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ARTICLE

Seven years of experience of preimplantation HLA typing: a clinical overview of 327 cycles

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Abstract Preimplantation human leukocyte antigen (HLA) typing allows the birth of healthy children who are potential donors of stem cells for their affected siblings. This technique can be used for acquired diseases such as leukaemia or can be used for single-gene disorders such as thalassaemia. This retrospective study presents clinical data obtained from 171 couples who had undergone 327 preimplantation HLA typing cycles: 262 cycles for HLA typing in combination with mutation analysis and 65 cycles for the sole purpose of HLA typing. Of the diagnosed embryos 17.6% were found to be HLA matched. Embryo transfer was performed in 212 cycles, 34.9% clinical pregnancy rate per transfer was achieved and 59 healthy and HLA-compatible children were born. Twenty-one sick children have been cured through haemopoietic stem cell transplantation. The effect of maternal age and ovarian reserve on reproductive outcome was assessed retrospectively. The data demonstrated that, once a mutation-free and HLA-compatible embryo was found, clinical pregnancy rate did not differ statistically significantly despite the presence of some cycle-related limitations such as advanced maternal age and/or diminished ovarian reserve. Preimplantation HLA typing is an effective therapeutic tool for curing an affected sibling even for poor-prognosis patients.

KEYWORDS: haemopoietic stem cell transplantation (HSCT), human leukocyte antigen (HLA) typing, preimplantation genetic diagnosis (PGD), single-gene disorders (SGD), β-thalassaemia

Introduction

Preimplantation genetic diagnosis (PGD) for single-gene disorders (SGD) combined with human leukocyte antigen (HLA) typing has emerged as a therapeutic tool since 2001 (Verlinsky et al., 2001). This technique does not only allow couples to have an unaffected child, but also to select a potential donor for stem cell transplantation for the affected sibling. HLA typing without mutation analysis has also been used for acquired diseases (Verlinsky et al., 2004), such as acute myeloid leukaemia and acute lymphoid leukaemia, which require allogenic haemopoietic stem cell

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transplantation (HSCT) from an HLA-identical donor for the cure of the disease.

Stem cells in the cord blood from an HLA-identical sibling can be used with a much higher success rate than a transplant from alternative donors (Gaziev et al., 2000; Orofino et al., 2003) and are therefore of great therapeutic value for haematopoietic and other life-threatening diseases (Fiorentino et al., 2006; Kahraman et al., 2004, 2007; Van de Velde et al., 2004, 2009; Verlinsky et al., 2001, 2004).

The selection of embryos for HLA typing necessitates the application of assisted reproduction treatment even though the vast majority of the couples are fertile. The successful outcome of assisted reproduction cycles is highly dependent on female age and ovarian reserve. This technique is made crucially important by the facts that the theoretical probability of finding an HLA-identical embryo in cases of acquired diseases is 25% (1/4) and the probability of finding both a HLA-identical and mutation-free embryo in cases of single-gene disorders is no more than 18% (3/16).

The aim of this retrospective study is to share the results of the study centre's experience on HLA typing with or without mutation analyses in 327 cycles and to evaluate the effect of clinical parameters, such as ovarian reserve and the age of the woman, on clinical outcomes.

Materials and methods

Couples/patient characteristics

Between 2003 and mid-2010 at Istanbul Memorial Hospital's In Vitro Fertilisation Unit and Reproductive Genetics Laboratory, 136 couples were referred for both mutation analysis for a specific genetic disorder and HLA typing, while 35 couples were referred for the sole purpose of HLA typing for acquired disorders. Of these, 166 couples had one affected child and five couples (four couples with β-thalassaemia and one with X-linked adrenoleukodystrophy) had two affected children requiring stem cell donation. During this 7-year period, the number of cycles undergone by each couple varied from one to nine. Of the 171 couples, 91 underwent only one trial, 43 had two trials, 18 had three trials, seven had four trials and 12 had five or more trials.

Preclinical work up and PGD study

First, a haplotype analysis of mother, father and child and, when available, of other family members, such as an unaffected child or grandparents, was performed for each family prior to preimplantation HLA typing. A panel of 50 different short tandem repeat markers were tested on genomic DNA to ensure the presence of enough informative markers to aid the identification of monosomy, trisomy, recombination, allele drop-out (ADO) and uniparental disomy of the analysed chromosomes and regions. For each family at least 12 heterozygous markers spanning the HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ regions (HLA classes I, II and III) were selected for PGD study.

This 7-year period was divided into two according to the methods used which included lysis of single cells, mutation testing, polymerase chain reaction (PCR) conditions and primers used in the PGD study. This was because the preclinical work-up studies were prepared in co-operation with two different genetic centres, from 2003 to 2008 with GENOMA Laboratory in Italy and from 2008 to the present with Reproductive Genetics Institute (RGI) in Chicago, USA.

Oocyte collection, insemination and culture, biopsy and transfer of embryos were performed in Istanbul Memorial Hospital's In Vitro Fertilisation Unit and all PGD studies and evaluations were performed in Istanbul Memorial Hospital's Reproductive Genetics Laboratory.

Cell lysis and PCR reactions and mutation analysis

In the first period, the alkaline lysis method was used as described previously (Fiorentino et al., 2005). Cells were lysed by incubation at 65°C for 10 min in a sterile PCR tube containing 5 μl lysis buffer (200 nmol/l KOH, 50 nmol/l dithiothreitol). The lysis buffer was then neutralized prior to adding the first-round PCR mix which contained all external primers for co-amplification of all selected HLA markers and mutation linked markers. Second-round PCR reaction for each locus was then performed using 2 μl of the first-round product. In the second period, the proteinase K method was used for lysis of the cells as described previously (Verlinsky et al., 2001). The biopsied single cells were placed into a lysis solution containing 0.5 μl 10× PCR buffer, 0.5 μl 1% Tween 20, 0.5 μl 1% Triton X-100, 3.5 μl water and 0.05 μl proteinase K. The lysis reaction was as follows: 45°C for 15 min for the lysis of the cells and 96°C for 20 min for inactivation of proteinase K. The first-round PCR master mix, which contained each dNTP (dATP, dCTP, dGTP, dUTP), PCR buffer, Taq polymerase (1.25 U), 1.5 mmol/l $MgCl_2$, 5% dimethylsulphoxide and 0.5 μmol/l of each set of outside primers was then added during a hot-start step, which was 72°C for 10 min.

Although the reaction conditions were different in the two periods, both can be briefly summarized as follows; DNA testing was performed by two rounds of PCR reactions: in the first round using multiplex PCR which allows simultaneous amplification of HLA regions and mutation-linked markers, and in the second round using single-plex PCR which is a fluorescent PCR with semi- or heminested primers. Primer sequences and PCR conditions used in this study have been reported previously (Fiorentino et al., 2004, 2005; Rechitsky et al., 2004; Verlinsky et al., 2001, 2004).

Between 2003 and 2008, mutation analysis was performed using the minisequencing technique, as described elsewhere (Fiorentino et al., 2003). After the middle of 2008, restriction enzyme digestion reactions and subsequently polyacrylamide gel electrophoresis analysis (Rechitsky et al., 2004) were used. Since the middle of 2009, both methods have been used according to preference.

IVF and embryo biopsy procedure

The stimulation protocols were as outlined previously (Kahraman et al., 2002, 2004). Gonadotrophin-releasing hormone (GnRH) analogue suppression (short or long) or GnRH antagonist protocol and human menopausal gonadotrophin or recombinant FSH were used for ovarian stimulation. Oocyte retrievals were performed 36 h after the injection of 10,000 IU human chorionic gonadotrophin by transvaginal ultrasound guidance. Approximately 3 ± 1 h after oocyte retrieval, cumulus cells were enzymically removed. Intracytoplasmic sperm

injection was applied to metaphase-II oocytes (Palermo et al., 1992). During 2003–2005, injected oocytes were placed in G1 and G3 media (Vitrolife, Gothenburg, Sweden) and during 2005–2009 they were placed in potassium simplex optimized medium (Life Global, Belgium). One blastomere was removed from cleavage-stage embryos (De Vos and Van Steirteghem, 2001) from an opening made using a laser (Saturn 3, Research Instruments, UK). Unless there was no diagnosis due to amplification failure, biopsy of a second blastomere was strictly avoided to prevent causing harm to the embryo through removing a considerable amount of embryonic volume (Cohen et al., 2007). Subsequently, embryo transfer was performed usually on day 4 but rarely on day 5. Recently, since 2009, trophectoderm tissue biopsies have also been performed. Blastocyst-stage biopsy was performed by making a hole in the zona pellucida on day 3 of embryonic development which allowed the developing trophectoderm cells to protrude after blastulation, facilitating the biopsy. On day 5 post fertilization, approximately 2–5 cells were excised using laser energy, without loss of inner cell mass. After diagnosis, the embryos were replaced during the same cycle, on day 5 or day 6. Pregnancy was first evaluated by serum human chorionic gonadotrophin assay 12 days after embryo transfer and clinical pregnancy was diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy (Zegers-Hochschild et al., 2009).

Informed consent was obtained from all patients prior to preclinical work up, during the treatment and before embryo-transfer procedures. The patients were informed about the possibilities of misdiagnosis and the cancellation of embryo transfer in the absence of HLA-matched embryos.

Data analysis

The data were analysed retrospectively. Statistical analysis was performed with the use of Pearson chi-squared, Kruskal–Wallis, Mann–Whitney *U* and Fisher's Exact tests. $P < 0.05$ was considered as significant.

Results

This study analysed 136 couples who underwent 262 cycles for both mutation analysis for a specific genetic disorder and for HLA typing (group I) and 35 couples who underwent 65 cycles for the sole purpose of HLA typing (group II) (**Table 1**).

In group I, out of 262 initiated cycles, 166 cycles reached the stage of embryo transfer (63.4%), 60 clinical pregnancies (36.1%) were achieved and 51 babies were born. Nineteen children were cured by HSCT (**Table 1**). The majority of couples in group I were carriers of β-thalassaemia ($n = 120$). Other couples were carrying Wiskott–Aldrich ($n = 3$), X-linked adrenoleukodystrophy ($n = 3$), Fanconi anaemia ($n = 2$), α-mannosidosis ($n = 1$), Gaucher syndrome ($n = 1$), Hurler syndrome ($n = 2$), hyper IgD ($n = 1$), thrombasthenia ($n = 1$), sickle cell anaemia ($n = 1$) and Diamond Blackfan anaemia ($n = 1$). For group II, 70.8% of the initiated cycles reached embryo transfer resulting in a 30.4% clinical pregnancy rate (CPR) per transfer cycle and 10 babies have been

Table 1 Overall clinical results of HLA typing.

Result	*Group I (HLA typing combined with PGD)*	*Group II (HLA typing only)*	*P-value*	*Total*
No. of patients/cycles	136/262	35/65		171/327
Maternal age (years, mean ± SD, min–max)	32.0 ± 4.83 (21–43)	34.2 ± 5.65 (23–45)	<0.005[a]	32.44 (21–45)
Body mass index (kg/m^2)	25.77 ± 3.96	24.56 ± 5.2	<0.05	25.52 ± 4.26
Total dosage of gonadotrophins	3359.1 ± 1340	2715 ± 1337	<0.001	3234.11 ± 1361
Gonadotrophin starting dose	372.25 ± 141.28	331.15 ± 165	<0.05	364.19 ± 146.8
Gonadotrophin dose per day of stimulation	350.70 ± 120.09	299.47 ± 123.90	<0.05	340.72 ± 122.34
No. of oocytes retrieved	15.87 ± 8.81	16.86 ± 7.86	NS	16.02
No. of mature oocytes injected	12.18 ± 6.73	12.86 ± 6.11	NS	12.31
No. of oocytes fertilized	87.90 ± 12.34	83.96 ± 13.65	<0.05[a]	87.33
Cycles with transfer (%)	63.4	70.8	NS	64.8
β-HCG positive (%)	41.6	37.0	NS	40.56
Clinical pregnancy rate per transfer (%)	36.1	30.4	NS	34.9
No. of clinical miscarriages	14	3	NS	17
Implantation rate (%, *n*/total)	27.5 (72/262)	21.9 (16/73)	NS	26.3 (88/335)
No. of embryos transferred	1.58 ± 0.71	1.59 ± 0.72	NS	1.58
No. of full-term pregnancies	43	8	–	52
No. of pregnancies ongoing[b]	3	3	–	5
No. of babies born	51	11	–	62
No. of successful transplantations	19	2	–	21[c]

Values are mean ± SD unless otherwise stated.
HCG = human chorionic gonadotrophin; HLA = human leukocyte antigen; HSC = human stem cell; PGD = preimplantation genetic diagnosis; NS = not signifigant.
[a]Statistical analysis was performed according to Mann–Whitney *U*-test.
[b]Number of pregnancies ongoing at the time of writing.
[c]23 children are awaiting an appropriate time for HSC transplantation.

Table 2 Summary of indications, PGD results and clinical outcomes.

Indications for HLA typing	*Patients/ cycles*	*Cells analysed/ diagnosed*	*HLA-compatible embryos*					*Transfer cycles*	*Clinical pregnancies*	*Births/ babies*	*Successful stem cell transplantation*
			Total	*Normal*	*Carrier*	*Affected*	*Other*				
Group I (HLA typing combined with PGD)	136/262	2367/2202 (93%)	394 (17.9%)	101 (4.6%)	175 (7.9%)	108 (4.9%)	10 (0.5%)	166	60	43/51	19
β-Thalassaemia	120/236	2132/1997	357	85	167	96	9	148	52	37/ 44[a,b,c]	16[d]
Wiscott Aldrich	3/4	49/43	7	3	2	1	1	4	3	3/3[e]	1
X-linked adrenoleukodystrophy	3/3	13/13	5	2	0	3	0	2	1	1/1	1
Fanconi anaemia	2/3	18/15	1	1	0	0	0	1	0		
α-Mannosidosis	1/4	35/25	2	1	0	1	0	1	0		
Gaucher syndrome	1/4	23/22	6	4	1	1	0	3	1[f]	0	
Hurler syndrome	2/3	35/28	5	2	1	2	0	2	0		
Hyper IgD	1/1	9/9	0	0	0	0	0	1[g]	0		
Glanzmann thrombasthenia	1/2	43/40	7	1	2	4	0	2	1	1/2	1
Sickle cell anaemia	1/1	5/5	2	0	2	0	0	1	1	1/1[h]	
Diamond Blackfan anaemia	1/1	5/5	2	2	0	0	0	1	1[i]		
Group II (HLA typing only)	35/65	622/539 (86.7%)	88 (16.3%)	–	–	–	–	46	14	9/11	2
Acute lymphoblastic leukaemia	15/23	215/189	26	–	–	–	–	14	5[f,i]	3/4[,e,j]	
Acute myeloid leukaemia	10/17	155/128	23	–	–	–	–	14	4	2/3[i,k,l]	1
Diamond Blackfan anaemia	3/11	130/114	21	–	–	–	–	9	2[f]	1/1	1
Histiocytosis	1/3	37/33	8	–	–	–	–	3	1	1/1[h]	0
Chronic myeloid leukaemia	1/2	15/15	2	–	–	–	–	1	0		
Burkitt's lymphoma	1/2	14/9	4	–	–	–	–	2	1	1/1[h]	0
Aplastic anaemia	2/2	21/18	0	–	–	–	–	0			
Anaplastic anaemia	1/3	28/27	3	–	–	–	–	2	0		
Myelodysplastic syndrome	1/2	7/6	1	–	–	–	–	1	1	1/1[l]	0
Total	171/327	2989/2741 (92%)	–	–	–	–	–	212	74	52/62	21

HLA = human leukocyte antigen; PCD = preimplantation genetic diagnosis.
[a]Eighteen children are awaiting an appropriate time for stem cell transplantation.
[b]One transplantation could not be performed since HLA-incompatible child was born (patient consent given).
[c]One transplantation could not be performed since HLA-incompatible twins were born (patient consent given).
[d]For one patient, successful stem cell transplantation was performed, but the source cells could not be specified and the patient was lost to follow up.
[e]Two children are awaiting an appropriate time for stem cell transplantation.
[f]One pregnancy ended with missed abortion.
[g]HLA nonidentical but healthy embryo was transferred in this cycle with patient consent.
[h]One child is awaiting an appropriate time for stem cell transplantation.
[i]One pregnancy is ongoing.
[j]Twins were born preterm and cord blood was insufficient.
[k]One pregnancy was unembryonic.
[l]One affected child died before transplantation.

Table 3 Clinical results of β-thalassaemia carriers according to maternal age and ovarian reserve.

Result	*Maternal age*			*Cumulus–oocyte–complexes*				
	<38 years	*≥38 years*	P-*value*	*≤5*	*6–10*	*11–20*	*>20*	P-*value*
Cycles initiated	201	35		20	55	93	68	
Maternal age (years)	30.4 ± 3.72	40.14 ± 1.74		35.1 ± 4.73	33.8 ± 5.09	31.3 ± 4.78	29.8 ± 3.47	<0.001[a]
No. of oocytes collected	17.30 ± 8.82	8.63 ± 4.16	<0.001[b]	3.80 ± 0.95	8.15 ± 1.42	15.20 ± 2.97	27.07 ± 6.32	<0.001[a]
Body mass index (kg/m^2)	25.39 ± 3.93	26.15 ± 5.5	NS	27.12 ± 3.9	25.46 ± 4.23	25.29 ± 4.05	25.47 ± 4.61	NS
Total dosage of gonadotrophins	3043.96 ± 1254.97	4152.59 ± 1488.80	<0.001[b]	4490 ± 1539	4216.51 ± 1462	2861.79 ± 1115.23	2657.55 ± 841.9	<0.001[a]
Gonadotrophin starting dose	349.76 ± 141	432.87 ± 155.8	<0.001[b]	492.39 ± 107.51	456.69 ± 181.11	334.30 ± 118.38	301.36 ± 101.59	<0.001[a]
Gonadotrophin dose per day of stimulation	324.63 ± 115.25	418.15 ± 126.89	<0.001[b]	470.17 ± 105.97	428.93 ± 121.05	311.35 ± 104.95	278.89 ± 80.74	<0.001[a]
No. of mature oocytes injected	13.15 ± 6.77	6.80 ± 3.30	<0.001[b]	3.45 ± 1.00	6.22 ± 1.41	11.72 ± 2.91	20.29 ± 5.28	<0.001[a]
Oocytes fertilized (%)	88.33 ± 11.63	85.90 ± 15.99	NS	89.42 ± 15.82	85.83 ± 15.33	88.61 ± 11.05	88.41 ± 10.13	NS
No. of embryos biopsied	10.06 ± 5.40	5.03 ± 3.07	<0.001[b]	2.5 ± 1.67	4.6 ± 1.70	9.2 ± 3.17	15.1 ± 4.80	<0.001[a]
No. of cycles with transfer (%)	133 (66.2)	15 (42.9)	<0.01[c]	8 (40.0)	22 (40.0)	64 (68.8)	53 (77.9)	<0.001[c]
No. of transferred embryos	1.60 ± 0.72	1.47 ± 0.74	NS	1 ± 0.00	1.2 ± 0.54	1.6 ± 0.68	1.8 ± 0.79	<0.005[a]
β-HCG positive per transfer cycles	40.6	40.0	NS	50.0	36.4	39.0	41.5	NS
CPR per transfer cycle	35.3	33.3	NS	50.0	31.8	32.8	35.8	NS
CPR per cycles initiated	23.4	14.3	NS	20.0	12.7	22.6	28	NS
No. of clinical miscarriages	7	3	<0.05[d]	1	2	3	4	NS
Implantation rate (%, *n*/total)	27.2 (58/213)	22.7 (5/22)	NS	50.0 (4/8)	27.6 (8/29)	24.5 (25/102)	27.1 (26/96)	NS

Values are mean ± SD unless otherwise stated.
CPR = clinical pregnancy rate; HCG = human chorionic gonadotrophin; NS = not significant.
[a]Statistical analysis was performed according to Kruskal–Wallis test.
[b]Statistical analysis was performed according to Mann–Whitney *U*-test.
[c]Statistical analysis was performed according to Pearson chi-squared test.
[d]Statistical analysis was performed according to Fisher's Exact test.

born to date. Of these, two have provided a cure for their affected siblings. The detailed distribution of indications and overall results for each disease type are shown in **Table 2**.

In total 2989 blastomeres were biopsied and in 2741 (92.0%) a full diagnosis was achieved. In group I, 17.9% of the analysed embryos were found to be HLA compatible. Out of these compatible embryos, 4.6% were found to be free of mutation, 7.9% were found to be carriers of the analysed disease and 4.9% were found to be affected (**Table 2**). In group II, 16.3% of embryos were found to be HLA matched and 70.4% HLA non-matched (**Table 2**).

A total of 74 clinical pregnancies (34.9%) were achieved from 212 embryo-transfer cycles. Six pregnancies are ongoing. To date, with 52 deliveries, 59 healthy and HLA-compatible children have been born. Twenty-one sick children have already been cured with cord blood cell and/or bone marrow transplantation and 23 children are waiting for their newborn siblings to gain sufficient weight and maturity for the donation of stem cells (**Tables 1 and 2**). Twenty-one successful transplantations have been performed for the following indications: β-thalassaemia ($n = 16$), Wiskott–Aldrich syndrome ($n = 1$), thrombasthenia ($n = 1$), X-linked adrenoleukodystrophy ($n = 1$), acute myeloid leukaemia ($n = 1$) and Diamond Blackfan anaemia ($n = 1$) (**Table 2**). Umbilical cord blood haemopoietic stem cells were transplanted to the affected siblings of five couples. In eight transplantations, only bone-marrow cells were used and in seven transplantations both cord blood and bone-marrow haemopoietic stem cells were used to cure the affected sibling. Additionally, the source cells of one transplantation process could not be identified as the patient did not specify when queried and did not participate in follow up.

Since the possibility of finding embryos, which are both HLA compatible and disease free is extremely low, the clinical parameters of the women are very important. The most limiting factors in an IVF cycle are maternal age and ovarian reserve. These factors were further examined in the β-thalassaemia group since these patients comprise the largest homogenous group which might allow clinical deductions. In β-thalassaemia patient group, a total of 2132 embryos were biopsied and 93.7% of these were diagnosed. According to mutation analysis, 25.4% of the diagnosed embryos were found to be normal, 45.0% of the embryos were carriers and 25.2% were found to be affected for the tested mutation. Finally 12.7% of embryos were found to be both HLA compatible and not affected and thus transferable (4.3% normal and healthy, 8.4% HLA compatible but carriers).

To evaluate any possible clinical correlations with maternal age and ovarian reserve; patients were first divided into two groups according to their maternal age: <38 years and ≥ 38 years. According to this analysis, the mean number of oocytes collected ($P < 0.001$), the number of matured oocytes injected ($P < 0.001$) and embryos biopsied ($P < 0.001$) differed significantly, as might be expected (**Table 3**). Although some parameters differed as percentage scores, neither CPR per embryo transfer cycle (35.3% versus 33.3%) nor CPR per initiated cycle (23.4% versus 14.3%) were statistically significantly different between these two groups. However, the number of cycles that reached embryo transfer was significantly lower ($P < 0.01$) and the miscarriage rate was significantly higher in the older age group than in the younger age group ($P < 0.05$) (**Table 3**).

In addition, the groups were divided into four according to the number of cumulus–oocyte–complexes (COC) retrieved in individual cycles (**Table 3**). These thresholds were defined according to conventional usage in the study clinic. Among these four groups, which were classified as ≤ 5, 6–10, 11–20 and >20 COC, although maternal age ($P < 0.001$), mean number of embryos biopsied ($P < 0.001$), cycles with embryo transfer ($P < 0.001$) and the mean number of embryos transferred ($P < 0.005$) were statistically different, CPR per embryo transfer cycles and per cycles initiated and implantation rates did not differ significantly (**Table 3**).

Discussion

It has been 10 years since the first PGD with HLA matching for Fanconi anaemia was reported (Verlinsky et al., 2001), allowing successful haemopoietic reconstitution in an affected sibling by transplantation of stem cells obtained from HLA-matched offspring. According to reports from the PGD International Society and PGD Consortium Steering Committee of the European Society of Human Reproduction and Endocrinology (ESHRE), the number of cycles performed for HLA typing is growing annually. This rise can be attributed to the patient awareness worldwide as well as to the success rates of combined assisted reproduction techniques and HLA applications. According to ESHRE PGD Consortium data collection IX (Goossens et al., 2009), which gathered results from 57 centres, in the period from January to December 2006, 28 cycles were performed for β-thalassaemia and/or sickle cell anaemia with HLA typing. In addition, there were 12 cycles for HLA compatibility typing plus a further 17 cycles for HLA typing along with a specific disorder. Another report, which included the HLA typing experience of two European PGD centres, was recently published (Van de Velde et al., 2009). According to this, 139 couples were treated in 284 cycles resulting in the birth of 51 healthy HLA-matched babies with a live-birth rate of 15.9%. The data were obtained from UZ Brussels and from Genoma and seven different collaborating assisted reproduction centres including Istanbul Memorial Hospital's In Vitro Fertilisation Unit and Reproductive Genetics Laboratory between 2003 and 2007. The present data were obtained from 171 couples and 327 cycles, the largest HLA series performed to date by one centre, which may thus provide valuable information for both clinical outcomes and patient counselling.

The majority of the HLA typing combined with PGD cases were for β-thalassaemia carriers (88.2%). Turkey is one of the Mediterranean countries in which thalassaemia mutations are commonly seen, with a carrier rate of 2.1% in the population (Aksoy et al., 1985). According to data collected by the Turkish Association of Thalassaemia, the heterozygosity rate may be higher than 10%, particularly in the southern regions of Turkey (Basak, 2007). A higher rate of consanguineous marriages in those regions is a factor which further increases the incidence of thalassaemia. Consistent with that, in the β-thalassaemia carrier patients, 45 of the 111 couples for whom background information could be obtained were consanguineous (40.5%). This high rate of consanguinity also results in technical challenges in the diagnostic procedure: for example, the higher the number

of shared alleles, the lower the number of informative markers available in the PGD studies.

It is inevitable that the group with both HLA typing and mutation analysis performed simultaneously is the one in which the percentage of transferable embryos (HLA compatible and not affected) is very low. According to the current data, for this particular group, only 12.5% of embryos were found to be transferable, which permitted transfer in only 63.4% (166/262) of the cycles initiated. Due to the presence of aneuploidies, uniparental disomies, recombinations and polyploidies, the percentage of embryos that were both healthy and HLA compatible was lower than theoretically expected (12.8% here versus 18%). Still, these results are consistent with previous studies (Kahraman et al., 2004; Rechitsky et al., 2004). However, in the HLA-typing-only group, the gap between theoretically expected and practically observed rates of HLA-compatible embryos was greater (25.0% versus 16.3%). The two groups (group I and group II) in the current study were not matched perfectly in several parameters that may affect the PGD results. Maternal age ($P < 0.005$) and the fertilization rate ($P < 0.05$) differed while clinical outcomes such as CPR, miscarriage rate and implantation rates are not significantly different (**Table 1**). The mean maternal age of group II was significantly higher than that of group I, and sperm parameters could be another factor, which might be more impaired in group II. These two parameters could be responsible for the relatively high rate of aneuploidies of the analysed chromosomes, which decreases the proportion of HLA-compatible and transferable embryos. In addition, the diagnosis rate in the HLA-typing-only group (86.7%) was considerably lower than the rate for the HLA-typing and mutation-analysis group (93.0%; **Table 2**), which might be explained by poor embryonic quality of one or more patients who were present in group II.

Out of 62 babies born to date, three were not HLA compatible with their affected siblings. Two were twins who were transferred by patient request, after being informed that they were not fully matched. In the remaining case, sufficient diagnosis was not possible due to the presence of ADO in three out of five markers of the HLA region. This was explained to the patient who nevertheless requested transfer. Although prenatal diagnosis was strongly recommended, the patients chose not to undergo chorionic villus sampling or amniocentesis; the resultant baby was a carrier for thalassaemia but not compatible with the affected sibling.

The single-cell PCR technique has several pitfalls, such as contamination by extraneous DNA, amplification failure, preferential amplification and ADO, which is the failure of PCR to amplify one of the two alleles. If ADO occurs, only a single allele is amplified and detected after PCR, giving a heterozygous cell the appearance of homozygosity. This may lead to harmful consequences in the case for a dominant disease as the failure to amplify the mutant allele may lead to the transfer of affected embryos. Piyamongkol et al. (2003) has indicated that ADO was found to be affected by amplicon size, amount of DNA degradation, freezing and thawing, the PCR programme and the number of cells simultaneously amplified. The ADO rate and the efficiency of amplification of targeted regions depend on efficient lysis methods (Shirazi et al., 2009) and also the type of cell analysed. With simultaneous usage of linked short tandem repeat markers with multiplex single-cell PCR techniques, the accuracy of single-cell PCR is approximately 98% (Rechitsky et al., 2001). The ADO rate in this study was 9%, which is acceptable for single blastomere analysis. ADO rates in single cells can be decreased by analysing more than one cell, which is possible with blastocyst-stage biopsy. Trophectoderm biopsy is a good alternative to cleavage-stage biopsy as it enables the evaluation of approximately 2–5 cells, thus decreasing both the rate of amplification failures and ADO associated with single-cell PCR (Kokkali et al., 2007; McArthur et al., 2008; Pangalos et al., 2008). Furthermore, a higher rate of implantation could be achieved by trophectoderm analysis (McArthur et al., 2008). Since the current results with trophectoderm biopsy are preliminary, comparison between the efficiency of the two biopsy methods and clinical outcomes could not be included in this report.

Recombination in the HLA region or in the mutation site is another problem which may decrease the accuracy of preimplantation HLA typing. In a previous study, the frequency of recombination in the HLA region has been reported as 4.3% (Rechitsky et al., 2004). Recombination or chromosomal crossover is a natural event in human gametogenesis which increases genetic diversity. The genetic material may be exchanged between homologous chromosomes during prophase I of meiosis and it may occur at any location along the chromosome. If recombination occurs between the mutation site and a linked microsatellite marker, the linkage between these two points will be altered. In this case, the information based on marker alone will be misleading for the mutation status. Also, a recombination in the HLA gene may cause misdiagnosis of HLA compatibility if not detected. For accurate diagnosis of HLA compatibility and mutation analysis, at least three informative markers should be analysed for each HLA class regions (class I, II and III), and four markers which are close to the mutation site (two upstream and two downstream) should be added to the PGD study.

Although HLA typing offers the best therapeutic option for genetic disease affecting the haemopoietic and immune systems in children, there may exist several patient- or cycle-specific limitations. To assess this, the effect of maternal age and ovarian reserve on the success rate was compared between β-thalassaemia carriers, since this group provided sufficient homogenous data. According to **Table 3**, maternal age has little impact on achieving pregnancy if at least one transferable embryo was found. However, miscarriage rates were different in these two groups ($P < 0.05$). The relatively high miscarriage rate could be attributable to aneuploidic conceptions, since the technique could not be combined with aneuploidy testing (Fiorentino et al., 2006; Rechitsky et al., 2006) in the majority of patients. When the groups were compared according to CPR per cycle initiated, whilst the difference became more pronounced (i.e. 23.4% versus 14.3%), it did not reach statistical significance (**Table 3**). However, the number of patients was not homogenous in both groups (201 patients aged <38 years and only 35 patients aged ≥ 38 years), which may have reduced the statistical power of the comparison. Similarly, when the numbers of retrieved oocytes were classified as ≤ 5, 6–10, 11–20 and >20 COC, the clinical results again seemed not to be affected. Although advanced age and diminished ovarian reserve reduces the chance of having an embryo transfer in the treatment cycles, clinical

pregnancy and implantation rates did not seem to be affected (**Table 3**). The probable reason for that may be the fertility status of the couples. The fact that all of these couples were fertile increased their chances of achieving pregnancy. On the other hand, the lack of use of morphological and developmental criteria for embryo transfer decreased the success of the outcome. In some cycles, relatively poor-quality or slow-growing embryos were transferred, since they were the only ones that were both disease free and HLA compatible. Vandervorst et al. (1998) examined the correlation between pregnancy rates and the number of COC retrieved in cycles performed for autosomal recessive and dominant disorders. They found a significant correlation between the number of transferable embryos and COC but the correlation with pregnancies did not reach statistical significance. The authors attributed this to the low numbers of pregnancies. Nevertheless, they maintained that cycles in which fewer than six COC are retrieved should be cancelled (Vandervorst et al., 1998). According to other reports, while the number of oocytes retrieved significantly affected the IVF/PGD outcome, a significant threshold level was not identifiable (Tur-Kaspa et al., 2007; Verpoest et al., 2009). The current study compared parameters according to the number of COC and found no correlation with pregnancy rates in cycles with transfer and cycles initiated. Moreover, clinical pregnancy was achieved in 20.0% of cycles initiated with five or less COC (**Table 3**), showing that there is no disadvantage for this group and therefore cycle cancellation due to a low number of retrieved oocytes is not meaningful.

The data demonstrate that, once a mutation-free and HLA-compatible embryo is found, acceptable pregnancy rates can be obtained with this approach, even in the presence of some cycle-related limitations such as advanced maternal age and/or diminished ovarian reserve. HLA typing with or without mutation analysis is a promising and effective therapeutic tool for curing an affected sibling.

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References

Aksoy, M., Kutlar, A., Kutlar, F., Dincol, G., Erdem, S., Bastesbihci, S., 1985. Survey on haemoglobin variants, beta thalassaemia, glucose-6-phosphate dehydrogenase deficiency, and haptoglobin types in Turks from western Thrace. J. Med. Genet. 22, 288–290.

Basak, A.N., 2007. The molecular pathology of beta-thalassemia in Turkey: the Boğaziçi university experience. Hemoglobin 31, 233–241.

Cohen, J., Wells, D., Munné, S., 2007. Removal of 2 cells from cleavage stage embryos is likely to reduce the efficacy of chromosomal tests that are used to enhance implantation rates. Fertil. Steril. 87, 496–503.

De Vos, A., Van Steirteghem, A., 2001. Aspects of biopsy procedures prior to preimplantation genetic diagnosis. Prenat. Diagn. 21, 767–780.

Fiorentino, F., Magli, M.C., Podini, D., Ferraretti, A.P., Nuccitelli, A., Vitale, N., Baldi, M., Gianaroli, L., 2003. The minisequencing method: an alternative strategy for preimplantation genetic diagnosis of single gene disorders. Mol. Hum. Reprod. 7, 399–410.

Fiorentino, F., Biricik, A., Karadayi, H., Berkil, H., Karlikaya, G., Sertyel, S., Podini, D., Baldi, M., Magli, M.C., Gianaroli, L., Kahraman, S., 2004. Development and clinical application of a strategy for preimplantation genetic diagnosis of single gene disorders combined with HLA matching. Mol. Hum. Reprod. 6, 445–460.

Fiorentino, F., Kahraman, S., Karadayi, H., Biricik, A., Sertyel, S., Karlikaya, G., Saglam, Y., Podini, D., Nuccitelli, A., Baldi, M., 2005. Short tandem repeats haplotyping of the HLA region in preimplantation HLA matching. Eur. J. Hum. Genet. 8, 953–958.

Fiorentino, F., Biricik, A., Nuccitelli, A., De Palma, R., Kahraman, S., Iacobelli, M., Trengia, V., Caserta, D., Bonu, M.A., Borini, A., Baldi, M., 2006. Strategies and clinical outcome of 250 cycles of preimplantation genetic diagnosis for single gene disorders. Hum. Reprod. 3, 670–684.

Gaziev, D., Galimberti, M., Lucarelli, G., Polchi, P., Giardini, C., Angelucci, E., Baronciani, D., Sodani, P., Erer, B., Biagi, M.D., Andreani, M., Agostinelli, F., Donati, M., Nesci, S., Talevi, N., 2000. Bone marrow transplantation from alternative donors for thalassemia: HLA-phenotypically identical relative and HLA-non-identical sibling or parent transplants. Bone Marrow Transplant. 25, 815–821.

Goossens, V., Harton, G., Moutou, C., Traeger-Synodinos, J., Van Rij, M., Harper, J.C., 2009. ESHRE PGD Consortium data collection IX: cycles from January to December 2006 with pregnancy follow-up to October 2007. Hum. Reprod. 8, 1786–1810.

Kahraman, S., Kumtepe, Y., Sertyel, S., Dönmez, E., Benkhalifa, M., Findikli, N., Vanderzwalmen, P., 2002. Pronuclear morphology scoring and chromosomal status of embryos in severe male infertility. Hum. Reprod. 12, 3193–3200.

Kahraman, S., Karlikaya, G., Sertyel, S., Karadayi, H., Findikli, N., 2004. Clinical aspects of preimplantation genetic diagnosis for single gene disorders combined with HLA typing. Reprod. Biomed. Online 5, 529–532.

Kahraman, S., Findikli, N., Karliklaya, G., Sertyel, S., Saglam, Y., Karadayi, H., Fiorentino, F., 2007. Medical and social perspectives of PGD for single gene disorders and human leukocyte antigen typing. Reprod. Biomed. Online 14, 104–108.

Kokkali, G., Traeger-Synodinos, J., Vrettou, C., Stavrou, D., Jones, G.M., Cram, D.S., Makrakis, E., Trounson, A.O., Kanavakis, E., Pantos, K., 2007. Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of beta-thalassaemia: a pilot study. Hum. Reprod. 5, 1443–1449.

McArthur, S.J., Leigh, D., Marshall, J.T., Gee, A.J., De Boer, K.A., Jansen, R.P., 2008. Blastocyst trophectoderm biopsy and preimplantation genetic diagnosis for familial monogenic disorders and chromosomal translocations. Prenat. Diagn. 5, 434–442.

Orofino, M.G., Argiolu, F., Sanna, M.A., Rosatelli, M.C., Tuveri, T., Scalas, M.T., Badiali, M., Cossu, P., Puddu, R., Lai, M.E., Cao, A., 2003. Fetal HLA typing in β thalassemia implication for hematopoitic stem cell transplantation. Lancet 362, 41–42.

Palermo, G., Joris, H., Devroey, P., Van Steirteghem, A.C., 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 4, 17–18.

Pangalos, C.G., Hagnefelt, B., Kokkali, G., Pantos, K., Konialis, C.P., 2008. Birth of a healthy histocompatible sibling following preimplantation genetic diagnosis for chronic granulomatous disease at the blastocyst stage coupled to HLA typing. Fetal Diagn. Ther. 24, 334–339.

Piyamongkol, W., Bermúdez, M.G., Harper, J.C., Wells, D., 2003. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. Mol. Hum. Reprod. 9, 411—420.

Rechitsky, S., Verlinsky, O., Amet, T., Rechitsky, M., Kouliev, T., Strom, C., Verlinsky, Y., 2001. Reliability of preimplantation diagnosis for single gene disorders. Mol. Cell. Endocrinol. 183, 65—68.

Rechitsky, S., Kuliev, A., Tur-Kaspa, I., Morris, R., Verlinsky, Y., 2004. Preimplantation genetic diagnosis with HLA matching. Reprod. Biomed. Online 2, 210—221.

Rechitsky, S., Kuliev, A., Sharapova, T., Laziuk, K., Ozen, S., Barsky, I., Verlinsky, O., Tur-Kaspa, I., Verlinsky, Y., 2006. Preimplantation HLA typing with aneuploidy testing. Reprod. Biomed. Online 1, 89—100.

Shirazi, A., Ahadi, A.M., Farzaneh, D.F., Sadeghizade, M., 2009. Effect of lysis strategy in accuracy and repeatability of sex determination by single cell polymerase chain reaction method. J. Biol. Sci. 9, 78—82.

Tur-Kaspa, I., Bernal, A., Tkachenko, N., Pawlowska, J., Rechitsky, S., Verlinsky, Y., 2007. To PGD or not to PGD: is there a magic number of oocytes to start with? Fertil. Steril. 88, S231—S232.

Vandervorst, M., Liebaers, I., Sermon, K., Staessen, C., De Vos, A., Van de Velde, H., Van Assche, E., Joris, H., Van Steirteghem, A., Devroey, P., 1998. Successful preimplantation genetic diagnosis is related to the number of available cumulus-oocyte complexes. Hum. Reprod. 11, 3169—3176.

Van de Velde, H., Georgiou, I., De Rycke, M., Schots, R., Sermon, K., Lissens, W., Devroey, P., Van Steirteghem, A., Liebaers, I., 2004. Novel universal approach for preimplantation genetic diagnosis of beta-thalassaemia in combination with HLA matching of embryos. Hum. Reprod. 3, 700—708.

Van de Velde, H., De Rycke, M., De Man, C., De Hauwere, K., Fiorentino, F., Kahraman, S., Pennings, G., Verpoest, W., Devroey, P., Liebaers, I., 2009. The experience of two European preimplantation genetic diagnosis centres on human leukocyte antigen typing. Hum. Reprod. 3, 732—740.

Verlinsky, Y., Rechitsky, S., Schoolcraft, W., Strom, C., Kuliev, A., 2001. Preimplantation diagnosis for Fanconi anemia combined with HLA matching. JAMA 285, 3130—3133.

Verlinsky, Y., Rechitsky, S., Sharapova, T., Morris, R., Taranissi, M., Kuliev, A., 2004. Preimplantation HLA testing. JAMA 291, 2079—2085.

Verpoest, W., Haentjens, P., De Rycke, M., Staessen, C., Sermon, K., Bonduelle, M., Devroey, P., Liebaers, I., 2009. Cumulative reproductive outcome after preimplantation genetic diagnosis: a report on 1498 couples. Hum. Reprod. 24, 2951—2959.

Zegers-Hochschild, F., Adamson, G.D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., Vanderpoel, S.International Committee for Monitoring Assisted Reproductive Technology; World Health Organization, 2009. International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology. Fertil. Steril. 92, 1520—1524.

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