J Assist Reprod Genet (2011) 28:597–602
DOI 10.1007/s10815-011-9556-2

GENETICS

Reliable preimplantation genetic diagnosis in thawed human embryos vitrified at cleavage stages without biopsy

Li Zhang · Ahmet Yilmaz · Ri-Cheng Chian · Weon-Young Son · Xiao Yun Zhang · Dewen Kong · Michael Dahan · Hannanel Holzer · Seang Lin Tan · Asangla Ao

Received: 17 November 2010 / Accepted: 7 March 2011 / Published online: 25 March 2011

Abstract
Purpose To evaluate preimplantation genetic diagnosis (PGD) efficiency in thawed human embryos vitrified without biopsy.
Methods In this retrospective clinical study, 21 PGD cycles were carried out using fresh and vitrified-thawed embryos collected from 21 patients.
Results One hundred and ninety-nine embryos from patients with aneuploidy, single gene defects, or chromosomal translocations were vitrified at the cleavage stages; 93.5% of the embryos survived the vitrification procedure. Conclusive FISH results were obtained in 98.4% of the fresh and 99% of the frozen-thawed embryos screened for aneuploidy or chromosomal translocations. Conclusive PCR test results were obtained in 100% of the fresh and 93.9% of the frozen-thawed embryos screened for single gene defects. The overall clinical pregnancy rate per cycle was 57.1%. To date, 13 healthy children have been born.
Conclusion Reliable genetic diagnosis can be performed in thawed human embryos vitrified without biopsy. However, further research is required to support this conclusion.

Keywords Human · In vitro fertilization (IVF) · Preimplantation genetic diagnosis (PGD) · Vitrification

Capsule Reliable preimplantation genetic diagnosis can be performed in vitrified-thawed human embryos.

L. Zhang · A. Yilmaz · R.-C. Chian · W.-Y. Son · X. Y. Zhang · D. Kong · M. Dahan · H. Holzer · S. L. Tan · A. Ao
MUHC Reproductive Center, Royal Victoria Hospital,
and Department of Obstetrics and Gynecology, McGill University,
Montreal, Quebec, Canada

A. Ao
Department of Human Genetics, McGill University,
Montreal, Quebec, Canada

A. Ao (✉)
Royal Victoria Hospital,
687 Pine Avenue West F3-16,
Montreal, Quebec, Canada H3A 1A1
e-mail: asangla.ao@muhc.mcgill.ca

Introduction

Preimplantation genetic diagnosis (PGD) is now being routinely used in many centers to prevent the birth of an affected child from couples with incurable genetic diseases resulting from single gene defects, chromosomal translocations, or aneuploidy [1]. These couples are required to undergo in vitro fertilization (IVF) treatment. In routine IVF-PGD procedures, embryos are biopsied on day 3 post-insemination, genetic diagnosis is performed, and, if unaffected, biopsied embryos are transferred back into the patient on day 4 or 5. However, in some circumstances, freezing the embryos and analyzing them later to complete the PGD procedure cannot be avoided if the patient develops ovarian hyperstimulation syndrome (OHSS) or the PGD tests fail due to technical difficulties. In addition, some patients do not respond adequately to ovarian stimulation and produce only a few embryos in each cycle, requiring serial vitrification to obtain sufficient number of embryos for genetic analysis. Embryo freezing has been routinely used in IVF laboratories for decades [2] and excellent post-thaw survival rates have been achieved [3–5]. Safe use of embryos stored frozen for as long as 9 years to initiate a healthy pregnancy has been reported in the literature [6].

Embryo freezing in a PGD cycle has been generally carried out after biopsy on day 3 post-insemination [7–9] or at the blastocyst stage [10–12]. However, several reports

suggest that post-thaw survival rates of embryos biopsied on day 3 are significantly lower than those not subjected to embryo biopsy before freezing [7, 9, 13–15]. The exact cause of this phenomenon has not been thoroughly investigated but increased susceptibility to cryodamage due to the hole in the zona pellucida created for biopsy may be a plausible explanation [10].

Biopsying after thawing frozen embryos would, therefore, not only increase post-thaw survival rates but may also provide the PGD operator with more flexibility. To the best of our knowledge, only three small studies evaluating PGD on thawed embryos frozen at cleavage stages have been published [15, 17, 18]. Successful pregnancies have been reported in one of those studies [17]. However, there is still a clear need for investigating the efficacy of PGD in frozen-thawed human embryos in clinical settings. The objective of this study was, therefore, to evaluate the efficiency and reliability of PGD in thawed embryos frozen at cleavage stages without biopsy.

Materials and methods

Patients

This retrospective study was carried out at a university-based reproductive center. Twenty-one couples underwent IVF and vitrification cycles from April 2004 to October 2009. The reasons that necessitated vitrification of embryos included development of OHSS in some patients, technical problems encountered during the PGD tests, and poor ovarian response that required repetitive stimulation and serial vitrification to obtain sufficient number of embryos for PGD. In addition, surplus embryos from patients with recurrent miscarriages, advanced maternal age, and recurrent implantation failure were vitrified after embryo transfer following assisted reproductive technology (ART) without preimplantation genetic screening (PGS). The patients had only frozen or both fresh and frozen embryos available for PGD. Only fresh, only frozen, or both fresh and frozen embryos were transferred based on diagnostic results and embryo quality.

Embryo production and culture conditions

Women were treated with GnRH agonist/antagonists and FSH/hMG in a long- or short-treatment protocol. A single dose of 10,000 IU hCG was administered when two or more follicles reached a diameter above 18 mm. Oocytes were retrieved transvaginally 35 h after hCG injection and were inseminated by either conventional IVF or intracytoplasmic sperm injection (ICSI). Fertilization was assessed 17–19 h after insemination by examining the presence and number of pronuclei. Embryos with two pronuclei were cultured in cleavage medium (Cook Canada Inc., Stouffville, ON, Canada) in an atmosphere containing 6% CO_2, 5% O_2, and 89% N_2. The fresh embryos were biopsied on day 3 and transferred on day 4 or 5. The remaining biopsied spare embryos with good quality were vitrified. However, data from these vitrified spare embryos were not included in this study because they were subjected to biopsy before freezing, and thus, were not consistent with our objective.

Vitrification and warming procedures

The embryos were resuspended in equilibration medium containing 7.5% (v/v) ethylene glycol and 7.5% (v/v) 1,2-propanediol for 5 min at room temperature. The embryos were transferred to vitrification medium containing 15% (v/v) ethylene glycol, 15% (v/v) 1,2-propanediol, and 0.5 M sucrose and incubated at room temperature for 45–60 s. The embryos were loaded onto a specially designed vitrification device, the McGill Cryoleaf, and were plunged immediately into liquid nitrogen for storage.

Embryos were thawed by directly inserting the McGill Cryoleaf into thawing medium containing 1.0 M sucrose in HEPES-buffered human tubal fluid (HTF) for 1 min at 37°C. Warmed embryos were transferred to diluent medium-I (0.5 M sucrose in HEPES-buffered HTF) and diluent medium-II (0.25 M sucrose), respectively, for 3 min each. The embryos were washed twice in washing medium (HEPES-buffered HTF containing 10% human serum albumin). Embryos were considered to have survived if more than 50% of the blastomeres were intact after thawing and culturing for additional 2 h.

Blastomere biopsy, FISH, and PCR protocol

All embryos used in this study were biopsied on day 3. Embryos frozen on day 2 were cultured for an additional day after thawing before the blastomere biopsy was performed as described previously [19, 20]. Embryos frozen on day 3 were thawed in the morning and biopsied on the same day after culturing for a few hours for equilibration. Briefly, each embryo was put in a droplet of Ca^{2+} and Mg^{2+}-free medium (Cook Canada Inc.), and a hole (15–20 μm in diameter) was drilled in the zona pellucida using 1.48 μm infrared diode laser in computer controlled non-contact mode (Hamilton Thorn Inc., MA, USA). One blastomere from each embryo was aspirated gently. After removal, each blastomere was washed three times with PBSA (phosphate-buffered saline with 4 mg/ml bovine serum albumin, Sigma Inc., Oakville, ON, Canada) before being used in FISH or PCR. Each biopsied embryo was washed three times with embryo culture medium and

placed in a clean labeled medium drop under oil. Embryos were incubated in separate dishes.

For PCR-based genetic analysis, washed blastomeres were transferred into a 0.2 ml PCR thin wall tube containing 5 μL alkaline lysis buffer (200 mM potassium hydroxide and 50 mM DTT). A sample of PBSA from the last wash drop was taken as a blank to test for contamination. Unique nested multiplex PCR protocols involving fluorescently labeled primers specific for mutated alleles and STR markers were developed specifically for each disease. The PCR products were analyzed on ALFexpress Automated DNA Sequencer (Amersham Pharmacia Biotech) or ABI 3130 Genetic Sequencer (Applied Biosystems Inc.). The GeneMapper software was used to analyze the data (Applied Biosystems Inc.).

For FISH-based genetic analysis, washed blastomeres were placed on poly-L-lysine coated glass slides and fixed with spreading buffer (0.01N HCl/Tween 20) under an inverted microscope. The slides were air-dried, washed in PBS, and dehydrated in serial dilutions of ethanol for 2 min each. Commercially available multicolor FISH probes for chromosomes 13, 15, 16, 18, 21, 22, X, and Y (Vysis, Downers Grove, IL, USA) were applied on nuclei of biopsied blastomeres in two consecutive rounds. Co-denaturation, hybridization, washing, and nuclear staining steps were carried out in each round. The signals were detected using a fluorescence microscope (Olympus CK60, Olympus Canada Inc.) with appropriate sets of filters [21].

The embryos diagnosed as unaffected were transferred into the patients 4 or 5 days after fertilization. This project was approved by the Research Ethics Board of Royal Victoria Hospital at the McGill University Health Center.

Statistical analysis

Chi-square test of independence was used to compare ratios using SPSS (SPSS Inc., Chicago, IL) with the level of significance set at $P=0.05$.

Results

Data were collected in 21 PGD cycles involving 21 couples with the average age of 37.2±4.1 (range 28–44 years; Table 1). Only fresh, only vitrified, and both fresh and vitrified embryos were transferred in 4, 9, and 8 cycles, respectively. The PGD was performed to screen for aneuploidy ($n=14$), single gene defects ($n=5$), or chromosomal translocations ($n=2$). The clinical pregnancy rate per cycle in fresh, frozen, and fresh and frozen embryos combined were 75%, 56%, and 50%, respectively. The implantation rate per cycle in the fresh, frozen, and both fresh and frozen groups were 42.9%, 31.6%, and 28.6%, respectively ($P>0.05$). Thirteen healthy babies are born in 10 deliveries.

Details of embryological data collected using fresh and vitrified embryos are shown in Table 2. Five hundred and fifty cumulus-oocyte complexes were retrieved; 455 of which reached metaphase II stage. Thirty three embryos were transferred without PGD in 9 cycles but pregnancy was not achieved. The patients then decided to undergo PGD for aneuploidy screening. Their frozen embryos from previous IVF cycles as well as fresh embryos were used in the same PGD cycle. In total, 25 fresh and 29 vitrified embryos were transferred after PGD in 35 IVF or IVM cycles.

Table 1 Summary of the clinical data based on type of embryos transferred and PGD test performed [a]

Variable	Embryos transferred			Type of PGD test performed			Total
	Only fresh	Only vitrified	Both fresh and vitrified	Aneuploidy screening	Single gene defects	Chromosomal translocation	
Average age (mean±SD)	38.5±3.7	36.0±4.4	38.3±6.1	38.8±3.5	34.2±4.0	33.5±2.1	37.2±4.1
PGD cycles	4	9	8	14	5	2	21
Embryos transferred	7	19	28	39	12	3	54
Average number of embryos transferred	1.75	2.11	3.5	2.8	2.4	1.5	2.6
Clinical pregnancy cycles	3	5	4	9	1	2	12
Clinical pregnancy rate per cycle (%)	75%	56%	50%	64.3%	20.0%	100%	57.1%
Implantation rate (%)	42.9% [b]	31.6% [b]	28.6% [b]	35.9%	8.3%	66.7%	31.5%
Babies born	2	5	6	11	0	2	13
Singletons	2	3	2	5	0	2	7
Twins	0	1	2	3	0	0	3

[a] One hundred eighty-six of the 199 embryos (93.5%) survived vitrification. Total number of fetal heart beat (FHB) and sacs in all patients were 15 and 17, respectively

[b] There was no significant difference in implantation rates when only fresh, only vitrified, and a combination of fresh and vitrified embryos were transferred into the patient ($P>0.05$)

Table 2 Details of embryological data collected using fresh and vitrified embryos[a]

Variables	Fresh	Vitrified	Total
Patients	13	21	21
IVF or IVM Cycles	13	22	35
Oocytes collected	177	373	550
Oocytes reaching M-II stage	136	319	455
Zygotes (% of oocytes reaching M-II stage)	96 (70.6%)	245 (76.8%)	341 (74.9%)
Embryos cleaved (% of zygotes)	96 (100.0%)	243 (99.2%)	339 (99.4%)
Embryos transferred without PGD	33	/	/
Embryos transferred after PGD	25	29	54

[a] *IVF* in vitro fertilization, *IVM* in vitro maturation

The impact of freezing embryos on day 2 or 3 post-fertilization is shown in Table 3. Survival rates of embryos vitrified on day 2 and 3 were 94.4% and 91.9%, respectively ($P>0.05$). Conclusive PGD test results were obtained in 97.5% of the embryos vitrified on day 2 and 98.2% of the embryos vitrified on day 3 ($P>0.05$).

The impact of vitrification on PGD outcome is shown in Table 4. In aneuploidy screening, conclusive FISH results were obtained in 98.4% of the fresh and 98.7% of the frozen embryos ($P>0.05$); 34.4% of the fresh and 34.2% of the frozen embryos were euploid. There were no fresh embryos transferred among translocation patients. However, successful test results were obtained in all embryos, 20.0% of which were free of genetic abnormalities for the specific chromosomes tested. In screening for single gene defects, conclusive PCR test results were obtained in 100% of the fresh and 93.9% of the frozen embryos. When results from all 21 PGD cycles were combined, conclusive PGD test results were obtained in 98.6% of the fresh and 97.8% of the frozen embryos ($P>0.05$).

Table 3 Details of embryological data collected using embryos vitrified on day 2 or 3 post-fertilization

Variable	Day 2	Day 3
Patients	11	10
Average age ± SD	35.6±4.2	39.0±3.3
PGD cycles	11	10
Embryos thawed	125	74
Embryos survived (survival rate)	118 (94.4%) [a]	68 (91.9%) [a]
Embryos biopsied	83	61
Embryos with nucleated blastomeres	80	55
Embryos with conclusive PGD test results (% of nucleated blastomeres)	78 (97.5%) [a]	54 (98.2%) [a]

[a] There was no significant difference between the day 2 and 3 groups of embryos ($P>0.05$)

Discussion

Preimplantation genetic diagnosis (PGD) has been used for over 20 years [22] to select healthy embryos to initiate a pregnancy especially in cases of parents with inherited genetic diseases. Recently, PGD has also been offered to patients with advanced maternal age, recurrent pregnancy failure, or recurrent miscarriages to screen chromosome abnormalities to improve the ART outcome. Embryos are obtained by IVF, one or more cells are removed from them at different developmental stages, and genetic analysis is performed for either specific mutations or chromosomal abnormalities. Only unaffected embryos are transferred back into the patient to initiate a healthy pregnancy. The most common method used for PGD has been the biopsy of cleavage stage embryos [23] when all cells are still totipotent.

Freezing and storing embryos for future use in a PGD cycle cannot be avoided if the patient develops OHSS or technical problems occur during execution of the test. Also in some poor responders, the number of embryos obtained following ovarian stimulation cycle may not be sufficient for PGD and repetitive stimulation and serial embryo vitrification may be a better and more cost effective alternative to canceling the whole PGD cycle.

Embryo freezing in PGD procedure has been generally performed after embryo biopsy at the cleavage stages to remove cell(s) for genetic analysis. However, survival rates of these biopsied embryos are significantly lower than those frozen without biopsy [7, 9, 13, 14]. Therefore, freezing intact embryos and performing PGD after thawing all of the embryos simultaneously would not only provide the PGD operator with more flexibility but may also increase post-thaw survival rates. On the other hand, a setback in biopsying thawed embryos is the hardening of zona pellucida due to freezing and thawing [24] probably because of irreversible denaturation of some unidentified proteins located in the zona. However, assisted hatching [25] or culturing embryos in protein-free medium [26] may improve implantation rates lowered due to zona hardening. The small hole that must be created in the zona for embryo biopsy after thawing may also help the embryo to hatch. Therefore, zona hardening should not present a major obstacle to performing PGD in frozen-thawed embryos.

To the best of our knowledge, only a single birth following PGD in thawed human embryos frozen at cleavage stages has been reported in the literature [17] although successful PGD in thawed embryos has been reported previously. Ciotti et al. [15] reported that biopsy and FISH in thawed embryos was

Table 4 Comparison of preimplantation genetic diagnosis (PGD) efficiency in fresh and vitrified-thawed embryos

Variable	Aneuploidy screening		Translocation screening		Single gene defects screening		All methods combined		Total
	Fresh	Vitrified	Fresh	Vitrified	Fresh	Vitrified	Fresh	Vitrified	
Embryos vitrified	/	114		38	/	47	/	199	199
Embryos survived vitrification (survival rate)	//	105 (92.1%)		38 (100%)	//	43 (91.5%)	//	186 (93.5%)	186 (93.5%)
Embryos biopsied	69	85		26	10	33	79	144	223
Embryos with nucleated blastomeres (% of biopsied embryos)	62 (89.9%)	77 (90.6%)		25 (96.2%)	10 (100%)	33 (100%)	72 (91.1%)[a]	135 (93.8%)[a]	207 (92.8%)
Embryos with conclusive PGD test results (% of nucleated blastomeres)	61 (98.4%)	76 (98.7%)		25 (100%)	10 (100%)	31 (93.9%)	71 (98.6%)[a]	132 (97.8%)[a]	203 (98.1%)
Embryos with failed test results (% of embryos with nucleated blastomeres)	0/	1 (1.30%)		0/	0/	1 (3.0%)	0/	2 (1.5%)	2 (1.0%)
Embryos with inconclusive PGD test results (% of embryos with nucleated blastomeres)	1 (1.6%)	0/		0/	0/	1 (3.0%)	1 (1.4%)	1 (0.7%)	2 (1%)
Embryos free of genetic abnormality (% of embryos with conclusive test results)	21 (34.4%)	26 (34.2%)		5 (20.0%)	9 (90.0%)	18 (58.1%)	30 (42.3%)	49 (37.1%)	79 (38.9%)
Embryos carrying genetic abnormality (% of embryos with conclusive test results)	40 (65.6%)	50 (65.8%)		20 (80.0%)	1 (10.0%)	13 (41.9%)	41 (57.7%)	83 (62.9%)	124 (61.1%)

[a] No significant statistical difference between fresh and vitrified embryos ($P>0.05$)

possible but technical improvements were needed. Baart et al. [18] were able to perform FISH on blastomeres removed from frozen-thawed embryos although no pregnancy was reported in that study. Two other studies reported successful PGD in trophectoderm cells removed from thawed blastocysts. In the first study, Lathi and Behr [11] reported a successful pregnancy outcome using this approach. In the second and a larger study reported by Feyereisen et al. [16], most of the blastocysts were degenerated after thawing and embryo biopsy was successful in only 7 of the 25 thawed blastocysts analyzed. Following embryo transfer, two biochemical and one clinical pregnancy were established.

In this study, we analyzed data collected from 21 patients in 21 PGD cycles and 35 IVF or IVM cycles (Tables 1 and 2). The clinical pregnancy and implantation rate per cycle were highest in the fresh embryos. However, the sample size in this study was too small to reach a conclusion on clinical outcome. Freezing the embryos on day 2 or 3 post-fertilization did not have any significant effect on embryo survival and PGD test success rates (Table 3). The survival rate was similar in embryos frozen on day 2 (94.4%) and 3 (91.9%). Percentage of embryos with conclusive PGD test results was also similar in embryos vitrified on day 2 (97.5%) and 3 (98.2%).

The proportion of aneuploid embryos was similar between embryos transferred fresh (65.6%) and frozen (65.8%), suggesting that vitrification did not have any significant adverse effects on the incidence of aneuploidy (Table 4). In screening for single gene defects, conclusive PGD test results were obtained in 100% of the fresh and 93.9% of the frozen embryos but the percentage of unaffected embryos was higher in the fresh (90%) than in the vitrified (58.1%) group. This result may have been due to random chance, the disease being recessive or dominant, or smaller sample size available in the fresh than in the vitrified group (10 vs. 33).

When results from all three PGD groups were combined, percentage of embryos with conclusive PGD test results was similar in fresh and frozen embryos. In total, 98.1% of the embryos were successfully tested for PGD.

Our results collectively suggest that reliable PGD testing may be performed after thawing embryos vitrified without biopsy; 93.5% of the embryos survived vitrification, a survival rate similar to those reported by others [27]. Proportion of embryos with nucleated blastomeres, percentage of embryos with conclusive PGD test results, and aneuploidy rates in fresh and frozen-thawed embryos were similar. Freezing the embryos on day 2 or 3 had little or no effect on PGD efficiency. Currently, 13 healthy babies have been born following this approach. To the best of our knowledge, this is the largest number of babies born

following PGD in thawed embryos vitrified without biopsy at cleavage stages. We conclude that reliable PGD may be performed in thawed cleavage stage embryos. The strategy used for PGD in this study is practical and effective, and should benefit PGD patients in a variety of situations.

References

1. Obradors A, Fernandez E, Oliver-Bonet M, Rius M, de la Fuente A, Wells D, et al. Birth of a healthy boy after a double factor PGD in a couple carrying a genetic disease and at risk for aneuploidy: case report. Hum Reprod. 2008;23:1949–56.
2. Edwards RG, Handyside AH. Future developments in IVF. Br Med Bull. 1990;46:823–41.
3. Rezazadeh Valojerdi M, Eftekhari-Yazdi P, Karimian L, Hassani F, Movaghar B. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. J Assist Reprod Genet. 2009;26:347–54.
4. Son WY, Chung JT, Gidoni Y, Holzer H, Levin D, Chian RC, et al. Comparison of survival rate of cleavage stage embryos produced from in vitro maturation cycles after slow freezing and after vitrification. Fertil Steril. 2009;92:956–8.
5. Cao YX, Xing Q, Li L, Cong L, Zhang ZG, Wei ZL, et al. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. Fertil Steril. 2009;92:1306–11.
6. Check ML, Check JH, Summers-Chase D. A successful pregnancy from zygotes cryopreserved for >9 years: case report. Clin Exp Obstet Gynecol. 2001;28:91.
7. Jericho H, Wilton L, Gook DA, Edgar DH. A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod. 2003;18:568–71.
8. Kahraman S, Candan ZN. Outcomes of vitrified-warmed day-4 embryos after day-3 cleavage-stage biopsy. Reprod Biomed Online. 2010;21:636–41.
9. Magli MC, Gianaroli L, Fortini D, Ferraretti AP, Munne S. Impact of blastomere biopsy and cryopreservation techniques on human embryo viability. Hum Reprod. 1999;14:770–3.
10. Escriba MJ, Zulategui JF, Galan A, Mercader A, Remohi J, de los Santos MJ. Vitrification of preimplantation genetically diagnosed human blastocysts and its contribution to the cumulative ongoing pregnancy rate per cycle by using a closed device. Fertil Steril. 2008;89:840–6.
11. Lathi RB, Behr B. Pregnancy after trophectoderm biopsy of frozen-thawed blastocyst. Fertil Steril. 2009;91:1938–40.
12. McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. Fertil Steril. 2005;84:1628–36.
13. Joris H, Van den Abbeel E, Vos AD, Van Steirteghem A. Reduced survival after human embryo biopsy and subsequent cryopreservation. Hum Reprod. 1999;14:2833–7.
14. Zheng WT, Zhuang GL, Zhou CQ, Fang C, Ou JP, Li T, et al. Comparison of the survival of human biopsied embryos after cryopreservation with four different methods using non-transferable embryos. Hum Reprod. 2005;20:1615–8.
15. Ciotti PM, Lagalla C, Ricco AS, Fabbri R, Forabosco A, Porcu E. Micromanipulation of cryopreserved embryos and cryopreservation of micromanipulated embryos in PGD. Mol Cell Endocrinol. 2000;169:63–7.
16. Feyereisen E, Steffann J, Romana S, Lelorc'h M, Ray P, Kerbrat V, et al. Five years' experience of preimplantation genetic diagnosis in the Parisian Center: outcome of the first 441 started cycles. Fertil Steril. 2007;87:60–73.
17. Frydman N, Ray P, Romana S, Fanchin R, Lelorc'h M, Kerbrat V, et al. First birth after preimplantation genetic diagnosis performed on thawed embryos. J Gynecol Obstet Biol Reprod (Paris). 2003;32:363–7.
18. Baart EB, Van Opstal D, Los FJ, Fauser BC, Martini E. Fluorescence in situ hybridization analysis of two blastomeres from day 3 frozen-thawed embryos followed by analysis of the remaining embryo on day 5. Hum Reprod. 2004;19:685–93.
19. Dean NL, Tan SL, Ao A. The development of preimplantation genetic diagnosis for myotonic dystrophy using multiplex fluorescent polymerase chain reaction and its clinical application. Mol Hum Reprod. 2001;7:895–901.
20. Zhang XY, Ata B, Son WY, Buckett WM, Tan SL, Ao A. Chromosome abnormality rates in human embryos obtained from in-vitro maturation and IVF treatment cycles. Reprod Biomed Online. 2010;21:552–9.
21. Bielanska M, Tan SL, Ao A. Chromosomal mosaicism throughout human preimplantation development in vitro: incidence, type, and relevance to embryo outcome. Hum Reprod. 2002;17:413–9.
22. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature. 1990;344:768–70.
23. Harper JC, Boelaert K, Geraedts J, Harton G, Kearns WG, Moutou C, et al. ESHRE PGD Consortium data collection V: cycles from January to December 2002 with pregnancy follow-up to October 2003. Hum Reprod. 2006;21:3–21.
24. Hiraoka K, Hiraoka K, Horiuchi T, Kusuda T, Okano S, Kinutani M, et al. Impact of the size of zona pellucida thinning area on vitrified-warmed cleavage-stage embryo transfers: a prospective, randomized study. J Assist Reprod Genet. 2009;26:515–21.
25. Gabrielsen A, Agerholm I, Toft B, Hald F, Petersen K, Aagaard J, et al. Assisted hatching improves implantation rates on cryopreserved-thawed embryos. A randomized prospective study. Hum Reprod. 2004;19:2258–62.
26. Van de Voort CA, Hung PH, Schramm RD. Prevention of zona hardening in non-human primate oocytes cultured in protein-free medium. J Med Primatol. 2007;36:10–16.
27. Balaban B, Urman B, Ata B, Isiklar A, Larman MG, Hamilton R, et al. A randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. Hum Reprod. 2008;23:1976–82.