In situ culture and harvest of amniocytes using coverslip processing method

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Genetic amniocenteses were performed in a series of 127 patients as a routine study. Samples from the patients were cultured by in situ method, flask method or both according to the state of amniotic fluid. The overall success rate of culture was 97.6% and no culture failure was observed in the flask method. It took 5 days first of all and 8.15 days average from set-up to harvest and there were 7.2 colonies per dish in in situ method. Therefore, it is suggested that in situ method which decreased the mean culture days and made clonal analyses possible, is a clinically available and even more reliable method in parallel with flask method in prenatal diagnosis.

Keywords: Amniocentesis, in situ method, prenatal diagnosis

INTRODUCTION

Several approaches are now available for the prenatal diagnosis of chromosomal abnormality of the fetus, which are chorionic villus sampling (CVS), amniocentesis and cordocentesis. Chorionic villus sampling as early as the eighth week provides a rapid prenatal assessment of the fetus, but a number of problems have emerged. The overall rate of fetal loss from CVS is 3.5% (Jackson and Wapner, 1987; Biscarello *et al.*, 1991). Another problem of CVS cytogenetic assessment is discordance of direct and culture analyses (Simoni *et al.*, 1985; Mikkelsen, 1985), pseudomosaicism (Kennerknecht *et al.*, 1992) and also discrepancy between the chromosomal analyses on chorionic villi and fetal karyotype because of placental confined mosaicism (Ledbetter *et al.*, 1990). Cordocentesis (fetal blood sampling) is used when rapid karyotyping is indicated, such as the discovery of a malformed fetus on ultrasound and also in failed amniocentesis. However, the technique of sampling is very difficult and risky. Prenatal chromosome analysis aims at a safe diagnosis of the fetal karyotype as early as possible. Chromosome analysis of cultured amniocytes is a well established procedure, which leads to reliable results in the second and third trimesters. But, with amniocentesis, the disadvantage is the lateness of the procedure, so that when an abnormal result is obtained the pregnancy is far advanced, usually about 20 weeks or more of gestation, making termination difficult from the patient's viewpoint. So the successful and rapid culture is very important in the analysis of fetal karyotype.

The culture flask on suspension harvest and coverslip dish or slide chamber on *in situ* harvest are usually used for amniocyte cultures. But in *in situ* harvest, we could lessen not only the culture duration until harvest because of the selective harvest of the well-grown colonies, but also the possibilities of pseudomosaicism because of clonal analysis. Our aim was to confirm *in situ* method to routine amniocyte culture by decreasing the culture duration of 1st harvest, report the possibilities of pseudomosaicism. This paper describes our experiences of *in situ* culture in the study of 127 consecutive patients.

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MATERIALS AND METHODS

127 Routine prenatal cytogenetic studies have been performed for this study. The indications for amniocentesis are listed in Table 1.

Amniotic fluid was aspirated transabdominally with a 22-gauge spinal needle under ultrasound guidance and its volume was measured in every sample. Cells isolated by centrifugation were resuspended in culture medium (Chang B, C, penicillin-streptomycin, *l*-glutamine) before being transferred to culture flask and coverslip dish. The

Table 1. Indications for amniocentesis.

Indication	No. of Cases(%)
Maternal age ≥ 35	16(12.6)
Abnormal low	83(65.4)
Triple M. high	12(9.4)
Previous pregnancy with congenital anomaly	5(3.9)
Abnormal USG	3(2.4)
Others	8(6.3)
Total	127(100)

cultured cells were trypsinized in flasks and harvested *in situ* on coverslip dish. Chromosome preparations were GTG-banded. Trypan blue was used to identify live cells as the vital stain; 0.1 ml of amniotic fluid was incubated with 20 μl trypan blue at 37°C for 5 min before being examined on a counting chamber (haemocytometer). Total and viable cells were counted using light microscopy (Byrne *et al.*, 1991) three times in every sample and a mean value was accepted for the statistical analysis of the results. The multiple regression analysis using SAS package was applied.

RESULTS

Amniocyte culture and karyotype analyses were successful in 124 of the 127 cases, and the overall success rate was 97.6%. Among these, cell cultures were performed in 89 cases by only *in situ* method and in 22 cases by both flask and *in situ* method. 3 cases of culture failure were observed in only *in situ* method (Table 2). The mean gestational age and volume of aspirated amniotic fluid were 19.2 week and 19.0 ml respectively. Total and live cell numbers of amniotic fluid were 5.94×10^4/ml and 3.83×10^4/ml (Table 3).

With respect to the total and live cell numbers, there is a little difference among the culture groups, as shown in Table 4.

The cultured cells had been harvested from the 5th day since set-up and the mean culture duration until harvest was 8.15 days. And the mean number of colony per coverslip was 7.2 (Table 5).

Table 2. 127 Consecutive cultures of amniocytes.

Result	Method	No. of Cases (%)
Success	*In situ*	89 (70.1)
	In situ + Flask	22 (17.3)
	Flask	13 (10.2)
Failure	*In situ*	3 (2.4)
	In situ + Flask	0 (0.0)
	Flask	0 (0.0)
	Total	127

Table 3. Means of gestational age, aspirated volume, total cell

	Mean ± SD	Range
Gestational age	19.2 ± 2.5	14.0-29.0
Aspirated Volume	19.0 ± 2.8	14.0-30.0
Total cell ($\times 10^4$/ml)	5.94 ± 6.94	0.50-51.2
Live cell ($\times 10^4$/ml)	3.83 ± 4.14	0.17-30.0

Table 4. Means of gestational age, aspirated volume, total cell

	Gestational Age	Aspirated Volume	Total Cell ($\times 10^4$/ml)	Live Cell ($\times 10^4$/ml)
In situ	19.5 ± 2.6 (15-29)	19.7 ± 2.3 (14-30)	7.96 ± 8.14 (1.0-51.3)	5.16 ± 4.71 (0.2-30.0)
In situ + Flask	15.7 ± 1.3 (14-19)	18.5 ± 3.0 (14-24)	2.34 ± 1.31 (1.0-5.5)	1.36 ± 0.89 (0.5-2.8)
Flask	18.8 ± 2.8 (14-24)	15.7 ± 2.2 (14-20)	2.00 ± 1.87 (0.5-7.3)	1.17 ± 1.13 (0.2-4.5)
Culture Failure	19.0 ± 3.0 (16-20)	16.7 ± 2.9 (15-20)	3.08 ± 0.38 (2.8-3.5)	1.63 ± 0.54 (1.3-2.3)

DISCUSSION

Not only are the fetal anomaly rates increasing but also are the techniques for prenatal diagnosis developing and the frequencies for the tests are on the increase more and more. Among them amniocentesis is the most frequent test and usually performed between 16 and 22 weeks of the gestation. If the abnormal result of amniocentesis is obtained, making termination difficult, the successive and rapid culture is very important in the analysis of fetal

Table 5. Number of dishes, culture duration and colony frequency in *in situ* culture method.

No. of Dishes	Days until Harvest	No. of Colonies
7.97 ± 2.15 (4.0-16.0)	8.15 ± 1.01 (5.0-10.0)	7.20 ± 3.26 (2.0-16.0)

karyotype.

The prenatal detection of chromosomal aberrations using cultured amniocytes has been practised worldwide with an acceptably low risk. There are two methods of chromosomal harvesting which are commonly in use: the '*in situ*' or 'coverslip culture' technique and the 'trypsinization technique' or 'flask culture' method. In numerous European laboratories it took 13.4 and 15.7 days for the '*in situ*' and 'trypsinization' technique respectively from amniocentesis to analysis. It takes longer in culture than any other procedure. Culture flasks (T-25) are usually used as the culture vessels for amniocytes culture and culture duration is 7-10 days as usual in suspension harvest which is not for clonal analysis. It was reported that early amniotic fluid samples as early as the 10th - 14th of gestational age (Nevin *et al.*, 1990; Djalali *et al.*, 1992; Jorgensen *et al.*, 1992) and even the samples of small volume (Byrne *et al.*,1991) could be cultured using *in situ* culture. But the overall fetal loss rate was reported to be about 4.2% (Dunn and Godmilow, 1990) and the frequency of pseudomosaicism was higher in early amniocentesis than the amniocentesis of mid-trimester (Kennerknecht *et al.*, 1992). In addition, the culture failure rate of *in situ* technique was higher than that of flask, even higher when the quality of amniotic fluid was not good and the cell numbers were few. But, we could decrease not only the culture duration until harvest, but also the possibilities of pseudomosaicism in *in situ* harvest because of selective harvest of the well-grown cells and clonal analysis.

We decided the culture method as a condition of amniotic fluid and the overall success rate was 97.6%. 3 cases of culture failure were observed only in *in situ* method. Total and live cell numbers were increasing with the gestational age but decreasing after the 21th week. The amniocyte culture which would be supposed to be failed owing to elasped time after sampling, volume and pellet of amniotic fluid and cell numbers was set up by both *in situ* and flask method. And the numbers were much higher than those of only *in situ* culture group. It is supposed that not only optimal culture condition, quality control of culture medium, and culture technique but also live cell numbers per dish are necessary for the success of the culture.

The cultured cells had been harvested from the 5th day since set-up in only *in situ* method. The gestational age, volume of amniotic fluid, total and live cell numbers did not have any effects on the mean harvest day. The factor that significantly influenced decreasing the mean culture days was the numbers of dish set (unpublished). The more were the dishes set, the earlier were the cultured cells harvested. It was supposed for the cultured cells to be harvested even in the small colonies. And the mean number of colony per coverslip was 7.2. It is supposed that we could perform not only *in situ* culture for correct and rapid result but also flask culture in parallel with it as a back-up in order to lessen the culture failure rate and the mean culture days.

Therefore, it is suggested that *in situ* technique is optimal for amniocyte culture necessary for the clonal analysis and more useful even when both techniques are simultaneously performed.

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