Journal of Assisted Reproduction and Genetics (2019) 36:159–164
https://doi.org/10.1007/s10815-018-1359-2

GENETICS

The effect of repeated biopsy on pre-implantation genetic testing for monogenic diseases (PGT-M) treatment outcome

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Received: 21 August 2018 / Accepted: 25 October 2018 / Published online: 6 November 2018

Abstract

Purpose To study the outcome of repeated biopsy for pre-implantation genetic testing in case of failed genetic diagnosis in the first biopsy.

Methods The study group included 81 cycles where embryos underwent re-biopsy because there were no transferable embryos after the first biopsy: in 55 cycles, the first procedure was polar body biopsy (PBs) and the second cleavage-stage (BB); in 26 cycles, the first was BB and the second trophectoderm (BLAST) biopsy. The control group included 77 cycles where embryos underwent successful genetic diagnosis following the first biopsy, matched by maternal age, egg number, genetic inheritance type, and embryonic stage at the first biopsy. We measured genetic diagnosis rate, clinical pregnancy rates (PRs), live-birth rates (LBRs), gestational age, and birth weight.

Results For repeated biopsy, genetic diagnosis was received in 67/81 cycles (82.7%); at a higher rate in PB + BB than in BB + BLAST (49/55, 89.1% and 18/26, 69.2% respectively, $p = 0.055$). Transferable embryos were found in 47 and 68 cycles in the study and the control groups. PRs/ET were 20/47 (42.6%) and 36/68 (52.9%) ($p = 0.27$), 16/36 (44.4%) following PB + BB, and 4/11 (36.4%) following BB + BLAST ($p = 0.74$). LBRs/ET were 13/47 (27.7%) in study group, and 28/68 (41.2%) in the controls ($p = 0.14$), 10/36 (27.8%) following PB + BB group, and 3/11 (27.3%) following BB + BLAST ($p > 0.99$). Gestational age and birth weight were similar in all groups.

Conclusions Re-biopsy of embryos when no genetic diagnosis could be reached following the first biopsy, achieved high rates of genetic diagnosis, pregnancies, and live births.

Keywords PGT-M · Repeated embryo biopsy · Polar body biopsy · Cleavage-stage biopsy · Blastocyst biopsy

Introduction

Pre-implantation genetic testing for monogenic diseases (PGT-M) is the use of techniques to genetically profile oocytes or embryos prior to implantation; PGT-M is performed as a part of the in vitro fertilization (IVF) process, in couples with hereditary genetic disorders. The biopsy is the first step specific to PGT-M, and successful execution is a pre-condition for the potential success of a PGT-M cycle. The biopsy has to effectively obtain genetic material in optimal conditions for genetic analysis while preventing any damage to embryo viability. The biopsy and subsequent genetic analysis may be performed at different stages after oocyte retrieval and fertilization. For each PGT-M cycle, the best timing for oocyte or embryo biopsy is selected according to the genetic analysis required (maternal X-linked and autosomal dominant (AD) versus paternal or autosomal recessive (AR) disease and the availability of informative polymorphic markers), number and quality of polar bodies (PBs) or embryos available and preferred timing of embryo transfer (ET) [1–4].

In 9–10% of embryos, genetic diagnosis is not available from the first biopsy of the PGT-M process [5, 6]. This fact can be due to different reasons related either to the biopsy process or to total or partial amplification failure. We also use stringent rules for

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diagnosis (requirement to detect at least three informative alleles of the analyzed polymorphic markers surrounding the mutation). All tests that do not meet this requirement are considered no result with the possibility to perform a new analysis on a second biopsy. In recent years for some embryos, biopsy was performed twice at different stages of embryonic development, in an attempt to obtain genetic diagnosis.

The literature about repeated biopsy is limited, and mainly describes a few studies that include freezing between the first and the second biopsy [7–10]. Repeated biopsy in the same cycle, without freezing, was described in only one case report [11], and in one retrospective study from 2004 which examined repeated biopsy for pre-implantation genetic testing for aneuploidy (PGT-A) [12].

The importance of repeated biopsy is mainly to preclude women from undergoing the controlled ovarian hyperstimulation (COH) and the egg retrieval process multiple times, and to avoid associated risks such as ovarian hyperstimulation syndrome (OHSS). In addition, it gives a woman a greater chance for pregnancy where, in previous cycles, genetic diagnosis and therefore embryo transfer were not possible.

This study examines whether the repeated biopsy helps to obtain genetic diagnosis that the first biopsy has not achieved, and whether it changes the pregnancy and live-birth rates and newborn outcome compared to the PGT-M process with successful genetic diagnosis from the first biopsy.

Materials and methods

Ethical approval

The study was approved by the Shaare Zedek Medical Center (SZMC) institutional review board in accordance with national regulations.

Patients

The study population analyzed 81 fresh PGT-M cycles where biopsy was performed twice during IVF treatments at SZMC. The study group included 81 cycles of women whose embryos underwent re-biopsy because there were no transferable embryos after the first biopsy, divided into two subgroups according to sampling timing: in 55 cycles, first sampling was taken from PBs and second sampling was taken from day-3 embryo (BB); in 26 cycles, first sampling was taken from BB and second sampling was taken from trophectoderm (BLAST). There was at least one transferable embryo in 47 cycles after the second biopsy. The control group included 77 cycles of women who had a successful genetic diagnosis in the first biopsy, with at least one transferable embryo in 68 cycles; matched in age, egg number, type of genetic inheritance, and embryonal stage at the time of biopsy to the women in the study group (51 cycles where first sample was taken from PBs, and 26 cycles where first sample was taken from BB). All biopsies were performed in the fresh cycle, without cryopreservation. It should be clarified that for each women no more than 1 cycle was examined in each study group.

Demographic and clinical data are presented in Table 1.

Laboratory methods

IVF and blastomere biopsy were performed by a standard protocol involving COH with recombinant FSH and GnRH analog. Vaginal ultrasound-guided ovum retrieval was performed 36 h after HCG injection. Oocytes were identified, washed, and transferred to organ culture dishes containing equilibrated culture medium (Global, Life Global) and placed in an incubator with 5% CO_2. Oocytes were denuded with hyaluronidase (Sage, Origio, Cooper Surgical, Denmark) 2 h after collection, and were allowed to recover in the incubator for a further 2 h. Intracytoplasmic sperm injection (ICSI) was then performed on each mature egg followed by first PB biopsy. Second PB biopsy was performed 16–18 h later upon fertilization stage assessment and oocyte second PB extraction. Each fertilized and biopsied oocyte was then cultured separately in fresh 30 μl droplets of Global medium (Life Global) under oil for further 48 h. Both PB and BB day 3 biopsies were performed accordingly to the "zona-slitting" technique [13]. Blastocyst trophectoderm biopsy was performed on day 5 cultured blastocysts by laser-assisted (Lykos, Hamilton Thorne) herniating technique [14]. Each sample was separately transferred to a 0.5-ml tube containing 5 μl of proteinase K lysis buffer [15].

A sample of culture medium (media blank) from each droplet that contained a biopsied cell was analyzed to verify that there was no DNA contamination in the culture medium that could affect the results. In addition, a no template control (NTC, reaction blank) was used to monitor absence of external contamination in each PCR reaction.

DNA was extracted from peripheral blood cells from couples, affected children, and first-degree family members. For each disease, polymorphic microsatellite markers surrounding the diseased gene were identified, and informative markers were used to construct haplotypes in each family. These markers and the family mutations were then used to the development of single cell multiplex assays. All cases were performed using a multiplex nested approach with inclusion of at least four informative polymorphic markers surrounding the gene of interest [16]. Only samples that were informative for a minimum of three polymorphic markers flanking the mutation were considered for diagnosis. Stringent precautions to avoid any source of contamination, as recommended by the European Society for Human Reproduction and Embryology (ESHRE) PGD consortium, were used during all steps [5, 17, 18].

Table 1 Demographic, clinical data, and treatment outcome in the study and the control groups

	Study group		Control group	*P* value
	PB + BB	BB + BLAST		
Age (y) (mean ± SD)	31.3 ± 4.8		30.7 ± 4.7	0.392*
No. OPU cycles	81		77	–
No. eggs (mean ± SD)	14.0 ± 6.9		14.1 ± 6.1	0.960*
Genetic outcome	67/81 (82.7%)		77/77 (100%)	< 0.001*
	49/55 (89.1%)	18/26 (69.2%)		0.055^
PR/ET	20/47 (42.6%)		36/68 (52.9%)	0.273*
	16/36 (44.4%)	4/11 (36.4%)		0.737^
LBR/ET	13/47 (27.7%)		28/68 (41.2%)	0.136 *
	10/36 (27.8%)	3/11 (27.3%)		> 0.990^
Miscarriage rate	7/20 (35.0%)		8/36 (22.2%)	0.353 *
GA[#] (weeks) (mean ± SD)	37.8 ± 2.0		38.4 ± 2.6	0.425[◊]
BW[#] (grams) (mean ± SD)	2949.6 ± 470.6		3210.1 ± 575.7	0.276[◊]

BB blastomere, *BLAST* blastocyst, *BW* birth weight, *ET* embryo transfer, *GA* gestational age, *LBR* live-birth rate, *PB* polar body, *PR* clinical pregnancy rate

**P* value between study and control groups (Pearson chi-square)

^*P* value between two study subgroups – PB + BB vs BB + BLAST (Fisher's exact test)

[◊] *P* value between study and control groups (*t* test)

[#] Analysis data for singletons, excluding twins

Data collection

The data was collected from computerized hospital databases and when needed, from the patient's files (IVF, delivery room, neonatal, and maternity units). This included maternal age, number of oocytes retrieved, the indication for undergoing the PGT-M process, and the type of genetic inheritance (see Table 2); whether a clinical pregnancy was achieved, whether there was a live birth, and if so, what was the gestational age and infant birth weight. In addition, we examined the type of biopsy or biopsies performed for PGT-M (PB, BB, PB + BB, or BB + BLAST) and for the study group we examined whether genetic results were received from the repeated biopsy.

Statistical analysis

In order to compare quantitative variables between two independent groups, the *t* test as well as the non-parametric Mann-Whitney test was applied. The comparison of quantitative variables between three groups or more was performed by using the non-parametric Kruskal-Wallis test. Non-parametric tests were used when subgroups compared were smaller than ten cases. The association between two categorical variables was assessed using the chi-square and the Fisher's exact tests. Ninety-five percent confidence intervals (CI) were calculated for percentages. All tests applied were two-tailed and a *p* value of 5% or less was considered statistically significant.

Results

The main study results are presented in Table 1.

There was no statistically significant difference in maternal age and number of eggs retrieved between the study and the control groups.

Genetic outcome

In the study group, after the repeated biopsy, genetic diagnosis was received in 67/81 cycles (82.7%, 95% CI: 72.7–90.2%). In the subgroup in which samples were taken from PB + BB, genetic diagnosis was received in 49/55 cycles (89.1%, 95% CI: 77.8–95.9%), whereas in the subgroup in which samples were taken from BB + BLAST, genetic diagnosis was received in 18/26 cycles (69.2%, 95% CI: 48.2–85.7%, $p = 0.055$).

In the study group, out of 67 cycles for which a genetic diagnosis was received, in 47 cycles there was at least one transferable embryo. In the control group, out of 77 cycles where there was a successful genetic diagnosis in the first biopsy, in 68 cycles there was at least one transferable embryo. In all cycles, the embryo or embryos transfer was performed the day after the biopsy was taken.

In the study group, 29 cycles included biopsied embryos that failed to yield the minimum number of polymorphic markers, 15 cycles included biopsied embryos that suffered from total amplification failure (TAF), and 37 cycles included

Table 2 Inheritance type and diseases

	Study group	Control group
AD	NF1 (6*)	MD (7)
	BRCA2 (4)	NF1 (4)
	MD (4)	BRCA1 (2)
	BRCA1 (3)	BRCA2 (2)
	TS (2)	ADPKD (1)
	ACHONDROPLASIA (1)	WOLFRAM (1)
	ALGS (1)	HOCM (1)
	HED (1)	MARFAN (1)
	HME (1)	MCSD (1)
	HOCM (1)	NOONAN (1)
	MEN2 (1)	PFEIFFER (1)
	OI (1)	RB (1)
		SMEI (1)
		SOTOS (1)
AR	CF (7)	CF (8)
	FD (3)	DEAFNESS (3)
	SMA (3)	ALBINISM (2)
	DEAFNESS (2)	DLDD (1)
	TAY-SACHS (2)	FA (1)
	ALBINISM (1)	FD (1)
	BT (1)	FMF (1)
	DKC (1)	GD (1)
	MLD (1)	NAGS (1)
	NESIDIOBLASTOSIS (1)	TAY-SACHS (1)
	ZELLWEGER (1)	ZELLWEGER (1)
X-linked	FXS (12)	FXS (14)
	DMD (4)	HUNTER (4)
	IP (3)	DMD (3)
	MRX (3)	EDMD (3)
	ALD (2)	ALD (2)
	EDMD (2)	AHDS (1)
	AHDS (1)	BCM (1)
	AHO (1)	FABRY (1)
	CdLS (1)	IP (1)
	HUNTER (1)	SBMA (1)
	XLAS (1)	
	XLHED (1)	

AD autosomal dominant, *ADPKD* autosomal dominant polycystic kidney disease, *AHDS* Allan Herndon Dudley syndrome, *AHO* Albright's hereditary osteodystrophy, *ALD* adrenoleukodystrophy, *ALGS* alagille syndrome, *AR* autosomal recessive, *BCM* blue cone monochromatism, *BT* beta thalassemia, *CdLS* cornelia de lange syndrome, *CF* cystic fibrosis, *DKC* dyskeratosis congenita, *DLDD* dihydrolipoamide dehydrogenase deficiency, *DMD* Duchenne muscular dystrophy, *EDMD* Emery-Dreifuss muscular dystrophy, *FA* Fanconi anemia, *FD* familial dysautonomia, *FMF* familial Mediterranean fever, *FXS* fragile x syndrome, *GD* Gaucher's disease, *HED* hypohidrotic ectodermal dysplasia, *HME* hereditary multiple exostoses, *HOCM* hypertrophic cardiomyopathy, *IP* incontinentia pigmenti, *MCSD* metaphyseal chondrodysplasia schmid type, *MD* myotonic dystrophy, *MEN2* multiple endocrine neoplasia type 2, *MLD* metachromatic leukodystrophy, *MRX* x-linked mental retardation, *NAGS* n-acetylglutamate synthetase deficiency, *NF1* neurofibromatosis type 1, *OI* osteogenesis imperfecta, *RB* retinoblastoma, *SBMA* spinal and bulbar muscular dystrophy, *SMA* spinal muscular atrophy, *SMEI* severe myoclonic epilepsy of infancy, *TS* tuberous sclerosis, *XLAS* x-linked Alport syndrome, *XLHED* x-linked hypohidrotic ectodermal dysplasia.

*Number of women who underwent PGD process due to this disease

some biopsied embryos that failed to yield enough markers and other biopsied embryos that suffered from TAF.

Clinical pregnancy rates

Clinical pregnancy rate per embryo transfer (PR/ET) was 20/47 (42.6%) in the study group and 36/68 (52.9%) in the control group ($p = 0.273$). Divided into subgroups, in the PB + BB group clinical pregnancy was achieved in 16/36 (44.4%), whereas in the BB + BLAST group, in 4/11 (36.4%) cycles clinical pregnancy was achieved ($p = 0.737$).

Live-birth rates

LBR/ET was 13/47 (27.7%) in the study group, and 28/68 (41.2%) in the control group ($p = 0.136$). Divided into subgroups, LBR/ET was 10/36 (27.8%) in the PB + BB group and 3/11 (27.3%) in the BB + BLAST group ($p > 0.990$). Comparing the miscarriage rate, there was no significant difference between the groups: 7/20 (35.0%) pregnancies in the study group and 8/36 (22.2%) pregnancies in the control group ($p = 0.353$).

Among live births in study group 2/13 (15.4%) women gave birth to twins, and in the control group 5/28 (17.9%) women gave birth to twins.

Gestational age and birth weight

No significant difference between groups regarding gestational age and birth weight was found, both in analyzing data for singletons apart and in analyzing data for twins apart (see Table 1). For singletons, the mean gestational age in the study group was 37.8 ± 2.0 weeks, and similar in the control group: 38.4 ± 2.6 weeks ($p = 0.425$); the mean birth weight in the study group was 2949.6 ± 470.6 g, and in the control group was 3210.1 ± 575.7 g ($p = 0.276$). For twins, the mean gestational age in the study group was 35.0 ± 1.4 weeks, and similar in the control group: 36.4 ± 1.1 weeks ($p = 0.381$); the mean birth weight in the study group twins was 2337.5 ± 265.2 g, and in the control group was 2437.0 ± 411.2 g ($p > 0.990$).

Discussion

The aims of our retrospective study were to examine whether there is clinical benefit in performing repeated biopsy for PGT-M when the first biopsy does not yield a genetic result, whether it helps to obtain genetic diagnosis, pregnancies and live births, and whether it affects newborn outcomes. Our study demonstrates that genetic diagnosis from repeated biopsies in cases of failed first diagnosis is high (82.7%). In addition, clinical pregnancies and live-birth rates per embryo transfer are similar to those of embryos that underwent only one successful biopsy. Repeated biopsy did not affect gestational length or birth weight. This benefit was seen when the failed first biopsy was PB and the second was BB, and also when the failed first biopsy was BB and the second was trophectoderm, with no statistically difference between the groups. Thus, we recommend considering re-biopsy on cleavage-stage embryos or trophectoderm, in a fresh cycle, achieved high rates of genetic diagnosis, pregnancies, and live births when no genetic diagnosis could be achieved following the first biopsy.

The literature about repeated biopsy is limited, and describes only few case reports and studies include freezing between the first and the second biopsy [7–11].

Magli et al. examined repeated biopsies from PB + BB for PGS for aneuploidy diagnosis by FISH, and showed that removal of a BB subsequent to PB biopsy did not seem to have negative effects on embryo viability [12]. No single-gene disorders were examined, and no BLAST biopsies were performed. The researchers suggested that a follow-up study should be conducted to examine repeated biopsies for single-gene disorders diagnosis, but since 2004 no such study has been documented. Our results reinforce Magli et al. results: repeated biopsy did not seem to harm embryonic development or implantation and pregnancy rates.

Our study is the first that examined multiple cycles in which repeated biopsies were performed at different stages of embryonic development in the same cycle, without freezing between biopsies. In addition, we compared the rates of pregnancies, live births, and newborn outcomes to cycles in which only one biopsy was performed.

The study's results and conclusions have significant importance in determining the recommendation about performing repeated biopsy in PGT-M cycles where no genetic diagnosis was achieved in the first biopsy. Repeated biopsy in the same fresh cycle prevents repeated frozen-thawed cycles and can shorten the time to pregnancy and delivery with no negative effects on pregnancy and newborn outcomes.

The only statistically significant difference between the groups of PB + BB and BB + BLAST was in the genetic diagnosis, with higher rates in the former (see Table 1). Since in cases of PB analysis, sequential PB1 and PB2 are analyzed, it is of importance to note that PB with no genetic result could be caused by successful genetic analysis of PB1 without a clear result of PB2, or by homozygous PB1 (in which allele drop out and therefore possible misdiagnosis could not be ruled out). These facts, particularly the partial results from the PB biopsy, could explain why the re-analysis of an embryo after PB biopsy was more successful than when compared to BB vs BLAST analysis. Another possible reason for the difference is that this analysis was undertaken when our laboratory was only starting to offer the option of blastocyst biopsy. It could be the technique had not yet been perfected and not enough cells were sent for analysis.

Previous research of infants born after standard PGT-M process showed that newborn outcomes (gestational age and birth weight) were lower than those born after a spontaneous pregnancy, suggesting that the biopsy itself may cause harm [19, 20]. Although the groups of newborns are small (13 and 28 live births in the study and the control group, respectively), repeated biopsy did not impact embryo development or harm the newborn outcomes, compared with newborns who underwent only one successful biopsy.

In conclusion, in the case of failed genetic diagnosis in the first biopsy, we demonstrate that there is clinical benefit taking a repeated biopsy either from blastomere or trophectoderm of blastocyst. This will provide an option to obtain genetic diagnosis, pregnancy, and live birth from the current cycle. It will shorten the time to delivery, prevent women from undergoing multiple egg retrievals, and reduce their potential side effects by obtaining a genetic diagnosis of as many embryos as possible in any specific cycle.

Acknowledgements We would like to thank Tali Bdolah-Abram, MS, for her skillful help with statistical analysis.

Author's roles The study was done in partial fulfillment of the requirements of SP for a medical degree of the School of Medicine in Jerusalem, under the guidance of TEG and GA who conceived the research concept. SP contributed to the data collection and interpretation as well of writing the manuscript. OS, ER, ND, and AP contributed to the acquisition and analysis of the data. HH contributed to the interpretation of the data. All authors approved the final version before publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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