

Article

Obtaining metaphase spreads from single blastomeres for PGD of chromosomal rearrangements



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Abstract

It has previously been shown that it is possible to obtain metaphase chromosomes from single blastomeres converted into metaphase in the cytoplasm of a mouse zygote. This method is highly labour intensive and cannot be performed outside the preimplantation genetic diagnosis (PGD) laboratory, so to overcome these limitations, a method was developed for obtaining metaphase spreads from single biopsied blastomeres using different chemicals. The substances tested were calyculin A, caffeine, paclitaxel and colcemid in a total of 496 disaggregated and 234 biopsied blastomeres from day 3 embryos. It was demonstrated that the optimal method involved a combined use of 'selective biopsy' (selection of the biopsied blastomere according to morphological criteria) and exposure to caffeine. This resulted in shortening the mean incubation time of biopsied blastomeres, with a metaphase formation rate of 80%. The method is simple for obtaining metaphases from single blastomeres, and may be implemented in clinical practice of PGD for structural rearrangements.

Keywords: blastomeres, caffeine, chromosomal analysis, chromosomal rearrangements, metaphase spreads, preimplantation genetic diagnosis

Introduction

Initially, preimplantation genetic diagnosis (PGD) for chromosomal rearrangements was performed using fluorescence in-situ hybridization (FISH) in interphase nuclei from individual blastomeres (Munné *et al.*, 1998; Pierce *et al.*, 1998). It has then become possible to apply FISH to metaphase chromosomes from single blastomeres converted into metaphase in the cytoplasm of a mouse zygote (Verlinsky and Evsikov, 1999a). In contrast to interphase FISH analysis, this technique made it possible to distinguish between embryos with balanced and normal chromosomal complements.

Although the efficacy of this 'conversion' method was over 90%, there may be some controversy regarding the use of mouse cytoplasm for converting the human blastomere nucleus into metaphase. In addition, it is not at all applicable for PGD

of translocations performed outside the PGD laboratory, and it is also expensive and highly labour intensive.

To overcome the above limitations, a method was developed for obtaining metaphase spreads from single biopsied blastomeres using different chemicals, which allows reliable testing for chromosomal rearrangements. This method is presented in this paper.

Materials and methods

The work was performed on 496 single blastomeres obtained from 72 disaggregated day 3 embryos donated for research, of which 234 were biopsied for PGD purposes as described

elsewhere (Verlinsky and Evsikov, 1999a). The embryos were disaggregated into single blastomeres in Quinn's Ca²⁺- and Mg²⁺-free medium with 5% Plasmanate (Bayer, USA), following the removal of zona pellucida by pronase (Sigma, UK) at a concentration of 2 mg/ml. Only blastomeres with a single nucleus were selected and cultured in a drop of 50 µl Global (LifeGlobal, USA) medium, with 10% Plasmanate, containing one of the following four components: calyculin A (Sigma) in concentrations of 5, 30 and 80 ng/ml; caffeine (Sigma) 1, 3 and 6 mmol/l, paclitaxel (Sigma) 125, 250 and 500 ng/ml, and colcemid (Sigma) 0.05–1 µg/ml. The blastomeres were observed for nucleus disappearance every hour, and after hypotonic treatment (0.7% citrate Na, 1 mg/ml BSA, 2–3 min), they were transferred onto a glass slide and fixed in 3:1 methanol/glacial acetic acid. FISH analysis was performed after metaphase chromosomes became completely devoid of cytoplasm, with special care taken not to 'overspread'.

For the day 3 blastomere biopsy, the larger blastomeres with no visible nucleus but clear 'transparent' cytoplasm were selected, and were fixed either directly or after a brief incubation in medium containing colcemid. Otherwise, the largest blastomeres with a single nucleus were removed and incubated individually under oil for 8–15 h, until nucleus envelope breakdown and clarification of cytoplasm in Global medium containing 0.05–0.1 µg/ml colcemid, observed every 60 min for interphase–metaphase transition.

The choice of the above agents was based on their known effect on the cell cycle. Calyculin A is an inhibitor of protein phosphatases type 1 and type 2A, increasing 10-fold cdc2/H1 kinase activity and facilitating premature chromosome condensation (PCC) (Yamashita *et al.*, 1990). The effect is similar to okadaic acid, known to overcome the S-phase checkpoint, shorten G₂ phase, and cause premature chromosome condensation in 1-cell mouse embryos (Dyban *et al.*, 1993; Ghosh *et al.*, 1996). Okadaic acid has previously been used for obtaining metaphases from human blastomeres and polar bodies (Verlinsky and Evsikov, 1999a,b; Hlinka *et al.*, 2001). Calyculin A was also shown to significantly raise mitotic index at a concentration of 50 nmol/l, without affecting the quality of resulting metaphases after incubation for 30 min (Kowalska *et al.*, 2003).

Caffeine is also similar in action to okadaic acid, preventing mitosis in cells with incompletely replicated or damaged DNA. By changing the cell cycle controlling mechanism, caffeine induces chromosome condensation of those cells that have not completed DNA duplication. At a concentration of 5 mmol/l and higher, caffeine may induce fragmentation of chromosomes and detachment of the centromere–kinetochore region, also affecting chromosomal morphology (Ouspenski *et al.*, 1993).

The use of paclitaxel is based on its ability to suppress spindle microtubule dynamics and block blastomeres at metaphase–anaphase transition. Paclitaxel is currently used for cancer treatment, to block cell division in many types of tumours. Although the precise mechanism of its action is not understood, significant accumulation of mitotic cells may be due to a suppressive action on the centromere dynamics (Kelling *et al.*, 2003).

Finally, colcemid or demecolcine is a very well known tubulin

inhibitor widely used for accumulation of cells in mitosis. Treatment of HeLa cells with colcemid at concentrations of 0.06–0.10 µg/ml leads to an irreversible arrest in mitosis (McGill and Brinkley, 1975). Colcemid is commonly used at a concentration of 1 µg/ml for 20 min or for as long as 2 h, while longer exposure requires a lower concentration of 0.1 µg/ml (Czepulkowski, 2001).

The original method tested in this work included a selective biopsy followed by incubation of single blastomeres in the medium supplemented with the above reagents, to accelerate the onset of mitosis, causing premature chromosome condensation, and retaining chromosomes in metaphase for extended periods.

Results

The lowest efficiency in obtaining metaphases was observed for calyculin A (5 ng/ml), resulting in conversion of only 16 (17%) of 94 disaggregated blastomeres into well-spread metaphase chromosomes. This figure excludes 37 (39%) of metaphases of poor quality, representing either chaotically condensed or overcondensed chromatin, unsuitable for chromosomal analysis. Higher concentrations of calyculin A of 30 ng/ml and above appeared to cause visible changes and damage to blastomeres following incubation for over 30 min.

The overall success rate with paclitaxel was not sufficiently high either, with only nine (36%) of 25 analysable metaphases obtained, using 125 ng/ml concentration. While doubling the concentration improved the efficiency, a further increase in concentration (500 ng/ml) resulted in a detrimental effect, shown by the arrested blastomeres with formation of multiple micronuclei.

Twenty-hour incubation of disaggregated blastomeres in medium with several colcemid concentrations revealed the lowest concentration (0.05 µg/ml) for accumulating metaphases for a prolonged period, resulted in the highest harvest of 74.8% (86 of 115) metaphase chromosomes (**Figure 1**).

As seen from **Table 1**, culturing of disaggregated blastomeres with and without caffeine (1 mmol/l), combined with colcemid, did not result in significant differences in the production of metaphase chromosomes (91.3 and 83.7%, respectively), but caffeine significantly accelerated the onset of mitosis, shortening the average incubation time to 9.8 h compared with 13.1 h without caffeine ($P < 0.05$).

The efficiency of caffeine exposure was further improved by selective biopsy of appropriate blastomeres based on the above morphological criteria (**Figure 2**). The data show that the optimal method of obtaining metaphases from single biopsied blastomeres involved selective biopsy, followed by incubation in caffeine and colcemid. **Table 2** summarizes the clinical experience of the application of this method in clinical cycles, involving the conversion of 35 biopsied blastomeres, which resulted in 80% metaphase success rate following a mean incubation time of 3.6 h per metaphase. As seen from **Figure 3**, the metaphases harvested by this method may be analysed by FISH.

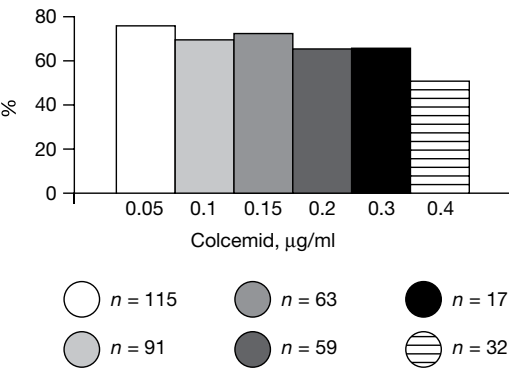


Figure 1. Conversion rate in blastomeres after 20 h incubation with colcemid. The figure shows metaphase formation rate in disaggregated blastomeres during 20 h incubation in the presence of various colcemid concentrations. Difference between the groups with 0.05 µg/ml and 0.4 µg/ml is statistically significant by Fisher's Exact test ($P < 0.05$).

Table 1. Acceleration of metaphase onset in blastomeres by caffeine.

Group	No. blastomeres	No. of metaphase harvest (%)	Mean incubation time \pm SD (hours)
No caffeine	43	36 (83.7)	13.1 \pm 7.1 ^a
With caffeine	46	42 (91.3)	9.8 \pm 6.7 ^b

^{a,b} $P < 0.05$ (Fisher's Exact test)

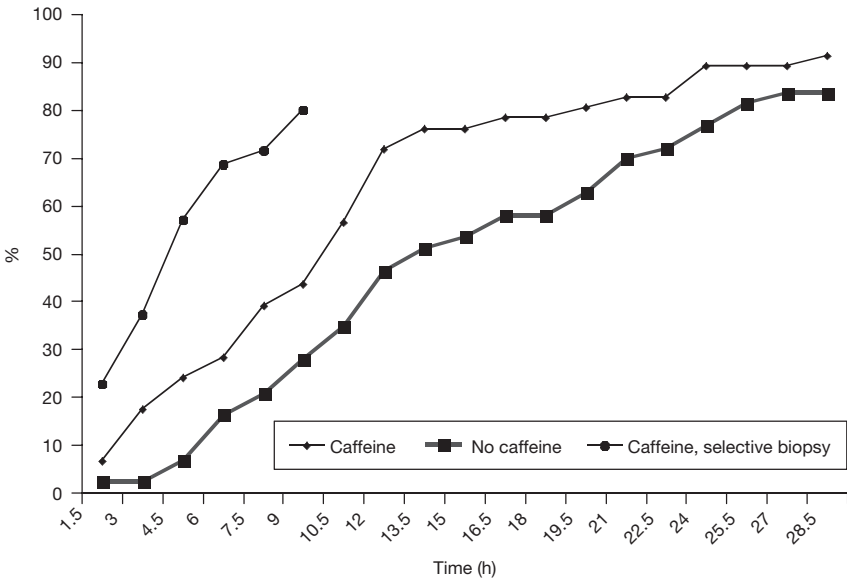


Figure 2. Dynamics of metaphase production by: caffeine, disaggregated blastomeres; no caffeine, disaggregated blastomeres; and caffeine, selective biopsy. The figure shows the percentages of metaphases generated over time using the different methods.

Table 2. Outcome of combined use of selective biopsy and caffeine.

<i>No. of embryos biopsied</i>	<i>No. in metaphase (%)</i>	<i>No. in interphase (%)</i>	<i>Mean time in hours of incubation per metaphase \pm SD (range)</i>
35	28 (80)	7(20)	3.6 ± 2.5 (0–8)

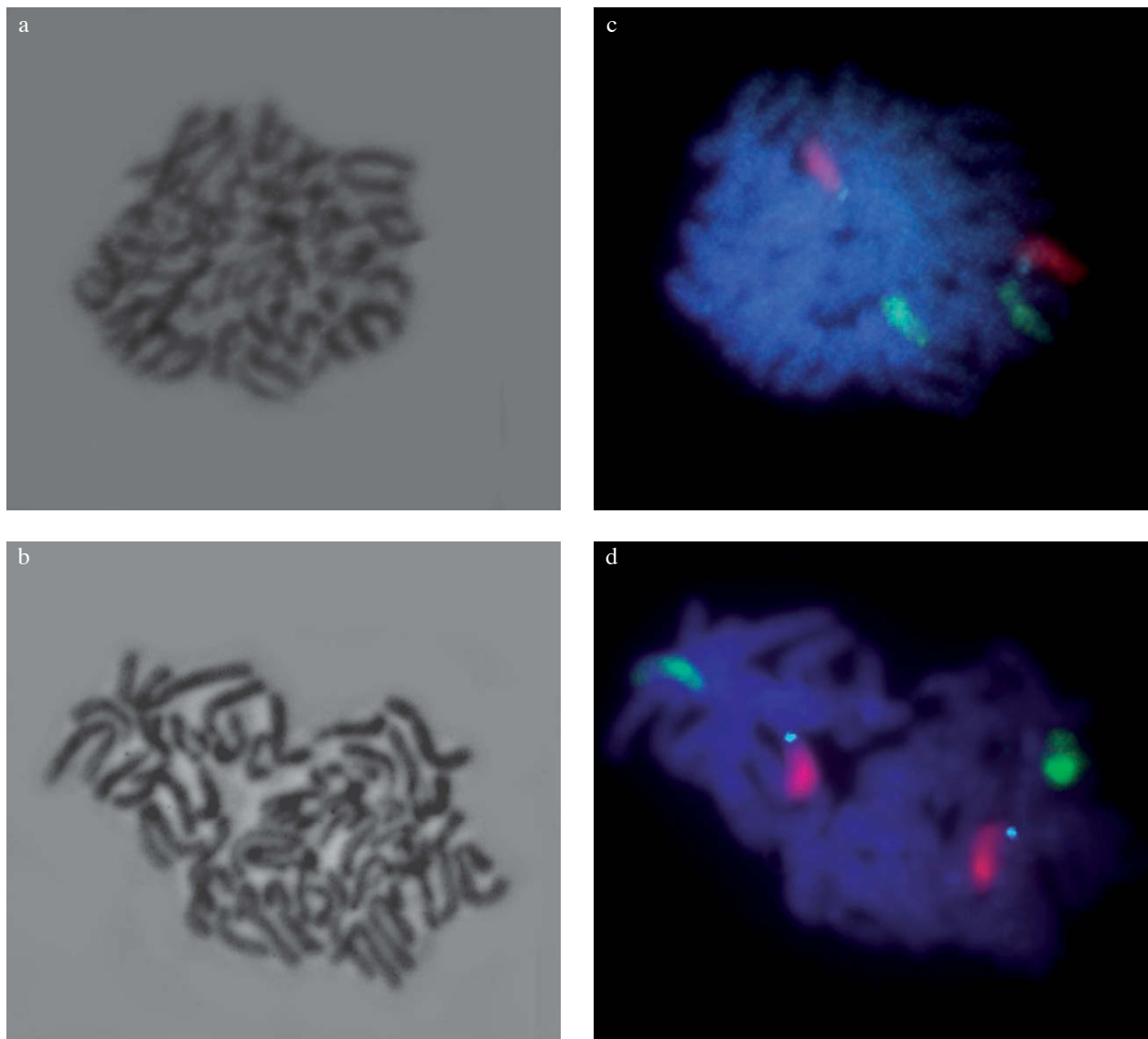


Figure 3. Two metaphases are shown, on the left under phase contrast (a and b) and on the right the same metaphases after fluorescence in-situ hybridization (FISH) (c and d). The metaphase chromosomes were obtained after incubation with caffeine. FISH probes applied: whole chromosome painting (WCP) 13 – green, WCP 15 – red, centromere specific (CEP) 15 – aqua.

Discussion

The data show that metaphase chromosomes may be reliably obtained from single biopsied blastomeres. First, the blastomere in mitosis may be selected and fixed after nuclear envelope breakdown. Alternatively, any blastomere can be removed, allowing it to enter mitosis under the influence of chemical factors promoting formation of metaphase. Clearly, combining these methods should be the most successful approach.

Tanaka *et al.* (2004) proposed that a 4- to 6-cell embryo be subjected to selective biopsy on day 2, to remove a blastomere already entering mitosis. As mitosis may last up to one hour in blastomeres from 4- to 8-cell human embryos, there was enough time to biopsy the selected cell. However, it requires a long time to check each embryo to identify the blastomere that may be close to division. Despite the fact that 4- to 6-cell embryos have higher mitotic potential compared with more advanced stages, the observation time may still require as long as 16 h, making the method extremely labour intensive for clinical application. It should also be noted that the removal of a blastomere from 4- to 6-cell embryos might affect the developmental potential of the biopsied embryo.

The data confirm that 'selection' of appropriate blastomeres should be an important part of the working algorithm. It is of note that at least one blastomere of 8-cell embryos may be readily undergoing mitosis at the time of embryo biopsy. However, the data show that not more than 20% of day 3 embryos have a cell ready to divide, so chemical exposure might be required to obtain metaphase in the majority of the embryos. Of course, the biopsy of blastomeres with multinucleation should be avoided, as this may indicate possible chromosomal errors in the cell (Hardy *et al.*, 1993; Pickering *et al.*, 1995). One of the morphological parameters for selection is the size of the blastomeres, an important indicator of a cell that is approaching mitosis. One of the distinct features of a mammalian cell approaching metaphase is also the configuration of nucleoli in the cell nucleus, specifically the number and the size of nucleoli (Anastassova-Kristeva, 1977; Jordan and McGovern, 1981). In the beginning of a new cell cycle (G1) nucleoli are smaller, with their number exceeding 5–8. They then fuse into 1–3 enlarged nucleoli during interphase, and only one or two in G2. The preliminary data in this study suggest that the largest blastomeres with 1–2 large nucleoli tend to enter mitosis earlier than the others. Incubation of the selected blastomeres for 11–15 h in medium containing a low colcemid dose of <0.1 µg/ml may ensure a high metaphase formation rate.

Alternatively, the most optimal strategy was shown to be blastomere culture in medium containing caffeine and colcemid, which significantly reduced the incubation time (from 13.1 to 9.8 h, $P < 0.05$) even when no appropriate blastomere could be found for preselection and biopsy. The rate of metaphase formation increased after incubation with caffeine, which also reduced the time required for blastomere culture after biopsy, increasing the percentage of blastomeres entering metaphase. The morphology of chromosomes obtained did not differ from those obtained with other methods.

In conclusion, it has been demonstrated that the optimal method involved the combined use of selective biopsy with exposure to caffeine. This resulted in a high metaphase formation rate of 80% and shortening of the average incubation time. The method is quite simple for practical application.

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