

## Articles

# Pregnancies from single normal embryo transfer in women older than 40 years



Michael Obasaju obtained his PhD in Comparative Pathology (Reproductive/Developmental Biology) from the University of California, Davis, CA, USA. He is currently the IVF/Andrology Laboratory Director at the New York Fertility Institute, New York, USA. Prior to this he was post-doctoral research fellow in the Dept of Obstetrics and Gynecology, University of California, Davis and Laboratory Director at Mount Sinai Medical Center and IVF America Program in New York, USA. Dr Obasaju's research interests include detection of sub-lethal damage to embryos, oocyte dysmorphisms, oocyte freezing, embryonic aneuploidies and implantation.

Dr Michael Obasaju

Michael Obasaju<sup>1</sup>, Arjun Kadam, Teresa Biancardi, Khalid Sultan, Majid Fateh, Santiago Munné<sup>2</sup>  
New York Fertility Institute, New York, New York, USA and, <sup>2</sup>The Institute for Reproductive Medicine and Science, Saint Barnabas Medical Center, Livingston, New Jersey, USA.

<sup>1</sup>Correspondence: New York Fertility Institute, 1016 Fifth Avenue, New York, NY10028, USA  
Tel. +212-734-5555; Fax 212-734-6059; e-mail: Obasaju@msn.com

### Abstract

The aim of this retrospective study was to determine the pregnancy rate from the transfer of single genetically normal embryos in patients of advanced reproductive age. The study group included 23 patients (mean age  $42.2 \pm 1.3$  years) who underwent 27 in-vitro fertilization (IVF) cycles in which preimplantation genetic diagnosis (PGD) was carried out on single blastomeres from day 3 embryos. The control group included 54 patients (mean age  $43.3 \pm 1.9$  years) who underwent 69 cycles of IVF without PGD. Ovarian stimulation in all patients consisted of follicular phase leuprolide acetate administration, followed by ovulation induction with gonadotrophins. The mean number of biopsied embryos was  $5.6 \pm 0.5$ . No embryo transfer occurred in six patients (10 cycles) because all embryos biopsied were abnormal. Seventeen patients (17 cycles) each had one genetically normal embryo transferred resulting in six on-going clinical pregnancies (35% per embryo transfer cycle). The mean number of embryos transferred in the control group was  $4.0 \pm 0.8$ . Nineteen clinical pregnancies were obtained in 69 transfer cycles in the control group (28% per embryo transfer cycle). The transfer of a single normal embryo in patients of advanced reproductive age can lead to acceptable pregnancy rates. Aneuploidy appears to be a major cause of reproductive failure in this group of patients.

**Keywords:** chromosomal abnormalities, embryo biopsy, pregnancy rate, preimplantation genetic diagnosis.

### Introduction

The poor reproductive outcomes in older patients undergoing IVF-embryo transfer with their own oocytes have been well documented (Van Blerkom *et al.*, 1995; Garside *et al.*, 1997; Janny and Menezo, 1996). Various contributory factors have been described including: diminished ovarian reserve leading to low numbers of retrievable oocytes, altered oocyte metabolism (Van Blerkom *et al.*, 1995), excessive deposition of zona glycoproteins (Garside *et al.*, 1997), diminished embryonic developmental capacity leading to cleavage arrest (Janny and Menezo, 1996), and aneuploidies in oocytes and cleavage stage embryos (Dailey *et al.*, 1996; Munné *et al.*, 1995; Márquez *et al.*, 2000). Due to these and other as yet undetermined factors, older women are at a higher risk of implantation failures and conception of genetically abnormal

fetuses, which are subsequently lost or aborted (Antonarakis, 1991; Fisher *et al.*, 1995). Recent studies focussing on women older than 40 years have reported no successful pregnancies above 43 years of age (**Table 1**) (Widra *et al.*, 1996; Laas *et al.*, 1998; Grimbizis *et al.*, 1998; Ron-El *et al.*, 2000).

High pregnancy rates have been reported in women of advanced reproductive age following the transfer of embryos obtained from oocytes from younger women (Navot *et al.*, 1994). This observation implied that endometrial receptivity was not compromised in the older patient and their sub-optimal reproductive performance was due largely to poor quality oocytes and embryos. Further studies have confirmed aneuploidy as the principal cause of the poor pregnancy prognosis observed in women of this age group (Dailey *et al.*, 1996; Munné *et al.*, 1995; Márquez *et al.*, 2000).

**Table 1.** Pregnancy and delivery rates in recent literature (from: Widra *et al.*, 1996; Lass *et al.*, 1998; Grimbizis *et al.*, 1998; Ron-El *et al.*, 2000).

Age of women (years)	41	42	43	44	45
Cycles ( <i>n</i> )	582	371	234	150	153
Pregnancies/cycle ( <i>n</i> )	49	55	60	9	0
Deliveries ( <i>n</i> ) <sup>a</sup>	15/25	17/36	32/50	0	0

<sup>a</sup>Not all studies reported deliveries. The values are from those studies reporting them.

An acceptable pregnancy rate in older patients undergoing IVF-embryo transfer with their own embryos is hypothetically possible if such embryos are diagnosed as genetically normal. Genetically normal embryos are currently clinically identified through preimplantation genetic diagnosis (PGD), after polar body or blastomere biopsy and analysis by fluorescent in-situ hybridization (FISH). The technique of PGD with single blastomere biopsy and FISH analysis was utilized in this study to detect genetically normal embryos in a group of patients with a mean maternal age of 42 years who underwent single-embryo transfers. Pregnancy results are presented for this group and a control group.

## Materials and methods

### Patients

A group of 23 patients of advanced reproductive age was retrospectively studied; they presented for IVF with embryo biopsy for aneuploidy analysis. Fifty-four patients in the same age group served as controls without undergoing embryo biopsy. The internal review board of the New York Fertility Institute approved the study. Written informed consent was obtained from all patients.

All patients were stimulated for ovulation using the short protocol of gonadotrophin-releasing hormone (GnRH) agonist (Lupron; TAP Pharmaceuticals, Deerfield, IL, USA) and the gonadotrophin, human menopausal gonadotrophin (HMG, Humegon.; Organon, West Orange, NJ, USA) or follicle-stimulating hormone (FSH, Metrodine; Serono, Randolph, MA, USA). The starting dose of Lupron was 0.2 ml administered daily by s.c. injection until the injection of human chorionic gonadotrophin (HCG, Profasi; Serono). Gonadotrophin administration was commenced usually at a starting dose of 225 IU per day. The dose of gonadotrophin was adjusted according to oestradiol concentration and ultrasound measurement of follicular development. HCG was administered when two to three or more follicles measuring 17 mm in size were observed. Oocytes were collected vaginally under ultrasound guidance 35 h later.

### Sperm preparation and insemination

In all cases semen samples were allowed to liquefy and motile spermatozoa were recovered by a modified swim-up procedure. Briefly, liquefied semen samples were manually assessed for concentration and motility. The semen was subsequently washed by gentle centrifugation at 300 *g*; the pellet resuspended in about 50–100  $\mu$ l of culture medium, and then

layered under 500  $\mu$ l of culture medium in a test tube. Motile spermatozoa were recovered from the culture medium layer after 15–20 min. Sperm concentration and motility of the recovered aliquot were determined and the concentration was adjusted if necessary before use for insemination at 4–6 h after egg collection.

The oocytes were inseminated with 100 000 motile spermatozoa/ml per oocyte and incubated in human tubal fluid (HTF) medium containing 0.5% human serum albumin (HSA: Irvine Scientific, CA, USA) under paraffin oil at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. In cases where intracytoplasmic sperm injection (ICSI) was performed, the methodology of cumulus–corona complex removal with hyaluronidase, preparation of oocytes for injection and the actual injection technique were as described previously (Van Steirteghem *et al.*, 1993). Inseminated or injected oocytes were assessed for fertilization 16–18 h later. Normally fertilized zygotes (two pronuclear (2PN); two polar bodies) were noted, transferred to fresh culture medium under oil and cultured for an additional 48 h. Abnormally fertilized zygotes (>2PN) were noted and discarded forthwith.

### Embryo biopsy

Embryo biopsy was performed on day 3 of in-vitro culture using acidic Tyrode's solution for zona digestion followed by gentle aspiration of a nucleated blastomere (Grifo, 1992). The biopsied cells were analysed on the same day with probes for chromosomes X, Y, 13, 15, 16, 18, 21, and 22 (Munné and Weir, 1996). The efficiency rate for the detection of numerical abnormalities in the intended chromosomes was about 85% (the error rate was ~10%). The biopsied embryos were cultured singly in individual numbered drops under oil. Embryos identified as genetically normal were transferred to the patient on the same day of the analysis. Embryo transfers were carried out on day 3 in the control group. Luteal phase support was provided in the form of progesterone in oil (50 mg i.m. daily) and commenced in the evening following oocyte retrieval and continued until either a negative pregnancy test or a sonographically confirmed embryonic viability was noted. A positive pregnancy was defined as the detection by ultrasound of a gestational sac with an embryo and cardiac activity.

### Statistical analysis

Statistical analysis was limited to calculations of mean values and standard deviations and was carried out using the Systat statistical package (Systat for Windows, version 5, IL, USA).

## Results

All the women studied were of advanced reproductive age with varying degrees of ovarian dysfunction and presented for IVF with embryo biopsy for detection of aneuploidy. Sixteen patients were in the 40–43 years age group and seven patients in the 43–46 years group. The overall results are presented in **Table 2**.

The majority of the biopsied blastomeres from embryos of all 23 patients showed abnormal genotype when analysed with probes for chromosomes X, Y, 13, 15, 16, 18, 21, and 22. The abnormalities detected included monosomies (18%), trisomies (34%), haploidy (29%), and complex numerical abnormalities

**Table 2.** Pregnancy outcome in women over 40 years.

Age group (years)	40–43	40–43	43–46	43–46
Treatment	Control	PGD	Control	PGD
No. of patients	33	16	21	7
No. of cycles	41	19	28	8
Mean age (years) <sup>a</sup>	41.2 ± 0.7	42.4 ± 1.2	44.6 ± 1.1	45.3 ± 0.9
Mean no. of oocytes retrieved <sup>a</sup>	7.4 ± 1.0	7.9 ± 0.4	5.9 ± 1.1	6.2 ± 1.3
Overall fertilization rate (%)	69	76	71	66
ICSI (%)	70	78	77	70
Routine IVF (%)	67	74	65	62
Mean no. of biopsied embryos <sup>a</sup>	0	5.6 ± 0.5	0	4.2 ± 1.0
No. of cycles with embryo transfer	41	12	28	5
Mean no. of embryos transferred <sup>a</sup>	4.2 ± 1.1	1 ± 0	3.8 ± 0.6	1 ± 0
No. clinically pregnant/transfer cycle <sup>b</sup>	13 (32.0)	4 (33.0)	6 (21.0)	2 (40.0)
No. implantations <sup>b</sup>	16 (8.7)	4 (33.0)	7 (6.3)	2 (40.0)
No. miscarriages <sup>b</sup>	3 (23.0)	1 (25.0)	5 (83.3)	0 (0.0)

<sup>a</sup>Values are means ± SD.

<sup>b</sup>Values in parentheses are percentages.

(18%). All four abnormal genotypes occurred randomly in embryos of each patient. Overall, abnormalities affected all chromosomes analysed. There was no consistent pattern in the aneuploidy detected in the chromosomes analysed from patient to patient. Some embryos had no results for particular chromosomes but were classified as abnormal due to abnormalities in the other chromosomes analysed. All normal embryos transferred had results for all the chromosomes analysed.

Six patients who underwent 10 cycles of IVF–embryo transfer had no embryo transfers because all the biopsied embryos were abnormal. Of the remaining 17 patients, each had one genetically normal embryo. Six clinical pregnancies were obtained from the transfer of these embryos (22% per cycle; 35% per embryo transfer cycle). Four pregnancies occurred in women 40–43 years of age, with one ending in a miscarriage (normal karyotype) and the others carried successfully to term. The two pregnancies in women 43–46 years of age were carried successfully to term. There were no remarkable differences in the parameters studied between the pregnant and non-pregnant patients. The mean ages, numbers of oocytes retrieved, fertilization rates either by ICSI or conventional IVF, and numbers of biopsied embryos were similar between the pregnant and non-pregnant groups.

In the control group, 19 pregnancies were obtained from a total of 69 transfer cycles (28% per embryo transfer cycle). The distribution of the pregnancies and miscarriages between the two age groups is depicted in Table 2. All parameters evaluated (mean age, number of retrieved oocytes, fertilization rates) were similar within the control groups and between the control and study groups. More embryos were transferred in the controls compared with the single embryo transfer in the study group. This resulted in four twin pregnancies (21%) in the control group compared with none in the PGD group.

## Discussion

PGD of aneuploidy using probes for eight chromosome pairs was performed in 27 cycles in women of advanced maternal age. A total of 17 women had single normal embryos transferred, of which six became clinically pregnant. The pregnancy rate was comparable to that obtained in control women of similar ages who underwent IVF–embryo transfer without embryo biopsy during the same period. The average number of embryos available for PGD selection (4–6 embryos) was the same as the average number of embryos transferred in the control group. The lack of improvement in pregnancy rate in the PGD group can be attributed to the low number of embryos available. In younger patients (35–40 years) usually with more embryos, PGD has been shown to improve pregnancy rates through the selection and transfer of normal embryos from a larger pool (Munné *et al.*, 1999; Gianaroli *et al.*, 1999). However, the present group of patients may have benefited from a lower chance of trisomic offspring, fewer spontaneous abortions (17% in the PGD group compared with 42% in the control group), and the avoidance of dizygotic twin and multiple pregnancies, which at this age are even more dangerous than in younger women. Furthermore, in those patients without normal embryos to transfer, PGD provides a better understanding of their reproductive failure. Interestingly the pregnancy rate observed in younger patients to whom selected single embryos were transferred (Vilksa *et al.*, 1999; Gerris *et al.*, 1999) was similar to the rate observed in the study group suggesting that PGD might offer even better pregnancy rates among all patients.

A high percentage of the embryos analysed were aneuploid. The different chromosome abnormalities observed included monosomies, trisomies, haploidy and complex numerical abnormalities. The aetiology of the embryonic aneuploidy is probably associated with the high rate of aneuploidy observed in oocytes from women of advanced reproductive age (Dailey

et al., 1996; Márquez et al., 2000). The contribution of sperm-derived factors to the observed aneuploidy in the embryos is unknown but probably limited. Recent studies have shown that paternal factors involving possibly the sperm centriole can adversely affect the quality of embryos resulting from IVF (Obasaju et al., 1999). The pattern of abnormalities observed in such cases was attributed to chaotic mosaicism due to disorganized centriolar function rather than aneuploidy. In this group of patients the predominant chromosomal abnormalities were numerical, lending further support to the oocyte as the major source of the aneuploidy.

The observations reported here raise important issues that warrant more advanced and detailed study. Firstly, improvement of pregnancy rates through PGD in this patient age group is still unproven, because a larger pool of embryos, above the 4–5 embryos usually transferred in these patients, is required to identify a potential pregnancy improvement through the selection of normal embryos. Secondly, transfer of embryos at the blastocyst stage may not be a viable alternative to PGD in this group of patients. Assuming blastocysts can be obtained from the limited number of embryos obtained in this group of patients, they may still be chromosomally abnormal, as both normal and abnormal embryos have been shown to develop to the blastocyst stage in vitro (Kadam et al., 1998; Sandalinas et al., 2001). Lastly, pregnancies in patients 43 years and older were carried to term in 38% of cases as opposed to previous reports regarding no live birth after 43 years of age (Table 1) (Widra et al., 1996; Lass et al., 1998; Grimbizis et al., 1998; Ron-El et al., 2000). This failure could be caused in part by embryonic aneuploidy which, while permissive to implantation was detrimental to continued development. The present results seem to indicate that failure to conceive in women of advanced maternal age is a result of diminished ovarian reserve and increased ovulation of aneuploid oocytes.

In conclusion, the present study demonstrates that acceptable pregnancy rates can be obtained from the transfer of single chromosomally normal embryos in women of advanced reproductive age despite the high rate of aneuploidy detected.

## Acknowledgements

The authors thank Mrs. Bea Mussolini for technical help in the preparation of the manuscript and the staff of our IVF programme for their enthusiastic support of this project.

## References

- Antonorakis SE 1991 Parental origin of the extra chromosome in trisomy 21: Downs syndrome collaborative group. *New England Journal of Medicine* **324**, 872–876.
- Dailey T, Dale B, Cohen J, Munné S 1996 Association between non-disjunction and maternal age in meiosis-II human oocytes detected by FISH analysis. *American Journal of Human Genetics* **59**:176–184.
- Fisher JM, Harvey JF, Morton NE et al. 1995 Trisomy 18: Studies of the parent and cell division of origin and the effect of aberrant recombination on non-disjunction. *American Journal of Human Genetics* **56**, 669–675.
- Garside WT, Loret de Mola JR, Bucci JA et al. 1997 Sequential analysis of zona thickness during in vitro culture of human zygotes: correlation with embryo quality, age, and implantation. *Molecular Reproduction and Development* **47**, 99–104.
- Gerris J, De Neubourg D, Mangelschots K et al. 1999 Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Human Reproduction* **14**, 2581–2587
- Gianaroli L, Magli C, Ferraretti AP et al. 1999 Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with a poor prognosis: identification of the categories for which it should be proposed. *Fertility and Sterility* **72**, 837–844
- Grifo JA 1992 Preconception and preimplantation genetic diagnosis: polar body blastomere, and trophectoderm biopsy. In: Cohen J, Malter HE, Talansky BE, Grifo J (eds) *Micromanipulation of gametes and embryos*. Raven Press, New York, USA, pp.223–249.
- Grimbizis G, Vandervorst M, Camus 1998 Intracytoplasmic sperm injection, results in women older than 39, according to age and the number of embryos replaced in selective or non-selective transfers. *Human Reproduction* **13**, 884–889.
- Janny L, Menezo YJR 1996 Maternal age effect on early human embryonic development and blastocyst formation. *Molecular Reproduction and Development* **45**, 31–37.
- Kadam A, Munné S, Obasaju M et al. 1998 Does human embryo chromosomal status influence blastocyst formation. In: Program and abstracts of the 54th Annual Meeting of the American Society for Reproductive Medicine. San Francisco, CA, USA, p.S75 (abstract no. O-202).
- Lass A, Croucher C, Duffy S et al. 1998 One thousand cycles of in vitro fertilization in women 40 years of age. *Fertility and Sterility* **70**, 1030–1034.
- Márquez C, Sandalinas M, Baççe M et al. 2000 Chromosome abnormalities in 1255 cleavage-stage human embryos. *Reproductive BioMedicine Online* **1**, 17–27.
- Munné S, Alikani M, Tomlin G et al. 1995 Embryo morphology, developmental rates and maternal age are correlated with chromosomal abnormalities. *Fertility and Sterility* **64**, 382–391.
- Munné S, Weier HUG 1996 Simultaneous enumeration of chromosomes 13, 18, 21, X and Y in interphase cells for preimplantation genetic diagnosis. *Cytogenetics and Cell Genetics* **75**, 263–270.
- Munné S, Magli C, Cohen J et al. 1999 Positive outcome after preimplantation diagnosis of aneuploidy in human embryos. *Human Reproduction* **14**, 2191–2199.
- Navot D, Drews MR, Bergh PA et al. 1994 Age related decline in female fertility is not due to diminished capacity of the uterus to sustain embryo implantation. *Fertility and Sterility* **61**, 97–101.
- Obasaju M, Kadam A, Sultan K et al. 1999 Sperm quality may adversely affect the chromosome constitution of embryos that result from intracytoplasmic sperm injection. *Fertility and Sterility* **72**, 1113–1115.
- Ron-El R, Eaziel A, Strassburger D et al. 2000 Outcome of assisted reproductive technology in women over the age of 41. *Fertility and Sterility* **74**, 471–475.
- Sandalinas M, Sadowy S, Alikani M et al. 2001 Developmental ability of chromosomally abnormal human embryos to reach blastocyst stage. *Human Reproduction* in press.
- Van Blerkom J, Davis PW, Lee J 1995 ATP content of human oocytes and developmental potential and outcome after in vitro fertilization and embryo transfer. *Human Reproduction* **10**, 415–424.
- Van Steirteghem AC, Liu J, Joris H et al. 1993 Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report on a second series of 300 consecutive treatment cycles. *Human Reproduction* **8**, 1055–1060.
- Vilksa S, Tiitinen A, Hyden-Granskog et al. 1999 Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. *Human Reproduction* **14**, 2392–2395.
- Widra AE, Gindoff RP, Smortich BD et al. 1996 Achieving multiple-order embryo transfer identifies women 40 years of age with improved in vitro fertilization outcome. *Fertility and Sterility* **65**, 1