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# Chromosome studies in first polar bodies from hamster and human oocytes

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**Most studies on preconception diagnosis published so far have used polymerase chain reaction (PCR) analysis to identify single gene defects. Although fluorescent DNA probes have been used to obtain a partial cytogenetic diagnosis of aneuploidies in first polar bodies without defined chromosome structures, the analysis of structural chromosome anomalies in the interphase nucleus is not adequate. We describe a procedure to obtain first polar body chromosome complements from hamster and human oocytes. In 63.6% (105 of 165) of hamster first polar bodies the chromosome complement showed a defined chromosome morphology and in 94.1% (16 of 17) of human oocytes fixed after follicular puncture it was possible to obtain high quality, well spread chromosome complements. First polar body chromosomes are fuzzy and shorter than oocyte chromosomes, but fluorescent in-situ hybridization results obtained in human first polar bodies clearly show that it is possible to detect whole chromosomes, centromeres and unique sequences, including the terminal regions of small chromosomes. This suggests that in fresh oocytes, DNA loss resulting from apoptotic chromosome fragmentation has not yet occurred. Using the procedure described, first polar bodies could be used to analyse the meiotic segregation of maternal structural abnormalities and to detect numerical chromosome anomalies in humans.**

*Key words:* aneuploidies/first polar body/hamster/human/preconception diagnosis/structural chromosome abnormalities

## Introduction

After the routine clinical application of prenatal diagnosis, preimplantation diagnostic techniques were developed to provide an even earlier warning to couples with a high risk of conceiving an affected child. This type of diagnosis allows the detection of some genetic or chromosome defects at the 6–8-cell stage, and enables only normal embryos to be transferred. Preimplantation diagnosis has been used in X-linked or single gene diseases (for a review see Harper, 1996) and to detect numerical chromosome abnormalities (Munné *et al*., 1993).

The next approach was the diagnosis of genetic anomalies in germ cells, before fertilization. The aneuploidy rate in males has recently been estimated by fluorescent in-situ hybridization (FISH) studies of decondensed sperm heads (Martinez-Pasarell *et al*., 1997). In females, the particular characteristics of gametogenesis allow the indirect characterization of the chromosome constitution of the gamete through the study of the first polar body (1PB) and this presents an opportunity for germ line analysis. The 1PB contains a chromosome set complementary to that of the oocyte. Thus, the 1PB can be analysed to obtain information on the chromosomal constitution of the corresponding oocyte, which is maintained in culture for insemination.

Although several studies on human 1PBs have been published, they have been limited to the use of polymerase chain reaction (PCR) to identify single gene defects (Verlinsky *et al*., 1990) and, more recently, Munné *et al*. (1995) and Verlinsky *et al*. (1995, 1996) have used centromeric DNA probes for partial preconception analysis of aneuploidy using FISH. The major problem with the use of 1PBs for cytogenetic evaluation of the oocyte is the difficulty in visualizing the 1PB chromosome complement (Verlinsky *et al*., 1996). 1PB chromosome complements with a good chromosome morphology have only been observed in some cases where the 1PB was fixed by chance with the oocyte (in the mouse: Dyvan *et al*., 1993; in humans: Angell *et al*., 1994; Benkhalifa *et al*., 1996; Dailey *et al*., 1996; Wall *et al*., 1996; Wójcik *et al*., 1996). In the present paper, we describe a procedure to obtain 1PB chromosome complements with defined chromosome morphology from hamster oocytes and we present our preliminary results using human 1PBs.

## Materials and methods

### *Source of hamster and human oocytes*

Ovulation was induced in adult female Syrian hamsters by i.p. injection of 30–40 IU of pregnant mare's serum gonadotrophin (PMSG; Sigma, St Louis, MO, USA) in the morning on day 1 of the oestrous cycle and 30–40 IU of human chorionic gonadotrophin (HCG; Lavet, Madrid, Spain) 16–17 h prior to killing them on day 3 of the cycle. Oocytes were obtained by flushing the dissected oviducts, and cumulus masses were transferred to drops of hyaluronidase 0.1% in Biggers–Whitten–Whittingham medium (BWW) (Sigma) until the oocytes dissociated. Immediately after, they were rinsed in BWW and maintained in an incubator at 37°C, 5% $CO_2$ and 95% humidity. The whole procedure was carried out under subdued light in $<$30 min.

Human samples were donated by patients undergoing in-vitro fertilization (IVF) cycles or gynaecological operations for different conditions. The Informed Consent Form and the protocol for the

study were approved by the Ethics Committee of the Universitat Autònoma de Barcelona. The oocytes were predominantly donated oocytes processed immediately after puncture.

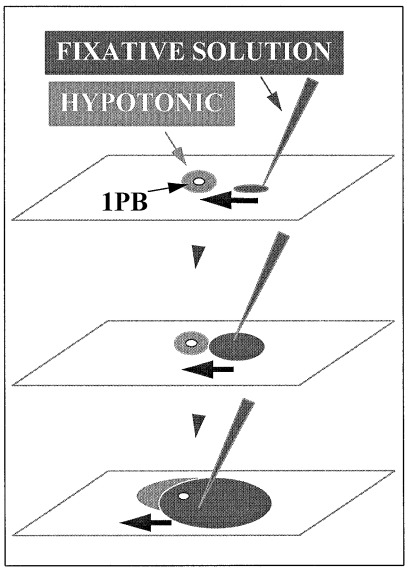


**Figure 1.** Graphic representation of the way in which the fixative solution should be applied. The fixative should be gently placed next to the hypotonic drop containing the first polar body (1PB), producing a one-way flux (large arrow) onto the slide. The fixative solution must be added under stereomicroscopic control.

**Figure 2.** Complete hamster first polar body complement from a mature oocyte showing 22 chromosomes with a well defined chromosome morphology.

**Table I.** Characteristics of the first polar body (1PB) chromosome complements from hamster and human oocytes

| | Hamster 1PB (%) | Human 1PB (%) |
|---|---|---|
| Defined chromosome morphology | 105 (63.6) | 16 (94.1) |
| Undefined chromosome morphology | 60 (36.4) | 1 (5.9) |
| Total | 165 (100) | 17 (100) |

### *Isolation of hamster and human 1PBs*

Hamster and human 1PBs were isolated enzymatically or mechanically as follows. (i) In the enzymatic procedure, trypsin was used at a concentration of 1 mg/ml BWW (Sigma) for hamster oocytes and of 30 mg/ml PBS (Difco, Detroit, MI, USA) for human oocytes, for 1–3 min under a stereomicroscope. When the zona began to disappear, the oocyte was rinsed in BWW and the 1PB was carefully separated from the oocyte. (ii) The mechanical procedure was carried out with a micromanipulator (Narishige, Tokyo, Japan); 1PBs from hamster oocytes were recovered after a local digestion of the zona with acid Tyrode (pH 2) and aspirated with a glass microneedle 20 µm in diameter. For human 1PBs the procedure used was the one previously described by Verlinsky *et al*. (1990).

### *1PB chromosome complement fixation*

In all cases the free 1PB was gently placed into a tiny well containing hypotonic solution (1% trisodium citrate). The hypotonic treatment was controlled under a stereomicroscope until the 1PB lost brightness (5–10 min). It was then gently placed onto a clean slide previously covered with a 1% trimethoxysilane film (Fluka, Buchs, Switzerland) (Ward *et al*., 1995). Using a micropipette, a 20 µl drop of ethanol:acetic acid (1:1) was gently added onto the slide beside the 1PB, for a gentle fixation. When the fixative solution covered the 1PB, a second drop was added and, if needed, one or two more drops (Figure 1). Finally the slides were air-dried and observed under a phase contrast microscope (×40).

### *Chromosome analysis*

Hamster 1PB complements were solid-stained with conventional Leishman's stain.The slides were observed and photographed with a Leitz Dialux 22 3-lambda Ploemopak photomicroscope (Leica, Madrid, Spain) using Agfa Copex Rapid AHU (50 ASA) film. Eventually, phase contrast microscopy was performed with an Olympus PROVIS-AX70 microscope and metaphases were captured and analysed using a Cytovision Ultra workstation (Applied Imaging, Sunderland, UK).

Human 1PB complements were first photographed under phase contrast and then analysed by FISH. We used directly labelled fluorescent commercial probes (Vysis, NewYork, NY, USA): whole chromosome 11 and whole chromosome 22 probes for single and double FISH (WCP 11 Spectrum Orange and WCP 22 Spectrum Green), and centromeric and locus specific DNA probes for dual FISH (LSI 21q22.13-q22.2 Spectrum Orange and CEP Xp11-q11 Spectrum Green), and for triple FISH (adding the double probe LSI 22q11.2/22q13.3, Spectrum Orange/Spectrum Green). FISH analysis was carried out immediately or in preparations kept at –20°C. The slides were treated with PBS for 5 min, 50 mM $MgCl_2$ /PBS for 5 min, 1% formaldehyde/50 mM $MgCl_2$/PBS for 10 min, PBS for 5 min and finally dehydrated in 70, 90 and 100% ethanol. A volume of 2.5 µl of the hybridization mix containing the probes was placed on 8×8 $mm^2$ coverslips and mounted to the slides. The probes and the slides were simultaneously denatured by placing the slides on to a hot plate for 3–5 min at 74°C. After overnight incubation at 37°C in a humidified chamber, the slides were washed using the protocol provided by Vysis. Phase contrast and fluorescence microscopy were performed with an Olympus PROVIS-AX70 microscope equipped with the appropriate filter set for FITC, Rhodamine and DAPI (Olympus, Hamburg, Germany). After direct analysis, phase contrast and FISH metaphases were captured and analysed using a Cytovision Ultra workstation (Applied Imaging).

## Results

The method was developed in an animal model. A total of 200 Syrian hamster oocytes were processed immediately after

recovery, 165 1PBs were successfully fixed and the remaining 35 were accidentally lost (Table I). In 105 (63.6%) 1PBs the chromosome complement showed a defined chromosome morphology similar to metaphase II chromosomes, with separated chromatids still joined by the centromere, but showing a 'hairy' aspect as a special feature (Figure 2). Of

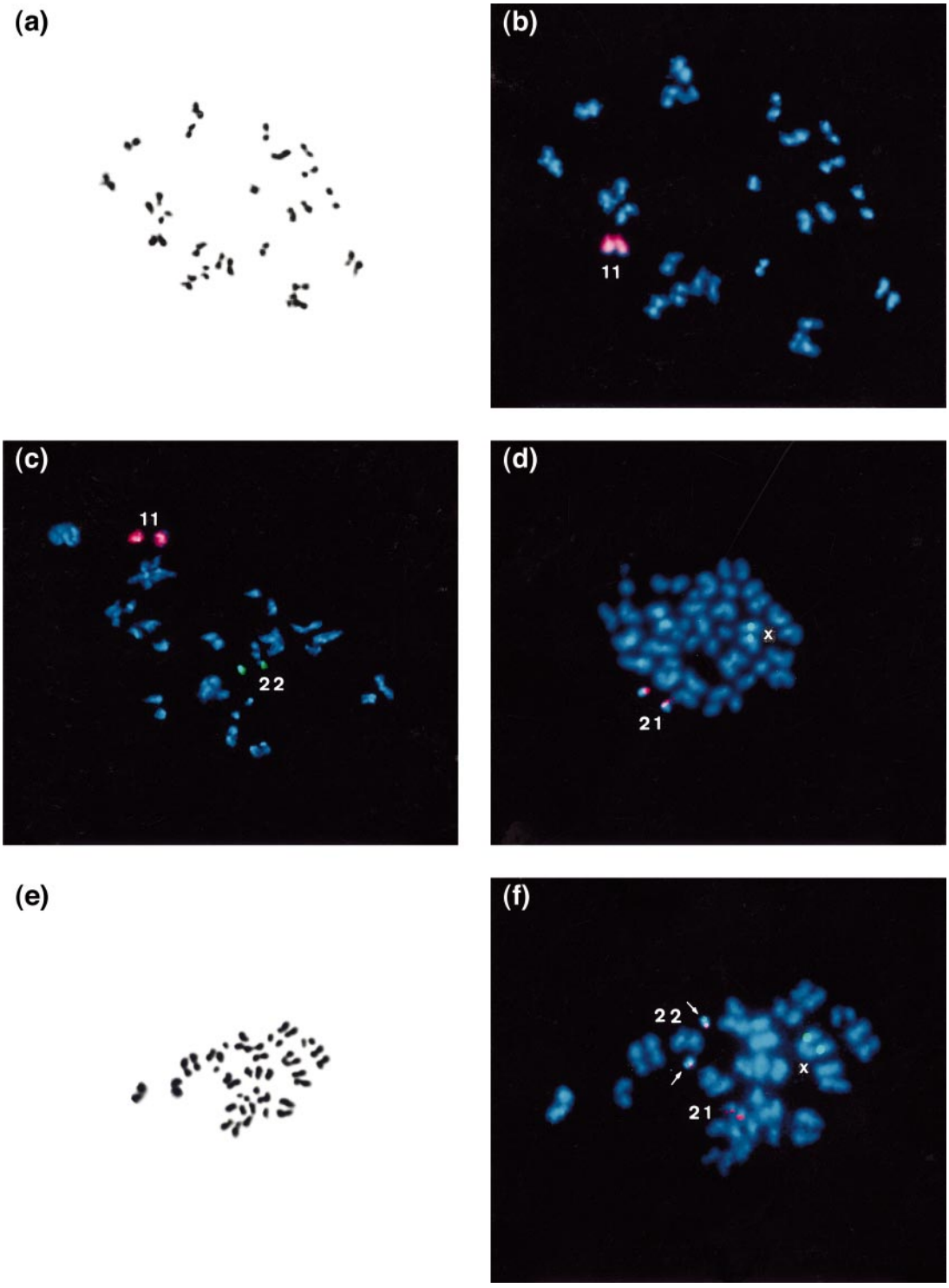


**Figure 3.** (**a**) Complete human first polar body (1PB) chromosome complement by phase contrast microscopy and (**b**) the same 1PB complement showing painting of chromosome 11 with the WCP 11 Spectrum Orange. (**c**) Complete human 1PB complement with double painting of chromosomes 11 and 22 with the whole chromosome probes WCP 11 Spectrum Orange and WCP 22 Spectrum Green. (**d**) Complete human 1PB complement showing hybridization with the LSI 21q22.13-q22.2 Spectrum Orange probe and CEP Xp11-q11 Spectrum Green probe for chromosomes 21 and X respectively. Note that one of the 21 chromatids shows a split LSI signal, which is common when using this probe. (**e**) Complete human 1PB complement by phase contrast microscopy and (**f**) the same complement hybridized with probes LSI 21q22.13-q22.2 Spectrum Orange, LSI 22q11.2/22q13.3, Spectrum Orange/Spectrum Green (arrows) and CEP Xp11-q11 Spectrum Green probes for chromosomes 21, 22 and X. The two signals for the double probe on chromosome 22 can be clearly distinguished.

these 105 1PBs, a total of 100 (95.2%) showed a complete complement with 22 chromosomes, while five complements (4.8%) were hypohaploid, three with 21 chromosomes and two with 20 chromosomes. The remaining 60 (36.4%) 1PBs showed no defined chromosome morphology.

Encouraged by the high frequency of good quality hamster 1PB chromosome complements obtained in this first approach, we decided to adapt the procedure to human oocytes. A total of 21 donated human oocytes were processed immediately after puncture. Seventeen of them were successfully fixed and the remaining four were accidentally lost during fixation during the early attempts to adapt the method to human oocytes. In 16 of the 17 1PB (94.1%) it was possible to obtain well spread, high quality chromosome complements (Table I). Human 1PB chromosomes are fuzzy and shorter than metaphase II chromosomes and also show separated centromere regions and chromatids. These 1PB chromosomes were suitable for in-situ hybridization analysis using fluorescent commercial probes. Figure 3a and b shows a complete human 1PB chromosome complement observed by phase contrast microscopy and hybridized with a whole chromosome probe for chromosome 11 (WCP 11 Spectrum Orange). Figure 3c shows a complete human 1PB chromosome complement hybridized with whole chromosome probes for chromosomes 11 and 22 (WCP 11 Spectrum Orange and WCP 22 Spectrum Green, respectively). Figure 3d shows a complete human 1PB complement hybridized with the LSI 21q22.13-q22.2 Spectrum Orange and CEP Xp11-q11 Spectrum Green probes for chromosomes 21 and X. Figure 3e and f shows a complete 1PB chromosome complement observed by phase contrast microscopy and hybridized with three probes (LSI 21q22.13-q22.2 Spectrum Orange, LSI 22q11.2/22q13.3 Spectrum Orange/Spectrum Green, and CEP Xp11-q11 Spectrum Green) for chromosomes 21, 22 and X.

## Discussion

So far it has been extremely difficult to obtain good quality chromosome complements from 1PBs (Verlinsky *et al*., 1996). For this reason, 1PBs have only been used for the detection of genetic defects by PCR and for the indirect diagnosis of numerical chromosome abnormalities using 1PB complements without any defined chromosome morphology. This material is not suitable for cytogenetic analysis of structural chromosome abnormalities. We have developed for the first time a hypotonic treatment and a fixative procedure to obtain good 1PB complements in a high proportion of cases. With this procedure it is possible to obtain well spread chromosome complements that can be accurately analysed by FISH for structural and numerical chromosome anomalies.

The reason for using FISH is that the high degree of contraction and fuzzy morphology of 1PB chromosomes makes it difficult or impossible to obtain G-banding. 1PB complements show a high sensitivity to the FISH denaturation step and therefore it is useful to obtain phase contrast images prior to the FISH procedure, to facilitate chromosome identification. FISH results in human 1PBs from fresh oocytes clearly show that, despite their fuzzy aspect, it is possible to detect whole chromosomes, centromeres and unique sequences, including interstitial regions (LSI 21q22.13-q22.2 and LSI 22q11.2) and very terminal regions (LSI 22q13.3) (Figure 3). Our results seem to indicate that the DNA loss related to DNA fragmentation involved in apoptosis, recently described by Takase *et al*. (1995) and Fujino *et al*. (1996) in incubated oocytes, has not yet taken place in fresh oocytes.

Only five hamster 1PBs of the group with defined chromosome morphology (Table I) showed an incomplete number of chromosomes and at least two of them, with 20 chromosomes, most probably resulted from the loss of chromosomes during fixation. The other three, with 21 chromosomes, could result from non-disjunction. The low level of artificial chromosome loss detected, when compared to studies of oocytes, could be explained by the characteristics of the fixative solution used [ethanol:acetic acid (1:1) instead of (3:1)] because the lower proportion of alcohol resulted in a slower evaporation of the fixative, and the increased proportion of acetic acid made the fixative solution more efficient in the disruption of the 1PB membranes. The way in which the fixative was applied, gently placing it next to the 1PB, and the use of the trimethoxysilane film also improved the results by reducing the area in which the chromosomes were spread, minimizing the artificial losses. In summary, the essential factors for routinely obtaining good chromosome spreads from 1PBs are: (i) to work with 1PBs from fresh oocytes, (ii) to use a long hypotonic treatment under stereomicroscopic control, (iii) to use a modified fixative solution (ethanol:acetic acid, 1:1), (iv) to produce a one-way flux of fixative on the slide and (v) to use trimethoxysilane coated slides.

The procedure described could be extended to clinical preconception cytogenetic diagnosis, because it allows the detection not only of aneuploidies (which are of maternal meiotic I origin in 80–95% of cases) but also of structural chromosome abnormalities, even when small segments of chromosomes are involved, because the transmission of chromosome anomalies to the offspring depends mainly on the segregation of the anomaly at meiosis I. The technique would be especially indicated in those couples where the woman is the carrier of a structural chromosome reorganization because, according to sperm chromosome studies, in these cases the risk of conceiving a chromosomally unbalanced child ranges from 15 to 90% (Templado *et al*., 1988). The analysis of 1PB chromosomes would allow the identification the type of segregation that has taken place and, consequently, predict if the embryo would be normal or not (Egozcue *et al*., 1996). Furthermore, the clinical application of 1PB chromosome analyses in preconception diagnosis would be especially indicated in those couples who, on moral grounds, would reject embryo selection after preimplantation diagnosis (Egozcue, 1994) or abortion after prenatal diagnosis.

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