The use of foetal ovarian stromal cell culture for cytogenetic diagnosis. Stromal ovarian culture cytogenetic diagnosis

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Abstract

Some studies have been carried out to analyze human female first meiotic prophase. Most of them use samples from fetuses collected after legal interruption of pregnancy. In some cases, a control population is needed and fetuses aborted for non-chromosomal reasons are used. The assumption of these samples as being euploids could perhaps represent an error. In this article, we describe an easy methodology to certify the euploidy of foetal ovarian tissue using an one-week somatic culture. Using this protocol, we have obtained a primary culture in 88.2% of the studied cases, material usable for being karyotyped in 93.3% of the cases, and a cytogenetic diagnosis was performed in 100% of these cases. Finding the same karyotype in cultured cells in cases in which we had a prenatal cytogenetic diagnosis has validated the technique, and in applying this protocol we have been able to check our prophase meiotic-study control population.

Introduction

Aneuploidy is one of the most frequent causes of spontaneous abortions and newborns with severe anomalies. Chromosomal aneuploidy has an incidence of 0.3% in newborns (Hassold et al. 1996). It mainly originates before fecundation, during gametogenesis, due to meiosis non-disjunction. Although many differences in terms of maternal-origin percentage have been found regarding the analyzed chromosome (Zaragoza et al. 1994), most of the published studies mention a predominant maternal origin (Hansmann and Probeck 1979; Kupke and Müller 1989; Nöthen et al. 1993; Ya-gang et al. 1993; Zaragoza et al. 1994; Fisher et al. 1995; Eggermann et al. 1996; Bugge et al. 1998; Nicolaidis and Petersen 1998). The cause of this difference in parental-origin in the etiology of some aneuploidies has been a topic of discussion amongst geneticists involved in human reproduction.

To study first meiotic stages or the pairing process of homologous chromosomes in the human female is one way to know about these reasons and implies the use of foetal samples. Meiosis starts around the 12th gestational week and it is blocked when it arrives at the dictyotene stage, also during the foetal time-period. In the majority of the human female meiosis I studies performed, samples are collected from induced therapeutic abortions (Speed 1984; Garcia et al. 1987, 1989; Cheng and Gartler 1994; Cheng et al. 1995; Barlow and Hultén 1997a, 1997b, 1998; Cheng et al. 1998, 1999; Hartshorne et al. 1999; Tease et al. 2002). This means that most of the aborted fetuses have an anatomical, physiological, genetic or chromosomal abnormality. Because a control population is needed (Cheng and Gartler 1994; Cheng et al. 1995; Barlow and Hultén 1997a, 1998; Cheng et al. 1998; Hartshorne et al. 1999; Tease et al. 2002) fetuses aborted for non-chromosomal reasons are used as controls. To assume that fetuses aborted, due to

non-chromosomal reasons, are euploids could cause some errors in the definition of 'normality' by classifying cases which may not be 46,XX as normal.

The aim of this work is to develop a suitable and reproducible technique that would allow us to classify as euploid, samples about whose karyotype we knew nothing, using samples of ovarian tissue available after extraction of oocytes. Cases, where the karyotype was known, were used as controls to check the efficiency of this technique. In addition, the cultures were used to establish cryopreserved stocks of somatic cells for future studies.

Materials and methods

Biological material

For the present study 17 fetuses were used (Table 1), all of which were collected after ethical approval for interruption of pregnancy: with permission of the family and according to the Ethical Rules Committee from the *Hospital Vall d'Hebron* and the *Hospital Clínic* of Barcelona, Spain. Gestational age was calculated from the last menstrual period, where available, by foot length and by ecography. In 11 cases a prenatal cytogenetic diagnosis was known.

Establishment of primary cultures

Ovaries were collected at the hospital in Phosphate Buffer Solution (PBS) 1% penicillin-streptomycin (Gibco-Life Technologies; Paisley, UK) within the first 6 hours after delivery and samples were transported within 30 min to the laboratory. The ovaries were dissected under sterile conditions from adjacent tissues and washed in PBS.

After extracting oocytes by needle puncture (Martínez Flores et al. 2003), the ovaries were disaggregated mechanically in PBS solution. The cellular solution was centrifuged for 7 min at 600 g and the supernatant removed. The cellular pellet was dissolved in pre-warmed AmnioMax™ medium (Gibco-Life Technologies; Paisley, UK) with and without 1% Fungizone (Gibco-Life Technologies; Paisley, UK) (Table 1) and 1.25 ml of the solution was cultured in a Petri dish (Nunc; Roskilde, Denmark) in 5% CO₂/95% air at 37 °C.

Evolution of the culture was observed every day under an Olympus CK2 inverted microscope (Olympus Optical Co.; Hamburg, Germany). On the second day of culture, new medium was added up to a final volume of 2.5 ml.

Obtaining material for cytogenetic analysis

After 7–8 days of culture, confluence was reached

Table 1. Relation of the cases used to apply the protocol. Origin, foot length, gestation weeks, the culture medium, the therapeutic abortion reason and karyotype obtained from the study of the stromal ovarian culture are shown per each one. Note that in some cases prenatal karyotype was known.

Case	Foot length ^a	G.W.	Culture Medium	Clinical Diagnosis	Stromal Culture Karyotype
V54	2.4	19	AmnioMax™ 1% Fungizone	Cardiopathology	46,XX
V57	2.5	19	AmnioMax™	Arnold Chiari S.	46,XX
V58	2.4	18	AmnioMax™ 1% Fungizone	47,XX,+21	47,XX,+21
V59	2.2	21	AmnioMax™ 1% Fungizone	46,XX,del(9)(q31-qter)	46,XX,del(9)(q31-qter)
V60	–	19	AmnioMax™ 1% Fungizone	47,XX,+21	47,XX,+21
V61	3.7	22	AmnioMax™	Oligoamnios; 46, XX	46,XX
V62	2.5	21	AmnioMax™	47,XX,+18	47,XX,+18
V63	2.6	20	AmnioMax™	47,XX,+18	47,XX,+18
V64	2.8	22	AmnioMax™	Foeto-foetal transfusion	46,XX
V65	4.5	22	AmnioMax™	Foeto-foetal transfusion	46,XX
V66	3.2	22	AmnioMax™	45,X0	45,X0
V67	3.9	22	AmnioMax™	Foeto-foetal transfusion	46,XX
V68	3.55	22	AmnioMax™	Foeto-foetal transfusion	46,XX
C1	2.5	20	AmnioMax™ 1% Fungizone	45,X0	45,X0
C2	1	13	AmnioMax™ 1% Fungizone	47,XX,+21	–
C3	–	18	AmnioMax™ 1% Fungizone	47,XX,+18	–
C4	1.9	15	AmnioMax™ 1% Fungizone	47,XX,+21	–

V: samples from Hospital de la Vall d'Hebron. C: from Hospital Clínic. G.W.: Gestational Weeks. ^aFoot length measured in centimetres.

and the culture was then divided into two parts. A smaller portion (around 30%) was cultured in a Petri dish (Nunc; Roskilde, Denmark) to freeze the cells and the remainder was transferred to a Falcon culture bottle (Corning Coastor Corporation; Cambridge, USA) in order to obtain metaphase stage cells from which to obtain the karyotype. Twenty-four hours after the transfer, extraction of the culture was performed. KaryoMax® Colcemid (Gibco-Life Technologies; Paisley, UK) was first applied for a period of 2 hours to the Falcon culture bottle followed by extraction and processing to obtain metaphase-cell 'spreads' using conventional cytogenetic procedures.

Preservation and recovery of somatic cells

Once a confluent monolayer was achieved in the Petri dish, extraction of the secondary culture was performed and the cells obtained were suspended in Foetal Calf Serum (Gibco-Life Technologies; Paisley, UK) with 10% dimethyl sulfoxide (Sigma Chemical Co; St. Louis, USA) and aliquotted in cryotubes (Nunc; Roskilde, Denmark). These aliquots were placed at -80°C for at least four hours and then transferred to storage in liquid nitrogen.

Some frozen material was thawed in order to check the survival rate. A cryotube of frozen cellular suspension was thawed at 37°C and DMEM medium (Gibco-Life Technologies; Paisley, UK) with 10% FCS was added to a final volume of 10 ml. After centrifugation, the cellular pellet was resuspended in fresh, pre-warmed AmnioMax™ (medium and cultured in 5% CO_2 / 95% air at 37°C).

The freezing protocol was applied in all cases in which a primary culture had been obtained. The thawing protocol was applied in 4 cases (V54, V57, V64 and C1) to check for survival of frozen cells.

Karyology

Slides were G-banded as described by Seabright (Seabright 1971) with slight modifications and analyzed in an Olympus AX70 brightfield photomicroscope (Olympus Optical Co.; Hamburg, Germany). Images were captured and produced by a Cytovision system (Applied Imaging; Sunderland, UK). At least 10 full karyotype cells were analyzed per case.

In some cases, FISH was performed to assess chromosomal abnormalities (V58) or to check chromosome morphology and particular banding pattern (V54), first found in G-banding analysis, using directly labelled whole-chromosome probes (Cambio; Cambridge, UK).

Results

Using the previously mentioned protocol, a primary culture was obtained from 15 ovaries out of 17 processed cases (88.2%) (Table 1). Morphology of the cultured cells was primarily fibroblast-like (Figure 1), although some round-shaped cells were present.

Material appropriated for karyology was obtained from 93.3% of the cultures performed. In addition a cytogenetic diagnosis was realized in 100% of the

Figure 1. Image of a cell culture from case V64, 2 days after thawing, at $100\times$ magnification (A). Detail of a group of fibroblast-like cells at $200\times$ magnification (B).

cases for which metaphase spreads were achieved (Table 1).

The efficiency with which primary cultures were obtained in the presence or absence of Fungizone showed significant differences. Efficiency of the culture technique appeared to be enhanced without Fungizone ($\chi^2=4.098$, $p=0.043$). However, other factors controlling primary culture establishment (see Discussion) could also explain these differences.

In general, a minimal cellular concentration of approximately 620,000 cells per milliliter was enough to initiate a primary culture. Attached cells were usually observed on the second or third day of culture. After one week of culture, a monolayer of attached fibroblast-like cells was present in the primary culture Petri dish covering approximately 90% of the surface.

The higher amount of cells in the metaphase stage was captured 24 h after the last transfer to the Falcon culture bottle and after applying colcemid treatment for 2 h.

In all cases in the trial a cryopreserved culture was established. In four cases (V54, V57, V64 and C1) the thawing protocol was applied, yielding a culture covering approximately 90% of the culturing surface in less than 4 d although some non-attached cells were present in the medium (Figure 1).

In all cases (V54 and V58) in which FISH was performed, the same results were achieved using G-banding analysis and chromosome painting.

Discussion

This paper, describes for the first time a somatic culture protocol to obtain material for Karyology from ovarian stroma collected from foetal samples after legal interruption of pregnancy. This methodology is straightforward, reproducible and suggests that an exact karyotype may be obtained from samples whose chromosome complement is unknown. Although there is still a small risk of diagnostic error originating in the different embryologic origin of somatic ovary cell-lines and germ cells, knowledge of the karyotype from ovarian stromal cells minimizes possible misinterpretation caused by the presence of a mosaicism in cytogenetic studies performed in oocytes I.

The establishment and evolution of the culture are subject to common factors like quality of the sample, cellular density, pH, osmolarity, temperature, chromosome complement of the cultured cells, etc. In this

particular case, some of the more critical factors are cell density and specimen quality.

In order to obtain a successful primary culture a minimal cellular density is needed in order to allow cellular paracrine interactions to work. We have seen that a concentration of about 620,000 cells per milliliter is enough to establish a primary culture. We think this could be one reason to explain why we were unsuccessful in obtaining a primary culture from cases C2 and C4 (Table 1). The availability of a small amount of tissue, due to the early developmental stages, added to a division of the cellular suspension in several fractions in order to perform different kinds of culture techniques (data not shown), did not permit the minimal critical cell density required to establish primary cultures.

In terms of specimen quality, the time between delivery and processing of the sample is an important factor. The ovary, in the foetal stage, is porous and capable of absorbing surrounding solutions. Therefore, once the foetus is expelled, the ovary must be removed as quickly as possible to prevent it from coming into contact with internal solutions, which could damage it irreversibly. These observations agree with what is published announcing the freshness of the ovaries as a critical factor for obtaining a high hybridization efficiency rate in oocyte I when Fluorescent *in situ* Hybridization (FISH) is performed (Cheng and Gartler 1994).

The influence of the presence of Fungizone in the medium is not yet clear. It may seem that the fungicide could be toxic for some cultures, but the data presented here are not sufficient to confirm this. A variety of factors relating to specimen quality and *in vitro* culture may influence data from primary cultures. However, for the diagnosis of our meiotic prophase study samples, we have excluded Fungizone from the culture medium.

The identification of the same karyotype in cells from foetal ovarian stromal cultures and clinical cytogenetic diagnosis (V58, V59, V60, V61, V62, V63, V66 and C1) (Table 1) has enabled us to propose this technique as an easy way to detect the euploidy of samples which enter a female meiotic prophase study.

Using this protocol, we have been able to classify different cases as control, which had an anatomical anomaly (V54, V57, V64, V65, V67 and V68). In this sense, we believe that it is very important to be able to certify the euploidy of a sample in order to define how meiotic prophase takes place in 'wild type'. Moreover, this is the case when an interchromosomal effect

has been postulated to interact with the normal evolution of meiosis in some aneuploidy cases.

This protocol is a further step towards better understanding of what happens in female meiotic prophase. Other developmental, physiological and anatomical factors, which may affect the evolution of first meiotic stages, must be detected in order to increase our knowledge of human female meiotic prophase.

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