Preimplantation genetic diagnostic (PGD) with multicolour FISH can significantly reduce the transfer of aneuploid embryos in human IVF. The preimplantation genetic screening (PGS) for aneuploidy is a technique that has used PGD technology to examine chromosomes in embryos from couples undergoing IVF with the aim of helping select the chromosomally 'best' embryo for transfer [15].

Clinically, PGS is advocated for older women [28, 35] and for patients with recurrent spontaneous abortion or repeated implantation failure [12, 32]. High rates of aneuploidy have been reported in these groups of women. Indeed, the most

commonly evaluated chromosomes are 13, 16, 18, 21 and 22, because these chromosomes are most frequently involved in embryo aneuploidies [26, 35] and spontaneous abortions [31].

The frequency of aneuploidy in human oocytes has been reported to range from 15.0 to 20.0 % [13]. The mechanisms underlying aneuploidy are still poorly understood. However, recent observations suggest that chromosome segregation failure in oocytes are often involved and that maternal meiosis errors increase with maternal age [16].

Preliminary observations suggest that aneuploidy in embryos may also be affected by ovarian stimulation regimens employed in IVF [18, 27]. Exogenous administration of gonadotrophins used for ovarian stimulation results in a higher concentration of circulating steroids, which may affect oocytes and/or embryos [14]. This is supported by studies where milder ovarian stimulation for IVF was associated with a reduced aneuploidy rate in the human preimplantation embryo [3]. It has also been suggested that oocyte yield after superovulation is associated with increased maternal meiotic segregation errors [13].

In addition to the ovarian stimulation regimen, other factors, such as in vitro maturation have also been proposed to be possible contributing factors for the incidence of aneuploidy in human embryos. Emery et al. [7] demonstrated that embryos created by intracytoplasmic sperm injection (ICSI) after 16–24 h of in vitro maturation have a higher rate of aneuploidy than those from standard ICSI. However, in this case, the ejaculated sperm sample was collected and prepared a day prior to the ICSI procedure; therefore, whether the increased aneuploidy incidence could be attributed to the in vitro culture itself or to defects in sperm-derived factors was not elucidated.

The aim for the present study was to evaluate different factors that might be related with the incidence of embryo aneuploidy in ICSI cycles.

Materials and methods

Study design

This study was conducted on 440 embryos recovered from 119 ICSI cycles in conjunction with PGS as a result of advanced maternal age (≥.38 y old), performed during January and December 2009, in a private assisted reproduction centre.

The influence of the following variables on the aneuploidy incidence was evaluated: (i) maternal age, (ii) paternal age, (iii) total dose of FSH administered for ovarian stimulation, (iv) dose of FSH per number of retrieved matured oocytes (FSH/MII), (v) serum 17β-oestradiol levels on the ovulation trigger day, (vi) number of aspirated follicles, (vii) number of retrieved oocytes, (viii) retrieved oocyte rate (number of retrieved oocytes divided by the number of aspirated follicles), and (ix) immature oocyte rate.

Written informed consent was obtained in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local Institute Review Board.

Controlled ovarian stimulation & laboratory procedures

Controlled ovarian stimulation was achieved by long-term pituitary downregulation using a GnRH agonist (Lupron Kit; Abbott S.A. Societé Française des Laboratoires, Paris, France) followed by ovarian stimulation with recombinant FSH (Gonal-F; Serono, Geneva, Switzerland).

Follicular dynamics were observed by transvaginal ultrasound examination, to track the follicular growth, starting on day 4 of gonadotropin administration. When adequate follicular growth and serum E2 levels were observed, recombinant hCG (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger final follicular maturation. Oocytes were collected 35 h after hCG administration by transvaginal ultrasound ovum pick-up.

The recovered oocytes were assessed for their nuclear status, and the oocytes in metaphase II were submitted to ICSI following routine procedures [29].

Normal fertilization status, indicated by the presence of two clearly distinct pronuclei, was assessed 18 h after ICSI (excluding abnormal fertilizations such as the presence of only one or more than two pronuclei).

Preimplantation genetic diagnosis and aneuploidy screening

On the morning of day 3 of embryo development, one cell per embryo was biopsied by laser zona drilling using a 1.48 mm Infrared Diode Laser (Octax Laser Shot System, MTG, Bruckberg, Germany).

After the biopsies, the embryos were returned to culture. The removed blastomere nuclei were spread using 0.1 N HCl and 0.01 % Tween 20 (Sigma, Dorset, UK). Briefly, the individual nuclei were placed on a slide in a drop of HCl-Tween spreading solution and observed until they had lysed. The slides were then air dried and dehydrated before fluorescent in situ hybridization (FISH) analysis was performed. All embryos were analysed for chromosomes X, Y, 13, 16, 18, 21, and 22 (Abbott Laboratories) following the manufacturer's instructions. Embryos with abnormal findings in biopsied nuclei were not submitted to reanalysis study.

Statistical analysis

Logistic regression models were used to evaluate the influence of the following variables on the aneuploidy incidence: (i) maternal age, (ii) paternal age, (iii) total dose of FSH administered for ovarian stimulation, (iv) dose of FSH per

number of retrieved matured oocytes (FSH/MII), (v) serum 17β-oestradiol levels on the ovulation trigger day, (vi) number of aspirated follicles, (vii) number of retrieved oocytes, (viii) retrieved oocyte rate and (ix) immature oocyte rate. The regression analyses were adjusted for all evaluated variables.

The results were expressed as a slope and p-value. The results were considered to be significant at the 5 % critical level ($p<0.05$). Data analysis was carried out using the Minitab (version 14) Statistical Program.

Results

A total of 440 embryos recovered from 119 ICSI/PGS cycles were successfully biopsied, of which 240 were considered euploid and 200 were considered aneuploid.

Among the aneuploid embryos, 37.0 % were chaotic (with two or more chromosomal numerical abnormalities), 35 % were monosomic (when only one copy of a chromosome was present in the embryo), and 28.0 % were trisomic (when three, instead of two, copies of a chromosome were present in the embryo).

The patients' characteristics are described as the mean± standard deviation for numeric variables and percentages (%) for categorical variables: maternal age: 40.3±4.4; paternal age: 43.5±9.3; total dose of FSH administered for ovarian stimulation: 2533 IU±712 (range:675–2850 IU); dose of FSH per number of retrieved matured oocytes: 338.8±102.6; serum 17β-oestradiol levels on the ovulation trigger day: 2740.7± 1344; number of aspirated follicles: 14.4±10.3; number of retrieved oocytes: 10.9±4.2; retrieved oocyte rate: 75.3 %; and immature oocyte rate: 28.4 %.

The presence of aneuploidy was not influenced by the paternal age, total dose of FSH, FSH/MII, 17β-oestradiol levels, retrieved oocyte rate and immature oocyte rate. However, the maternal age, number of aspirated follicles and number of retrieved oocytes negatively influenced the incidence of aneuploidy (Table 1).

When the chromosomal numerical abnormalities were evaluated separately, the incidence of chaotic embryos, trisomy or monosomy were not influenced by the presence of any of the evaluated variable (Table 2).

Table 1 Multivariate regression analysis of factors contributing to the incidence embryo aneuploidy for the following variables: maternal age, paternal age, total dose of FSH, dose of FSH per number of retrieved mature oocytes, serum 17β-oestradiol levels, number of aspirated follicles, number of retrieved oocytes, retrieved oocyte rate and immature oocyte rate

Response variable	Predictor variable	Slope	P
Embryo aneuploidy	Maternal age	1.7	< 0.01
	Paternal age	0.2	0.372
	Total dose of FSH	0.2	0.218
	FSH/MII	0.1	0.296
	17β-oestradiol levels	0.2	0.378
	Number of follicles	1.9	< 0.001
	Retrieved oocytes	2.6	< 0.001
	Retrieved oocyte rate	0.4	0.078
	Immature oocytes	0.2	0.172

Discussion

Data from numerous studies using a wide variety of techniques have demonstrated that a high proportion of human oocytes are affected by chromosomal abnormalities. Embryos produced from chromosomally abnormal gametes display aneuploidy in all of their cells. Although such embryos occasionally produce children affected by chromosomal abnormalities, the vast majority either fail to implant or culminate in a miscarriage [38].

The identification of factors that may contribute to the incidence of embryo aneuploidy is one of the most studied areas in ART. Until now, except for the advanced maternal age, there are no clearly established factors for the development of aneuploidy. For a woman over 40 years of age undergoing IVF treatment, it is typical for more than half of the oocytes retrieved to be chromosomally abnormal [9, 10]. This is in agreement with our findings, which demonstrate a negative influence of maternal age on the incidence of aneuploidy. In contrast, the effect of paternal age on embryo aneuploidy occurrence was not demonstrated. Although a decline in semen parameters has been observed in men of increasing age [1, 19], at present, there is controversy regarding the role of paternal age on clinical outcomes after IVF [8, 30].

There is growing evidence in animal studies to support the hypothesis that elevated FSH is one underlying cause of aneuploidy [5, 6, 34]. However, while animal studies seem to support an association between FSH exposure and embryonic aneuploidy, human studies have been more conflicting [21, 22, 27, 33, 37].

In the present study. no association between exogenous FSH or the FSH/MII rate and aneuploidy was noted. It has been argued that the elevated gonadotrophins levels associated with advanced maternal age, due to both the decreased ovarian reserve and the high doses of gonadotrophins used for controlled ovarian stimulation, may be in part cause of the high incidence of aneuploidy observed in older women [4]. In our study, the logistic regression models were adjusted for maternal age and other variables that could influence the results, which, in part, could explain the lack of influence of FSH on aneuploidy incidence.

Table 2 Multivariate regression analysis of factors contributing to the incidence chaotic embryos, monosomy and trisomy for the following variables: maternal age, paternal age, total dose of FSH, dose of FSH per number of retrieved matured oocytes, serum 17β-oestradiol levels, number of aspirated follicles, number of retrieved oocytes, retrieved oocyte rate and immature oocyte rate

Response variable	Predictor variable	Slope	P
Chaotic embryos Incidence	Maternal age	1.3	0.123
	Paternal age	0.4	0.112
	Total dose of FSH	1.2	0.228
	FSH/MII	1.2	0.326
	17β-oestradiol levels	0.2	0.293
	Number of follicles	1.8	0.089
	Retrieved oocytes	1.4	0.098
	Retrieved oocyte rate	0.3	0.342
	Immature oocytes	0.9	0.283
Embryo monosomy	Maternal age	1.3	0.242
	Paternal age	1.2	0.283
	Total dose of FSH	0.2	0.435
	FSH/MII	0.1	0.393
	17β-oestradiol levels	0.2	0.173
	Number of follicles	1.9	0.243
	Retrieved oocytes	1.3	0.078
	Retrieved oocyte rate	0.2	0.387
	Immature oocytes	0.4	0.556
Embryo trisomy	Maternal age	0.9	0.354
	Paternal age	1.2	0.144
	Total dose of FSH	1.3	0.265
	FSH/MII	0.9	0.222
	17β-oestradiol levels	0.8	0.323
	Number of follicles	1.6	0.122
	Retrieved oocytes	1.6	0.323
	Retrieved oocyte rate	0.9	0.324
	Immature oocytes	0.8	0.184

However, while the FSH dose was not related to embryonic aneuploidy, a negative correlation of oocyte yield on aneuploidy incidence was noted. Recent studies suggest that a low number of oocytes in ovarian response following ovarian stimulation are associated with a higher chance of conceiving [17, 36]. In fact mild ovarian stimulation leading to a lower oocyte yield has been previously associated with a decrease in the proportion of aneuploid embryos [3]. A previous study also showed that mild stimulation results in high-quality embryos for transfer, as indicated by embryo morphology [17]. Moreover, Haaf et al. [13] have provided evidence that oocyte yield after superovulation affects maternal meiotic segregation errors.

However, in the above mentioned studies, other variables that could be correlated with the results, such as maternal and paternal ages and FSH/MII rate were not evaluated. In our study, the data was analyzed by logistic regression models and the regression analyses were adjusted for all evaluated variables. In this model, it could be demonstrated that independently on the maternal age and on the dose of FSH used for ovarian stimulation, a lower oocyte yield may lead to a decreased incidence of abnormal embryos.

Why, in the present study, there was no influence of the FSH dose nor the oestradiol levels on the incidence of aneuploidy while a significant negative relation between oocyte yield and embryo aneuploidy was observed is still to be elucidated. In a study by Mizunuma et al. [25], with the same dose of FSH, patients with a higher response to stimulation had higher circulating FSH levels suggesting that women with a lower clearance rate of FSH may therefore have higher circulating gonadotropin levels and a higher response.

In addition, higher doses of FSH are usually administered in older women in order to obtain a reasonable number of retrieved oocytes. This is due to changes in multiple neuroendocrine axes, which are accompanied with ovarian aging ([20]). However, in the present study, very high doses of FSH were never used as a clinical approach of our centre.

During conventional controlled ovarian stimulation, natural follicle recruitment and selection is completely overridden, allowing the non-discriminate growth of many follicles at different developmental stages, which in natural conditions would undergo atresia. Independent of the exogenous FSH dose, interindividual differences in ovarian response among ART patients are usually noted [2]. A low number of oocytes obtained following a mild stimulation may represent a physiological response to the subtle interference with single dominant follicle selection and not a pathological reduction in ovarian response associated with ovarian ageing.

For the last 20 years, PGS has been mostly performed on cleavage stage embryos after the biopsy of 1–2 cells and PCR and FISH have been used for the diagnosis. However, it has been described that the prevalence of mosaicism is highly relevant for PGS, in which selection of embryos for transfer into the uterus is often based on the chromosomal analysis of this one aspirated blastomere [23, 24] In fact, at the cleavage stage, the cells biopsied from the embryo may not be representative of the rest of the embryo. There has therefore been a move towards blastocyst and polar body biopsy, depending on the indication and regulations in specific countries.

In the present study embryos were biopsied on day three and chromosomes were analyzed by FISH. Clearly, this is a limitation of the study and we cannot guarantee that abnormal blastomeres represent abnormal embryos in all the cases. However, mosaicism is also observed in blastocysts. There have been dramatic changes in the method of diagnosing small numbers of cells for PGS. Both array-comparative

genomic hybridisation and single nucleotide polymorphism arrays have been introduced clinically for PGS. These techniques are quite new and has recently been incorporated in assisted reproduction centers.

Together with the previously described studies, our evidence raises the question of whether is it worth trying to increase the ovarian response to the detriment of oocyte quality, even in older patients. In summary, our study suggests that independent of the exogenous FSH used for ovarian stimulation and of the maternal age, lower oocyte yields may represent a more appropriate response to ovarian stimulation, even in older patients, allowing the most competent follicles and oocytes to develop and thereby reducing the occurrence oocyte aneuploidy.

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