Preimplantation genetic diagnosis for chromosome rearrangements – one blastomere biopsy versus two blastomere biopsy

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Abstract

Purpose Preimplantation Genetic Diagnosis (PGD) has proven to be a useful reproductive option for carriers of some chromosome rearrangements. The data presented in this study compares the impact of one versus two blastomere biopsy on the likelihood of achieving a PGD result, as well as the effect on subsequent embryo development and clinical outcomes.

Methods IVF-PGD couples had either one or two blastomeres biopsied from all embryos with ≥ 7 blastomeres on day 3 post oocyte collection. These blastomeres were assessed for the specific chromosome rearrangement using Fluorescent In-situ Hybridisation (FISH). Further embryo development was monitored on days 4 and 5. Clinical outcomes were assessed retrospectively.

Results The data shows that statistically more embryos achieved a PGD result following two blastomere biopsy, compared with one blastomere biopsy (92 % versus 88 %, respectively). Furthermore it was found that embryo development and clinical outcomes were similar between the two biopsy groups.

Conclusions Based on this analysis it appears that the biopsy of two blastomeres from embryos with ≥ 7 blastomeres on day 3 is a valid and successful approach for couples presenting for IVF-PGD for a chromosome rearrangement.

Keywords In vitro Fertilisation (IVF) · Preimplantation Genetic Diagnosis (PGD) · Fluorescent In Situ Hybridisation (FISH) · Embryo biopsy · Translocation

Capsule The number of blastomeres biopsied from an embryo must be a careful balance between obtaining highly accuracy PGD results without compromising embryo quality and clinical outcomes.

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Introduction

Carriers of balanced chromosome rearrangements (e.g. translocations or inversions) are at increased risk of producing chromosomally unbalanced offspring or suffering recurrent pregnancy loss as a result of meiotic malsegregation of the chromosomes involved in the chromosome rearrangement. Preimplantation Genetic Diagnosis (PGD) is a reproductive option available to carriers of balanced chromosome rearrangements. PGD provides the opportunity to distinguish between normal/balanced versus unbalanced embryos prior to implantation, thereby increasing a couple's chance of a viable pregnancy.

In 2010, Harper et al [7] estimated that approximately 90 % of in vitro fertilisation (IVF) clinics perform embryo biopsy and PGD on day 3 of the embryos' development, when the embryo is typically composed of 6–8 blastomere cells. Despite this, there is currently no consensus between clinics as to how many blastomeres should be removed from the embryo for genetic analysis. Some clinics advocate the removal of a single blastomere [4,6], while others recommend that two blastomeres be biopsied in order to obtain a conclusive PGD result [1,9,13]. Given that embryo biopsy is an invasive procedure, it has long been recognised that this procedure could affect the subsequent growth and development of the embryo [3]. The European Society for Human Reproduction and Embryology (ESHRE) PGD consortium guidelines (2010) state that while the removal of more than one blastomere can have detrimental affects on clinical outcomes, the removal of two blastomeres may be required in some cases in order to improve the diagnostic accuracy of the PGD test. To help maintain both embryo viability and diagnostic accuracy, ESHRE recommend that two blastomeres only be taken from embryos that consist of six or more blastomeres on day 3 [8]. While the removal of multiple blastomeres is not thought to affect the processes of compaction and blastulation, it has been hypothesised that this process may interfere with

blastomere polarisation and the allocation of blastomeres to the trophectoderm and inner cell mass [13]. This raises the question, does more harm than good come from two blastomere biopsy compared to one blastomere biopsy? Any decision on this issue must be based on the need to obtain highly accurate and reliable PGD results without significantly compromising embryo quality and clinical outcomes.

Our clinic operates a large IVF program and offers PGD as a clinical procedure to both internal patients and patients cycling at referring IVF units throughout Australia and New Zealand. Here we present data from 170 IVF-PGD cycles for chromosome rearrangements that were performed at our clinic and referring clinics from June 2004 to September 2009. The first aim of this study was to investigate whether the number of blastomeres biopsied from a day 3 embryo influenced the percentage of embryos with a conclusive PGD result. The second aim was to determine whether there was any significant difference between one blastomere biopsy compared with two blastomere biopsy in relation to ongoing embryo development and patient clinical outcomes.

Materials and methods

Study design

This study involved an analysis of 898 embryos from 170 PGD cycles for 114 couples having PGD for a chromosome rearrangement at our clinic from June 2004 to September 2009. Between June 2004 and September 2008 all couples presenting for chromosome rearrangement testing had two blastomeres biopsied from their embryos, providing that the embryo had ≥ 7 blastomeres on day 3 post oocyte collection. In 2008, a preliminary analysis showed that 92 % of embryos that achieved a PGD result following two blastomere biopsy could have achieved a conclusive PGD result from the first biopsied blastomere alone. Based on these findings, and the assumption that the removal of only one blastomere would significantly improve clinical outcomes, single blastomere biopsy commenced from September 2008. For this study, only embryos ≥ 7 blastomeres on day 3 were included in the data sets (despite the fact that embryos ≥ 5 blastomeres were considered suitable for day 3 biopsy), as these embryos were of suitable size for either one or two blastomeres to be removed. This allowed for a more accurate comparison between the one and two blastomere biopsy groups.

PGD test development

Before commencing an IVF-PGD cycle, all couples underwent genetic counselling and test development to determine if PGD was possible for their particular chromosome rearrangement. A specific fluorescence *in-situ* hybridisation (FISH) test was

designed and developed for each couple using probes commercially available from Vysis Inc (Abbott Molecular, Illinois, America) and Poseidon (Kreatech, Amsterdam, The Netherlands). The FISH probes were specifically selected to enable all segregates of the chromosomal rearrangement to be distinguished.

FISH conditions were initially optimised on peripheral blood lymphocytes from each partner. The expected binding location of each probe was confirmed by metaphase analysis. A measurement of uncertainty was performed to ensure that the FISH probes were providing reliable results with a sensitivity of ideally ≥ 95 % [12]. The optimised test was validated on single blastomeres from embryos donated to research and training, once the embryos had been allowed to succumb. Once an accurate test had been developed, couples were able to commence an IVF-PGD cycle.

Ovarian stimulation and embryo development

Each couple underwent controlled ovarian hyperstimulation to generate multiple oocytes. Oocyte retrieval occurred 36–38 h post human chorionic gonadotrophin (hCG) administration (either 250 mg of recombinant [rhCG] Ovidrel (Merck Serono, Australia) or 10 000 IU of urinary [uhCG] Pregnyl (Organon, Australia)). The oocytes were inseminated using either standard IVF or intracytoplasmic sperm injection (ICSI). Oocytes destined for ICSI were assessed for maturity, with only those at metaphase II considered suitable for insemination. The success of insemination was assessed 18 h post insemination with normal fertilisation confirmed by the presence of two pronuclei. Between day 3 to day 5 post insemination, the embryo development was evaluated on a daily basis and the developmental stage recorded. Embryo development stages were recorded as ‘the number of blastomeres’, morula, early blastocyst, blastocyst, expanding blastocyst, hatching blastocyst or hatched blastocyst. For the purpose of this paper, these embryo development stages were grouped into three categories, namely (1) the number of blastomeres present for cleavage stage embryo, (2) morula, defined as the presence of compaction and (3) blastocyst, defined as the presence of a blastocoel cavity and inner cell mass.

Genetically and morphologically suitable embryos were transferred to the uterus on either day 4 (providing they had continued to develop post biopsy) or day 5 (providing they had developed to the compacting stage or beyond). Luteal support consisted of either Crinone (Merck Serono, Australia) or progesterone pessaries (Orion, Australia).

Embryo biopsy and PGD

Embryos were de-compacted in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free biopsy media (COOK Medical, Australia). A hole was drilled in the zona pellucida using a ZILOS laser (Hamilton Thorne

Research, Massachusetts, America) and one or two blastomeres gently aspirated for PGD analysis. The embryo was washed in blastocyst culture media (COOK Medical, Australia) and transferred into a fresh drop of media for ongoing culture.

Single blastomeres were fixed using Carnoy's fixative as per the method described by Velilla et al. [14]. Fixed slides were pre-treated (if required) using the protocol recommended by Vysis Inc. The relevant probe mix (1 μ l) was applied to the fixed blastomeres and a 10 mm round coverslip added and sealed with paraffin film. Slides were hybridised in a Hybrite (Vysis Inc) using the optimised FISH conditions. Post hybridisation washes were performed and 4.5 μ l of DAPI counterstain (Vysis Inc) added. Nuclei were analysed independently by two PGD scientists using an Olympus BX51 fluorescent microscope (Olympus Optical CO. LTD, Tokyo, Japan).

Embryos were considered normal/balanced for the autosomal chromosomes when two signals were present for each of the probes used. Embryos were considered normal/balanced for the sex chromosomes when two signals were present for X chromosome probes and no signals were present for Y chromosome probes, or when one signal was present for each of the X and Y chromosome probes. Extra or missing signals were considered to represent an unbalanced chromosome complement which renders the embryo not suitable for transfer. Two signals were considered to represent two homologue chromosomes when the signals were at least two domains apart, a domain being the diameter of one signal [10]. Signals were considered split if the signals were less than two domains apart and hence counted as one signal [10]. For embryos that underwent biopsy of two blastomeres, the embryo was considered mosaic if one blastomere was defined as normal/balanced and the other blastomere as unbalanced. The overall result for these embryos was recorded as unbalanced and not suitable for transfer. Two blastomeres were considered to be concordant if both blastomeres gave the same result of either normal/balanced or unbalanced.

Throughout the study period, all technical procedures involving embryo biopsy and FISH were rigorously monitored by quality control programs of International Organisation for Standardisation (ISO 9001:2000) and National Association of Testing Authorities, Australia (NATA). Other than the change in the number of blastomeres biopsied from an embryo, all aspects of embryo biopsy and FISH techniques remained unchanged during the study period.

Outcome measures

PGD results were measured in terms of 'diagnostic efficiency', which was defined as the number of embryos with a

result per number of embryos biopsied. This outcome measure was further assessed in terms of the percentage of embryos with a result from the first biopsied blastomere alone. For the one blastomere biopsy group this was defined as the number of embryos with a result per the number of embryos biopsied, while for the two blastomere biopsy group, this was defined as the number of embryos with a result from the first biopsied blastomere alone per the number of embryos biopsied. The two blastomere biopsy group was also assessed for concordance which was defined as the number of embryos that achieved the same result from both blastomeres per the number of embryos with a result from two blastomeres.

Embryos were grouped according to the expected developmental milestones on days 3, 4 and 5. The developmental milestones were as follows: day 3 (number of blastomeres), day 4 (morula) and day 5 (any of the blastocyst stages). The number of embryos at each developmental stage on days 3, 4 and 5 was compared between the one and two blastomere biopsy groups.

Clinical outcomes were measured as 'implantation rate' (number of fetal heart beats/total number of embryos transferred), 'pregnancy rate per PGD cycle' (number of viable clinical pregnancies/number of initiated cycles), 'pregnancy rate per transfer' (number of viable clinical pregnancies/number of embryo transfers), 'miscarriage rate' (number of fetal heart beats lost/number of fetal heart beats), 'live birth rate' (number of delivery events/number of embryo transfer procedures).

Clinical pregnancies were defined as the presence on ultrasound of a viable gestational sac (with fetal heart beat) at 6–7 weeks gestation.

Statistical analysis

χ^2 , Fisher's exact and t-tests were used for statistical comparisons between the one and two blastomere biopsy groups. A p-value ≤ 0.05 was considered statistically significant.

Results

Patient characteristics and clinical outcomes

The patient characteristics for each group are outlined in Table 1. There was no significant difference between the one blastomere and the two blastomere biopsy groups in terms of the number of PGD cycles assessed, number of couples, mean maternal age, mean number of IVF cycles with oocyte retrieval, mean number of PGD cycles and the total number of embryos biopsied. The one blastomere biopsy group had significantly more embryos biopsied per PGD cycle when

Table 1 Patient characteristics and clinical outcomes

	Number of blastomeres biopsied		p- value
	One blastomere	Two blastomeres	
Number of PGD cycles assessed (number of couples)	64 (48)	106 (66)	
Mean maternal age	34.33	33.96	
Mean number of IVF cycles with oocyte retrieval	4.67	3.25	
Mean number of PGD cycles	1.36	1.61	
Number of embryos biopsied (mean per PGD cycle)	400 (6.25)	498 (4.70)	(0.0027)
Number of normal/balanced embryos (%)	110 (28)	109 (22)	
Number of embryos suitable for transfer (%) ^a	95 (86)	100 (92)	
Number of PGD cycles with transfer (%)	43 (67.2)	60 (56.6)	
Mean number of normal/balanced embryos available per transfer	2.2	1.66	0.0001
Mean number of normal/balanced embryos transferred per cycle	1.5	0.9	0.0001
Implantation rate (%)	11 (21.2)	20 (25)	
Pregnancy rate per PGD cycle (%)	11 (17.2)	20 (18.9)	
Pregnancy rate per transfer (%)	11 (25.6)	20 (33.3)	
Miscarriage rate (%)	1 (9.1)	2 (10)	
Live birth rate (%)	10 (23.3)	16 (26.6)	

^aSuitability for transfer was based on an assessment of embryo morphology in conjunction with a genetic diagnosis of normal/balanced

compared to the two blastomere biopsy group (6.25 versus 4.7 respectively, $p=0.0027$).

Diagnostic efficiency and PGD results

Between June 2004 and September 2009, 898 day 3 embryos ≥ 7 blastomeres in size were biopsied. One blastomere was biopsied from 400/898 (45 %) embryos and two blastomeres were biopsied from the remaining 498/898 (55 %) embryos. Over the study period, testing was performed for a total of 71 different chromosome rearrangements (64 reciprocal translocations, 5 Robertsonian translocations and 2 pericentric inversions). Overall, significantly more embryos in the two blastomere biopsy group achieved a PGD result compared with the one blastomere biopsy group (92 % versus 88 % respectively, $p=0.0269$) (Fig. 1). However, when assessing the first biopsied blastomere alone (i.e. comparing the one blastomere biopsy group with the first biopsied blastomere from the two

blastomere biopsy group) there was no significant difference between the two biopsy groups in terms of the percentage of embryos with a conclusive result (88 % one blastomere biopsy versus 85 % two blastomere biopsy) (Fig. 1).

Analysis of the two blastomere biopsy group revealed that 378/498 (76 %) of embryos obtained a result from both biopsied blastomeres, with 321/378 (85 %) of these embryos achieving a concordant result from both blastomeres (i.e. both blastomeres were normal/balanced or both blastomeres were unbalanced). A non-concordant result was obtained for the remaining 57/378 (15 %) of embryos.

To further assess the level of concordance we have performed a small reanalysis of embryos that underwent PGD for a chromosome rearrangement. The reanalysis involved ten embryos that were originally diagnosed as unbalanced following PGD. Upon reanalysis of these embryos, nine embryos were confirmed to be unbalanced for the chromosome rearrangement. One embryo was classified as normal/balanced following reanalysis. This particular embryo originally had two blastomeres biopsied, one of which gave a normal/balanced result and the other an unbalanced result. Due to the presence of the unbalanced blastomere this embryo was classified as unbalanced and not suitable for transfer.

Embryo development

There was no significant difference in embryo development between the one blastomere and two blastomere biopsy groups on days 3 and 4. There was also no difference between the groups on day 5 in terms of the number of embryos reaching the blastocyst stage (Table 2). Further

Fig. 1 Comparison of the percentage of embryos (≥ 7 blastomeres on day 3) that achieved a PGD result for one blastomere versus two blastomere biopsy groups

Table 2 Comparisons between the one blastomere and two blastomere biopsy groups for the number of embryos at the blastocyst stage on day 5

	One blastomere	Two blastomeres
Number of embryos on day 5	213	225
Number of embryos at the blastocyst stage (%)	96 (45)	91 (40)
Number of normal/balanced embryos (%)	54 (56)	47 (52)
Number of unbalanced embryos (%)	42 (44)	44 (48)

assessment also showed that there was no significant difference between the one blastomere and two blastomere biopsy groups in terms of the number of normal/balanced or the number of unbalanced embryos at the blastocyst stage on day 5 (Table 2).

Clinical outcomes

Clinical outcomes are summarised in Table 1. There was no significant difference between the two biopsy groups in terms of number of normal/balanced embryos, number of normal/balanced embryos suitable for transfer (based on embryo morphology) or number of cycles with a transfer. Transfer of morphologically and genetically suitable embryos occurred on day 4 for 15 (25%) patients from the two blastomere biopsy group while all remaining transfers were performed on day 5. The one blastomere biopsy group had a significantly higher number of normal/balanced embryos available per transfer compared to the two blastomere biopsy group (2.2 versus 1.66 respectively, $p=0.0001$). Subsequently, the one blastomere biopsy group had a significantly higher number of normal/balanced embryos transferred per cycle compared to the two blastomere biopsy group (1.5 versus 0.9 respectively, $p=0.0001$). Despite this, there was no significant difference in implantation rate, pregnancy rate, miscarriage rate or live birth rate between the two biopsy groups.

Discussion

Since the introduction of embryo biopsy and PGD, the methodology used in the laboratory has been closely monitored to ensure an ongoing quality of service. The number of blastomeres biopsied from a day 3 embryo requires careful consideration and must be a compromise between achieving high diagnostic efficiency while minimising any detrimental affects on subsequent embryo development and clinical outcomes. The data presented in this paper indicates that the number of blastomeres biopsied from a day 3 embryo is associated with the percentage of embryos with

a conclusive PGD result. Significantly more embryos achieved a conclusive PGD result following two blastomere biopsy compared to one blastomere biopsy (92 % and 88 % respectively). In regards to the two blastomere biopsy group, some instances arose where a second blastomere was required to clarify a difficult analysis from the first biopsied blastomere. In other instances the availability of a second blastomere may have allowed for the detection of mosaicism which may in turn have prevented an embryo with a mosaic unbalanced chromosome complement from being transferred.

A study published by Michiels et al. [9] also compared the biopsy of one versus two blastomeres and explored the accuracy and reliability of PGD results. They similarly found that significantly more embryos achieved a PGD result following two blastomere biopsy when compared to embryos that had one blastomere biopsied. They did not however, assess ongoing embryo development following biopsy. Furthermore, there was a large difference in size between the one blastomere biopsy group which had only 118 (6.3 %) embryos biopsied compared to the two blastomere biopsy group which had 1711 (90.6 %) embryos biopsied. An additional 59 embryos (3.1 %) had three blastomeres biopsied. This was in contrast to the current study, where the one blastomere biopsy group had 400 (44.5 %) embryos and the two blastomere biopsy group had 498 (55.5 %) embryos. Another difference between our study and that of Michiels et al. [9] is that their study included data from day 3 embryos that varied in size from five blastomeres up to compacting. They biopsied one blastomere if the embryo contained five blastomeres on day 3, or two blastomeres if the embryo contained ≥ 6 blastomeres on day 3. Our study limited the data set to the assessment of embryos ≥ 7 blastomeres in size on day 3, which allowed us to evaluate ongoing embryo development and clinical outcomes without the one blastomere biopsy group being skewed by the inclusion of day 3 embryos that were deemed to be of poorer quality.

The two blastomere biopsy group was further analysed in relation to concordance of PGD results between blastomeres. This analysis showed that 85 % of embryos had a concordant result from both biopsied blastomeres, while the remaining 15 % of embryos tested had a non-concordant result (i.e. one blastomere was normal/balanced and the other blastomere was unbalanced). One possible explanation for a non-concordant FISH results is embryo mosaicism which has been reported by some groups to be as high as 40–60 % [8]. Other factors that may contribute to non-concordant results include technical errors, which may result from poor blastomere quality, FISH probe error and/or incorrect FISH interpretation (due to overlapping signals, split signals, signal bleed through and the presence of auto-fluorescent debris on the slide) [10]. While FISH

interpretation can be subjective and scoring differences can occur, the implementation of rigorous FISH training programs and the adherence to specific FISH scoring criteria are used to mitigate these effects. Furthermore, the possibility of FISH probe error should be minimised during the test development process, given that a measurement of uncertainty is performed to confirm low probe error rates (i.e. $\leq 5\%$) prior to the test being offered clinically. Given that all possible measures had been taken to minimise technical errors, one could assume that a significant proportion of non-concordant results are in fact due to mosaicism. Our results indicate that the biopsy of two blastomeres increases the ability to detect mosaicism and aids in avoiding the transfer of a mosaic unbalanced embryo.

It was interesting to note that the one blastomere biopsy group had a significantly higher mean number of embryos available per transfer than the two blastomere biopsy group (2.2 versus 1.66 respectively). While this may in part be due to the fact that the one blastomere biopsy group had a significantly higher number of embryos biopsied per PGD cycle, it is also possible that the one blastomere biopsy group contained embryos which would have been identified as mosaic unbalanced if two blastomeres had been biopsied. Sandalinas et al. [11] and Emiliani et al. [5] reported that mosaic embryos are capable of developing to the blastocyst stage. Based on this, it could be hypothesised that a mosaic unbalanced embryo incorrectly diagnosed as “normal/balanced” following one blastomere biopsy has the potential to reach the blastocyst stage and be transferred. Depending on the chromosomal imbalance involved and the proportion of unbalanced blastomeres, this may result in implantation failure, miscarriage, or the birth of a chromosomally abnormal child. This could help explain why the one blastomere biopsy group did not have a higher pregnancy rate than the two blastomere biopsy group, despite having more embryos available for transfer.

The current study has shown that there is no significant difference in embryo development on days 3, 4 and 5 between the one blastomere and two blastomere biopsy groups. Furthermore, our data indicates that there is no significant difference in clinical outcomes following the removal of one versus two blastomeres. While these findings support previous data reported by Van de Velde et al. [13] and Michiels et al. [9], they are in contrast to studies performed by other groups [4,6]. The laboratory of Goossens et al. [6] and De Vos et al. [4] reported that the removal of more than one blastomere from a day 3 embryo has a detrimental affect on developmental potential and day 5 embryo quality, and subsequently on clinical outcomes. This difference in findings may be attributed to different biopsy criteria being used by different laboratories. In this study, we only considered embryos ≥ 7 blastomeres in size suitable for two blastomere biopsy. This contrasts to Goossens et al. [6] and De Vos et al. [4] who biopsied one or two

blastomeres from embryos ≥ 6 blastomeres in size. It is possible that embryos with fewer blastomeres on day 3 respond more poorly to the biopsy of two blastomeres than embryos of greater size, presumably due to a larger percentage of their cellular mass being removed. It is also interesting to note that De Vos et al. [4] included embryos consisting of 5 blastomeres in their one blastomere biopsy data set, despite the fact that these embryos were not considered suitable for two blastomere biopsy and were therefore not included in the two blastomere biopsy group. By only including these poorer quality embryos in the one blastomere biopsy group and not the two blastomere biopsy group, the data becomes biased and it is not possible to perform an appropriate comparison between the two groups. By applying a day 3 embryo size restriction (embryos ≥ 7 blastomeres in size) to our data we have been able to make a true comparison between the two data sets.

Another area of difference between the current study and the studies performed by Goossens et al. [6] and De Vos et al. [4] was the embryo culture media that was used. We have previously shown that the type of embryo culture media used in the laboratory can have a significant impact on embryo development and clinical outcomes [2]. Furthermore, our previous data has demonstrated that biopsied embryos are more susceptible to suboptimal culture conditions than non biopsied embryos [2]. It is possible that subtle variations in culture media between laboratories could therefore impact on the embryo's ability to withstand the biopsy of two blastomeres. Based on this, it is advisable that each laboratory considering two blastomere biopsy validate the process in house to ensure that their culture system is capable of supporting two blastomere biopsy. Our laboratory has seen very little difference in embryo development and clinical outcomes between the two biopsy groups which suggests that our culture system has been optimised to the level required for either one or two blastomere biopsy.

To conclude, the results of the current study indicate that a conclusive PGD result is more likely to be achieved following two blastomere biopsy compared with one blastomere biopsy. Furthermore, the biopsy of two blastomeres allows for increased confidence in the PGD result and an increased ability to detect mosaicism. Given that the removal of two blastomeres does not affect embryo development or patient clinical outcomes, it appears that two blastomere biopsy is a valid and successful approach for couples with a chromosome rearrangement presenting for IVF-PGD.

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