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Full Length Research Paper

Identification of cytogenetic alterations in infertile couples experiencing repeated spontaneous abortions - using Giemsa Trypsin Giemsa banding (GTG)

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The most significant complication of pregnancy is repeated spontaneous abortions. The incidence of Chromosomal Instability (CI) association is high in couple experiencing two or more recurrent miscarriages. The altered centromere functions may have an increased risk for CI and this leads to spontaneous abortion due to cell division errors. In this study we aimed to screen karyotype results in couples who were referred for infertility and also to find the rate of Chromosomal Abnormalities (CA) in couples with recurrent pregnancy loss. To find out this anomaly blood cultures were performed in a series of 30 women with repeated spontaneous abortions and also in their husbands. Therefore, this study was designed to identify the frequency of cytogenetic abnormalities in infertile couples. Peripheral blood cultures were set up according to standard protocols and 50 G-banded metaphases were analyzed in each case according to ISCN (1995). Numerical and structural Chromosomal Abnormalities (CA) were detected in infertile cases. Among 30 couples, 10 (80%) showed structural aberrations, and 2 (10%) showed numerical aberrations. In addition 2 (10%) individuals were found to have chromosome variants. Among structural abnormalities that formed the largest group of chromosomal anomalies, reciprocal translocations were seen in 4 cases (20%), which frequently involved chromosomes 4, 8, 9, 13, 14, 17, 18 and Y. Apart from these major chromosomal abnormalities, chromosome variants were found in 2 cases, which includes variations in Y chromosome. The observation in the present study shows the patients with genetic alterations may be predispose to cell division errors due to chromosome instability and thus may lead to spontaneous abortion.

Key words: Infertility, Chromosomal Abnormality (CA), metaphase analysis, spontaneous abortion.

INTRODUCTION

Infertility is a condition where there is a failure to conceive after at least one year of unprotected intercourse. It affects approximately 15% of couples in reproductive age (Kleiman et al., 1999). In half of the couples, causes are male-related, associated with impaired spermatogenesis (de Kretser, 1997). Consensus is that an understanding of the fundamentals of (MI) is an important part of providing complete urologic care to MI (Quallich, 2006). Among the variety of reasons for male infertility, genetic factors in about 30% of infertile males should be taken into account, including Chromosomal Abnormalities (CAs) and gene mutations. The importance of human reproductive wastage has been increasingly emphasized during recent years. Various studies have

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confirmed that the total frequency of chromosome anomalies was 50-60%.The highest frequency of chromosome defects is seen in early spontaneous abortions.

The overall incidence of a chromosomal factor in infertile males ranges between 2 to 8%, with a mean value of 5% (Foresta et al., 2002). Approximately 5 -10% oligozoospermic cases and 15 - 20% azoospermic cases harbor genetic abnormalities. The most common type of karyotype abnormality observed in infertility is represented by Klinefelter's Syndrome (KS) and also Y chromosome long arm micro deletions which is described as the most frequent chromosomal alteration (Yoshida et al., 1996). Infertile men have a higher incidence of CA than the general population (Zhang and Lu, 2004). Genetic abnormalities may arrest germ cell production and maturation or lead to the production of nonfunctional spermatozoa. They could involve gene abnormalities affecting numerous genes localized on several chromosomes, 40 - 50% have qualitative or quantitative abnormality in sperm production. In 4 - 8% of couples with recurrent pregnancy loss, at least one of the partners has CA that probably contains chromosomal abnormallities. Maternal problems consist of uteral malformations, immunological factors, endocrine problems and so on (Hassold and Chiu, 1985). Most women with a history of recurrent abortion will be under the care of a gynecologist, who will have already searched for a gynecological cause and will have excluded most serious maternal disorders (Cowchock et al., 1993). However, most spontaneous miscarriages are caused by chromosomal abnormalities in the embryo or fetus (Nybo-Andersen et al., 2004; Tiepolo and Zuffardi, 1976). Results of the numerous studies showed that approximately 50 to 80% of all pregnancy losses depend on the maternal and gestational age at the time of loss, caused by chromosomal abnormalities (Kobayashi et al., 1994; Reijo et al., 1996; Dohle et al., 2002; Boue and Gallano, 1984; Mattei et al., 1981; Lee and silver, 2000). The present study aims to evaluate the karyotypic results from peripheral blood of couples who were referred to Human genetics lab due to secondary infertility in women, Oligozoospermia in male, and also to find the rate of CAs in couples with repeated spontaneous abortions.

MATERIALS AND METHODS

Subject recruitment

The infertile subjects were recruited from the South Indian population from various infertility clinics. A total of 30 infertile couples experiencing three years of infertility were examined. The age of the referred female subjects ranged from 21-40 yrs, while the age of male subjects ranged from 25-42 yrs.

Cytogenetic study

5 milliliter of peripheral blood from experimentals and control subjects were collected using heparinized syringe for leucocyte culture following the method of Moorhead (1960) and Hungerford (1978). Whole blood cultures were cultivated using 4 ml of Rosewell Park Memorial Institute (RPMI 1640) culture medium, 1 ml Fetal Bovine Serum (FBS), Streptomycin, 50 µl PHA, 0.5 ml of blood was added and culture vials were incubated in 5% CO_2 at 37°C for 48 h. At 46^{th} h the cells were arrested by adding Colchicine at final concentration of 1 mg/1 ml. The cultures were further incubated for 2 h. The samples were then harvested by centrifuging at 1000 rpm for 10 min. The supernatant was discarded and the cell pellet was treated with 0.45% of 5 ml prewarmed potassium chloride and incubated at 37°C for 20 min.

The sample was again centrifuged at 1000 rpm for 10 min. The supernatant was discarded and cell pellet was washed with Carnoy's fixative thrice. Care was taken to completely homogenize the solution using vortex to avoid clumping of cells. After third wash the Carnoy's fixative was added and then the cell pellet was suspended in a known volume of fixative. From the cell suspension three drops, were allowed to fall on a clean pre-cooled microscopic slide at a height of 1 m, and warmed on a slide warmer at 35 to 50°C. Slides were air dried. Slides were subjected to Giemse Trypsin Giemsa (GTG) banding. The slides bearing chromosome spreads were treated with 0.25% trypsin for 3 to 10 s. This enabled the digestion of the cell membrane and the cytoplasm and enhanced good exposure of the metaphase chromosomes. After trypsin treatment, the slides were stained in 4% buffered Giemsa solution for 3 min, washed with distilled water and then air-dried. Metaphases were screened and interpreted according to the International System for Human Cytogenetic Nomenclature (ISCN 1995).

RESULTS

Among 30 couples (60 cases) studied, CAs were found in 14 subjects including 9 males and 5 females. Among 14 subjects, 10 (80%) showed structural aberrations, and 2 (10%) showed numerical aberrations. In addition 2 (10%) individuals were found to have chromosome variants. Among structural abnormalities that formed the largest group of chromosomal anomalies, reciprocal translocations were seen in 4 cases (20%), which frequently involved chromosomes 4, 8, 9, 13, 14, 17, 18. Apart from these major CAs, chromosome variants were found in 2 cases, which included variations in Y chromosome (Table 1).

Table 2 shows 8 cases with 1 abortion (20.00%), 12 cases with 2 abortions (50.00%), 6 cases with 3 abortions (10.00%), 2 cases with 4 abortions (5.00%), 2 cases with 5 abortions (5.00%). It has been observed that more number of couples has 2 spontaneous abortions. From the Table 3 it was evident that three males whose wives have experienced 2, 1 and 3 SA showed 12, 15 and 24% of premature centromeric division cells respectively. A female with 2 spontaneous abortions shows 45% of PCD Cells. In the present study it was observed that PCD cells were observed in 4 individuals out of 30 spontaneous abortion couples (17.5%). Only few cells are showing PCD. But they showed normal 46, XX (female) and 46, XY (male) chromosomal complement in other cells. Table 4 shows the frequency of CAs in which 30% showing abnormal karyotype whereas 70% shows normal karyotype.

Table 1. Chromosomal abnormalities in Infertility patients.

Particulars	Sex/Age	No. of abortions	Group	Mosaic	Balanced translocation	Deletion	Inversion	Chromosome abnormalities
1	Male/35	2	3	*				47,XXY /46, XY
2	Female/30	3	2			*		46, XX,14P$^-$
3	Male/31	2	2	*				46, XY,7P- /46,XY
4	Male/33	2	2			*		46, XY,Yq-
5	Male/32	1	1			*		46, XY,Yq-
6	Female/26	1	1		*			46, XX, t(13;14) (p11;q11)
7	Male/28	3	1				*	46, XY inv (9) (p15:q13)
8	Male /30	3	2		*			46, XY t(8;18) (q23:p23)
9	Female/35	5	3	*				46, XX /45, X
10	Female/32	4	2	*				46, XX, /45, X
11	Male/36	2	3		*			46, XY t(7;8) (q11:p11)
12	Female/36	1	3		*			46, XX, t(4;17) (p16;q24)
13	Male/30	3	2	*				46, XY /45,X
14	Male/34	2	2			*		46, XY,Yq -

Group 1 - 25-29, Group 2 - 29-34, Group 3 - >34.

Table 2. Number of abortions in 30 women with the history of spontaneous abortions

No. of abortions	Number	Percentage
1	8	20.00
2	12	50.00
3	6	10.00
4	2	5.00
5	2	5.00

Table 3. Percentage of premature centromeric division (PCD) cells in lymphocyte cultures.

S/No.	Sex	% of PCD cells	No. of abortions	Karyotype
1	Male	12	2	46, XY
2	Male	15	1	46, XY
3	Male	24	3	46, XY
4	Female	45	2	46, XX

DISCUSSION

Recurrent abortion is a difficult medical problem happening in about 1-2% of fertile women (Yoshida et al., 1996). Male infertility can be the result of abnormalities in hormonal control, spermatogenic disorders, disruption of sperm transport and maturation, failure of sperm oocyte interactions and coital disorders that limit the exposure of the oocyte to sperm (Irvine, 1998). The eukaryotic cell genome is unstable. This instability of some crucial genes may cause errors with pathological consequences such as infertility and mental retardation. MI due to severe oligozoospermia and azoospermia has been associated with a number of genetic risk factors. Genetic analysis

Table 4. Frequency of the chromosomal abnormality.

Cytogenetic result	No.	Percentage
Mosaic	5	8.3
Balance translocation	4	6.7
Deletion	4	6.7
Premature centromere	4	6.7
Inversion	1	1.67 (30)
Normal	42	70
Sum	60	100

identified 16/150 (10.6%) abnormal karyotypes, 8/150 (5.3%) AZFc deletions and 14/150 (9.3%) cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations. Microdeletions in the azoospermia factor (AZF) region of the Y chromosome were first reported in azoospermic patients by Tiepolo and Zuffardi 1976. It was then found that patients with severe oligozoospermia also might result from severe deletion of the AZF gene on the Y chromosome (Kobayashi et al., 1994; Reijo et al., 1996).

Recent studies also have shown that mutations in the mitochondrial genome in the genes involved for oxidative phosphorylation may lead to oligospermia and production of morphologically abnormal sperms with impaired motility (Simoni et al., 2004). Different abnormal karyotypes, sex-chromosome aneuploidies, translocations and marker chromosome have been reported in male and female infertility (Dohle et al., 2002). Interestingly we found one case with t (13p; 14q) was observed. Studies indicate that when the Robertsonian translocation is maternal, there is greater risk that the fetus will exhibit an unbalanced phenotype (Boue et al., 1984).

Deletions were observed in four cases but there was no loss of deleted fragment as it was retained in all the metaphases analyzed. It is thus assumed that gain or loss of this fragment during gametogenesis could have led to the chromosomal imbalance in the fetus resulting in spontaneous abortion. Numerical aberrations were found in one case, which included mosaics with two or three cell lines. In the present study a number of major CAs were observed (Table 1). Inversions were very rare. Only one case was found to have pericentric region of chromosome 4. The risk of pregnancy loss with a chromosome inversion is not known. It has been estimated that the risk of miscarriage in couples with reciprocal translocations is approximately 25-50% whereas with Robertsonian translocation it is approximately 25% (Lee and Silver, 2000). In one case pericentric inversion of chromosome 9 was identified but in most of the studies pericentric inversion of chromosome 4 has been observed in cases without any history of RSA (Mattei et al., 1981). In 1990, Murthy and Prabhakara reported a female with a history of spontaneous abortion. Her chromosomal analysis revealed 46, XX with pericentric inversion of 9q while her husband was normal. Metaphases analysis of the female showed 20.5% cells with premature centromere division, 4% cells with endoreduplication and 2% with polyploidy. These frequencies were considerably higher as compared to a normal control. This indicates the possibility of inversion 9 to have a role in the etiology of RSA. But more data and molecular genetic studies are needed to confirm this possibility.

In future, to complete this study, cytogenetic analysis of the abortuses should be done, which help the family in other pregnancies. Peripheral blood karyotyping of both partners should be considered as a required examination of couples with recurrent miscarriage but, the influence of other factors, such as family history of miscarriages, should be considered when deciding who should be karyotyped. When a balanced translocation is recognized, it should be recommended to have a triple screening test followed by prenatal diagnosis to see if they are the result of unbalanced translocations. In conclusion, CA is one of the main causative factors for spontaneous abortion and this association is higher as maternal age increases. Therefore, cytogenetic tests are recommended for patients with a history of abortion, especially in women with a higher age to identify chromosomal alterations. Chromosomal analysis is strongly suggested, particularly in those who suffer fertility problems. This study strongly point out the importance of karyotyping in evaluating couples who need assisted reproductive technologies for genetic counseling.

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