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Journal of Women Health Care and Issues
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Research Article

The Effects of Sample Quality in the Long-Term Culture and Chromosome Abnormalities Found in Early Pregnancy Loss

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Received date: June 12, 2023; Accepted date: July 17, 2023; Published date: July 24, 2023

Citation: Lydia Pratanu, Jeanne Adiwinata P, Damar Prasmusinto, Johanes Edy Siswanto (2023), The Effects of Sample Quality in the Long-Term Culture and Chromosome Abnormalities Found in Early Pregnancy Loss, *J. Women Health Care and Issues.* 6(4); **DOI:**10.31579/2642-9756/153

Abstract

Background: Blighted ovum and fetal death are conditions when the zygote fails to develop during the first trimester and are classified as early pregnancy loss (EPL). Fetal chromosomal abnormalities cause more than 50% of EPL; therefore, every EPL case should undergo chromosomal analysis. Conventional cytogenetic analysis, as the gold standard, can only be done by culturing living cells undergoing mitosis. A chromosomal diagnosis cannot be established if the tissue condition is not good.

Objective: This study aimed to identify the effects of sample quality associated with the successful long-term future growth of the tissue and chromosome abnormalities in early pregnancy loss specimens.

Method: The design of this study design was cross-sectional. The long-term culture cytogenetic technique was done by applying collagenase enzyme as pre-treatment in fibroblast culture from curettage tissue. Chromosomal analysis was done using Wright staining with G-banding and analyzed using the Applied Imaging Computerized Cytogenetic system according to the International System for Human Cytogenetic Nomenclature 2016.

Result: A total of 32 tissue specimens were enrolled in this study. The success rate of fetal fibroblast culture in this study was 56.3% (18 samples). Sample quality did not have a significant association with culture growth. Still, the presence of necrotic tissue ($p<0.001$), blood clots ($p=0.005$) and mucous ($p=0.016$) was significantly associated with bad quality of the chromosomes. Based on chromosome analysis done in successfully grown samples, we obtained seven samples (38.8%) with chromosomal abnormalities, such as aneuploidies and mosaicism.

Conclusion: This study concluded that the successful growth of tissue culture is not affected by the sample quality. However, the quality of chromosomes valid for diagnosis is influenced by the sample quality, i.e. the presence of necrotic tissue, blood clot, and mucous.

Key words: early pregnancy loss; sample quality; chromosome abnormalities

Introduction

According to Cunningham GF et al., abortion is the termination of pregnancy before 20 weeks gestation or termination of pregnancy with a fetus weighing less than 500 grams. Based on the gestation age, abortion can be classified as early abortion / early pregnancy loss (EPL), namely abortion that occurs under 12 weeks and late abortion, which occurs above 12 weeks gestation [1]. It is estimated that around 20-30% of normal pregnancies will experience bleeding before 20 weeks' gestation, and half of those numbers end in spontaneous abortion [2]. Various factors, including blighted ovum and fetal, maternal and environmental factors, can cause early pregnancy loss. Blighted ovum – known as anembryonic pregnancy, happens when the zygote (fertilized egg) does not develop as it should. The gestational sac is formed in a pregnancy with a blighted ovum, but the embryo fails to develop, so fetal echo does not detect it [2]. Early pregnancy loss caused by fetal factors can be caused by bacterial, viral or parasitic infections in the fetus or due to chromosomal abnormalities in the fetus. Chromosomal abnormalities account for more than 50% of EPL. These chromosomal abnormalities can be in the form of numeric abnormalities or structural abnormalities.

Abnormalities in the number of chromosomes can cause aneuploidy and polyploidy. More than 50% of chromosomal abnormalities in ELP are aneuploidy – the addition of one or two chromosomes (2n+1 or 2n-1) or polyploidy – the addition of a haploid set of chromosomes (e.g. triploid). Aneuploidy is most often found in the form of autosomal trisomy (> 50%), and a small number (0.21%-2.8%) are in the form of double trisomy [3,4]. Structural chromosomal abnormalities can be translocations (exchange of intrachromosomal segments), deletions (reduction of segments in one chromosome), inversions, insertions, isochromosomes, dicentric, and ring chromosomes. According to Friedman JM et al. and Ledbetter DH et al., chromosomal structural abnormalities are more common in infertile couples (2-4%) and newborns with congenital abnormalities [5,6]. Maternal factors that affect EPL include maternal age. Advanced maternal age (over 35 years) is often associated with a non-disjunction process in oogenesis. This results in nullisomic or disomic gametes, which, when fertilized, produce a trisomic or monosomic zygote. Environmental factors that affect EPL include smoking habits, alcoholic beverages, therapeutic radiation for malignancy treatment, and intrauterine contraceptives. Chromosomal abnormalities in EPL tissue can be examined through conventional cytogenetic tests using the long-term culture method. The chorionic villi will be separated from the decidual tissue and other debris during this process. Afterwards, collagenase is given as an enzyme that dissociates stromal core villus cells into fibroblasts, proliferating rapidly beyond maternal cells. In long-term culture, maternal decidual cells are not viable in culture media, so maternal cell contamination (MCC) can be reduced. According to Songster et al. (1992), using long-term culture, it is estimated that MCC can be reduced by up to 0.5% [7].

One thing that influences the success rate of the cell culture until the release of analysis results is the quality of the tissue sample. Sample quality is influenced by several factors, starting from the sampling method to the time factor. One of the sampling techniques for the conception product is ERPC – the evacuation of retained products of conception. This technique carries out the curette process while monitoring using abdominal ultrasound. This technique can reduce MCC and bacterial contamination. If the sample is processed immediately, then the success rate of chromosome analysis of the conception product is 48-59%. Based on several previous studies, it was also found that samples mixed with blood, maternal decidual cells, mucus and other debris would affect the success rate of cytogenetic culture [8-10].

It is important to perform sample processing as soon as the laboratory receives the transport media with the conception product. Blood clots, mucus and other debris, are good media for bacterial growth. In addition, necrotic tissue must be cleaned immediately so the sample can grow properly. Samples that remain in the laboratory for a long time will show a higher failure rate.

Methodology

The research design was descriptive and cross-sectional descriptive on the product of conception (PoC). This research was conducted at the Cytogenetics Laboratory, Harapan Kita Women and Children Hospital. The study was held from January 2010 to June 2012.

This research design and proposal have been approved by the Institutional Review Board of Faculty of Medicine University of Indonesia with the record document: 144/pt02.FK/ETIK/2010. The research sample was the product of the conception of the study population that is, EPL cases in Indonesia. The sample is not limited, whether it comes from pregnancy with IVF or not. The sample is also not limited, whether it comes from single or multiple pregnancies. The research sample was obtained from EPL cases in several hospitals in Jakarta from March 2010 to December 2011. The minimum sample size, 35 people, is calculated using the hypothesis test formula to get the proportion in one population. The inclusion criteria of this study were abortive tissue from every pregnant woman who experienced EPL in the first trimester, the cause of EPL was unknown, and the patients were willing to be included in the study. While the exclusion criteria from this study were pregnant women experiencing infection, trauma, using drugs, herbs, teratogenic substances, exposure to chemicals and pregnant women with anatomic abnormalities of the internal genitalia before the EPL incident. Recruitment of research subjects was carried out consecutively in 7 hospitals in Jakarta. The subject was asked to sign an informed consent before taking the tissue specimen. The medical history is obtained from the patient's medical record.

The main research materials are tissue specimens of a blighted ovum or dead fetus removed by dilation and curettage. Not using the Betadine solution is required when obtaining the samples in the curette room. If samples are obtained using suction curettage, the sample must be taken from the end of the suction pipe to remain aseptic. The tissue sample will then be placed on the previously prepared transport medium. The villus tissue was cleaned and isolated from the curette scraping tissue, then dissociated in two stages using the enzyme collagenase (=pretreatment). Furthermore, cleaning procedures, selection and isolation of the chorionic villi were examined under a dissection microscope. The final result is expected to be a suspension of fibroblasts from villi that appear dissociated in the form of sand/dots/debris or fine particles. The fibroblast suspension was then cultured and saved in an incubator with 5% CO_2 at 37ºC with 95% humidity. For several days to get fibroblast culture growth was observed periodically using an inverted microscope. The media was replaced every two days until the culture showed sufficient confluency. After the confluence reached almost 70-80%, monolayer/subculture fibroblast cells with new culture bottles and media were performed. After incubation, these fibroblast cultures will be harvested and spread on glass objects. After that, the object glass was stored in an oven at 50ºC overnight, and then the slides were ready to be stained with trypsinization of G-banding. Each glass object that has been G-banded is ready to be examined and analyzed with Cytovision CDI and prepared to be diagnosed based on the International System for Human Cytogenetic Nomenclature (ISCN) 2005 [6,7]. A total of 20 cells from two or more glass objects were analyzed. If mosaicism is found, ten more cells are added for observation and counting. The results are recorded in the analysis sheet and then photographed with the CDI to be concluded in the interpretation results sheet.

Result

A total of 32 pairs of research subjects were studied. The mean age of the mother was 33.8 + 5.3 years, with a range of 25 to 42 years. The mean age of fathers was 36.2 + 5.5 years, ranging from 27 to 45 years. We found 13 samples with advanced maternal age (aged >35 years old) and ten samples with advanced paternal age (aged >40 years old). The mean gestational age was 9.8 weeks + 1.5 weeks, ranging from 6 to 12 weeks. Most of the gestational age is at 9-10 weeks. Three pairs were excluded from the study from the 35 pairs collected for this research period due to the wrong transport medium, which caused tissue damage (Table 1.).

Variable	n	%
Maternal age		
• ≤ 35 years old	19	59.3
• > 35 years old	13	40.6
Paternal age		
• ≤ 40 years old	22	68.8
• > 40 years old	10	31.2

Gestational age		
• 5-6 weeks	1	3.1
• 7-8 weeks	4	12.5
• 9-10 weeks	19	59.4
• 11-12 weeks	8	25

Table 1. Subject Characteristics

Category	n (total)
Good culture growth	
• Poor chromosome quality	10
• Good chromosome quality	
○ Normal	11
○ Abnormal	
➢ Mosaic	3
➢ Aneuploidy	4
Poor culture growth	4
Total	32

Table 2: Final EPL tissue culture results and their diagnosis

Table 2 shows that out of 32 samples, there were 28 viable samples. A total of 18 samples showed good chromosome quality, and ten samples showed poor chromosome quality, so the diagnosis could not be made.

Variable	Good Culture Growth (n=28)		Poor Culture Growth (n=4)		*p-value*
	n	%	n	%	
Necrotic Tissue					
• visible	11	78.6	3	21.4	**0.295**
• not visible	17	94.4	1	5.6	
Blood clot					
• visible	12	75	4	25	**0.101**
• not visible	16	100	0	-	
Mucus					
• visible	13	92,9	1	7.1	**0.613**
• not visible	15	83,3	3	16.7	

Table 3: Factors Affecting the culture growth

Table 3 shows that samples with necrotic tissue and blood clots show slightly lower growth rates. Based on this study, we can see that mucus existence in the tissues did not show a significant difference in success rates, but still, there are higher growth rates found in tissues without mucus.

Variable	Chromosome quality				*p-value*	Odds Ratio (CI 95%)
	Good quality (n=18)		Poor quality (n=10)			
	n	%	n	%		
Necrotic tissue						
▪ Not visible	17	100.0	0	0	**<0.001**	-
▪ Visible	1	9.1	10	90.0		
Blood clot						
▪ Not visible	14	87.5	2	12.5	**0.005**	14.00 (2.08-94.24)
▪ Visible	4	33.3	8	66.7		
Mucus						
▪ Not visible	13	86.7	2	13.3	**0.016**	10.40 (1.62-66.90)
▪ Visible	5	38.5	8	61.5		

CI: confidence interval

Table 4: Factors affecting chromosome quality

Table 4 shows that contamination of necrotic tissue, blood clots and mucus is related to chromosome quality. Samples without necrotic tissue produced good-quality chromosomes, while blood clots and mucus contamination led to poor-quality chromosomes.

Karyotype	n	%
46, XX	6	33.3
46, XY	5	27.8
45, X	2	11.1
47, XY, +12	1	5.5
46 XX, +21, -16	1	5.5
Mos 46, XY / 46, XX	1	5.5
Mos 47, XX, +21 / 46, XX / 46, XY	1	5.5
Mos 47, XX, +12 / 46, XX	1	5.5
Total	32	100

Table 5: Karyotype results

Out of 18 samples with high-quality chromosomes, we discovered that 11 had a normal karyotype (61.1%), while four exhibited aneuploidy (22.2%), and three presented mosaic patterns (16.7%) (Table 5.)

Discussion

Theoretically, EPL is related to the age of the mother and father. A mother's age over 35 and a father's age above 45 statistically increase the likelihood of EPL. In this study, EPL was higher in mothers aged <35 and fathers aged <40. This is probably due to the limited number of samples. Further analysis still needs to be carried out with a larger sample size to assess further the correlation between the ages of the father and mother with EPL.

Out of 32 samples, we found 28 (87.5%) successful culture growth EPL tissue samples. The rest of the samples (12.5%) did not grow. We observed a fragile macroscopic appearance with contamination in blood clots, mucous tissue and other debris. Sometimes, these contaminants are difficult to separate from the villi properly. When these samples are cultured, these contaminants tend to be carried away. During the cleaning and isolation/separation stages, although the tissue has been treated with an aseptic technique using media added with antibiotics and antimycotics, the possibility of bacterial or fungal contamination can still exist [8,9].

Poor sampling techniques can also cause culture failure [10-12]. Theoretically, it is stated that the tissue sampling technique through the ERPC procedure guided by transabdominal ultrasonography is proven to improve sample quality in terms of MCC and bacterial contamination [10]. However, the ultrasound-guided ERPC procedure in Indonesia has yet to be performed and thus cannot be compared.

Unfavorable cell culture growth can occur due to non-optimal enzymatic pretreatment processes. The pretreatment process with collagenase is crucial to determine whether the culture will succeed [13]. The collagenase enzyme is packaged in the form of hygroscopic lyophilized protein. Care must be taken when opening the enzyme package so that it is not exposed to a room with high humidity. It is recommended that when removed from the refrigerator, the enzyme should be placed in a desiccator at room temperature and can only be used after the bottle is no longer cold. Tissues that grow poorly can also occur because villi undergo extraordinary apoptosis (defective apoptosis) in embryogenesis. This apoptotic tissue is not visible macroscopically, so there is still a possibility that villi tissue macroscopically looks good and viable but has undergone apoptosis. Various studies stated that apoptosis might be involved in the pathophysiology of IUGR. Placental apoptosis increases in first-trimester spontaneous abortion, and trophoblast apoptosis in the placenta increases in growth-restricted fetuses. [14,15].

The success rate of diagnosis in this study was 56.3%. This is quite significant compared to the culture success rate, which is much higher, 87.5%. From this study, we observed that the success rate for diagnosis is higher in samples that do not contain necrotic tissue, blood clots and mucus. This is because samples not polluted by necrotic tissue, blood clots and mucus contain good villi, so cells are capable of mitosis and produce good chromosomes that can be analyzed. Chromosomal abnormalities in the sample can also cause diagnostic failure. There is literature which states a correlation between the proliferation and mitosis of cells with chromosomal abnormalities [16]. In a cytogenetic study, it was found that cells with chromosomal abnormalities were still able to proliferate at the beginning of the culture period but were unable to show a perfect mitotic stage, so the number of metaphases that can be analyzed is under 20cells, the standard requirements [15-18].

The normal karyotype found in this study was 34.4%. This number is much lower than data from various studies, which state that around 50% of spontaneous abortions in the first trimester show normal chromosomes. This number may go higher or lower than stated above because chromosome samples cannot be assessed due to poor quality [1,3].

The most aneuploidy seen in this study was 45, X (monosomy X). This is in accordance with various studies which state that monosomy X is quite common in embryos that experience spontaneous abortion, which can be seen in 10% of total cases. A small proportion of these embryos will develop to full-term gestation but still be born as stillbirth, and very few are born alive [3,5,6]. Trisomy 12, found in this study, is a lethal chromosomal abnormality and often ends up as a first-trimester spontaneous abortion. This is due to the very disorganized development of the embryo in the early stages of embryogenesis [3,6]. The trisomy 21 karyotype accompanied by monosomy 16 found in this study is also a lethal aneuploidy. Isolated monosomy 16 is already a lethal disorder, especially with the addition of trisomy 21, which causes this disorder to worsen, and the fetus experiences spontaneous abortion [3,19,20].

In this study, three samples showed Mos 46, XY/46, XX karyotype, which could be confined placental mosaicism (CPM) in a single fetus or twin pregnancy with both male (46, XY) and female fetus (46, XX) or karyotype of a male fetus (XY) with maternal cell contamination (XX). Confined placental mosaicism is a dis-concordance between the placenta's and fetus's karyotypes [21-23]. If we analyze the three patients with CPM, we find several possibilities. In samples with Mos 47, XX,+21/46, XX/46, XY, it can be caused by pregnancy with a single fetus with a three cell-line CPM or a triplet with one XX cell-line, one XY cell-line, and one XX with trisomy 21 cell-line; or it can also be twin pregnancies with karyotypes 46, XY and 47, XX,+21 accompanied by MCC. The Mos 47, XX,+12/46XX sample can be interpreted as the possibility of a CPM in a single pregnancy or a female fetus with trisomy 12 who has MCC. In samples with 46, XY/46, XX, it can be interpreted as MCC or twin pregnancy. Because overall analysis shows the majority of XX karyotypes, this sample is more likely to be a twin pregnancy.

Conclusion

The results of this study indicated that the success rates for culture and diagnosis were higher in good samples (no necrotic tissue, blood clots and mucus were found). Chromosomal abnormalities in this study included Turner Syndrome, trisomy 12, trisomy 21 with monosomy 16 and mosaicism. We find that the better the sample quality, the higher the

diagnostic success rate. Therefore, it is necessary to make requirements for sample acceptance, and when samples are received, a macroscopic examination must be carried out to determine whether the sample can be processed. In addition, an examination must also be carried out with a dissection microscope to see the presence of chorionic villi. If no chorionic villi are found, culture should not be continued because of the high failure rate.

Acknowledgement

We would like to thank all for providing assistance with the manuscript preparation. We also thank everyone involved in the collection and interpretation of data.

Conflict of Interest

There are no conflicts of interest to declare.

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DOI: 10.31579/2642-9756/153