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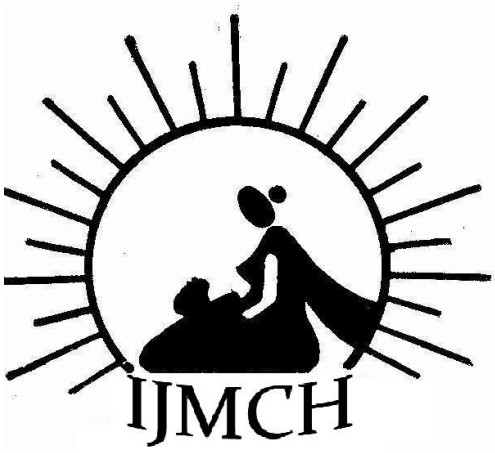

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Recurrent birth losses besides disorders of fertility are important issues in pregnancy wastage especially as about one-fifth of all conceptuses abort spontaneously with chromosomal anomalies noticeably accounting for the early fetal loss.

Direct chromosomal analysis of chorionic villi in spontaneous abortions

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Recurrent birth losses besides disorders of fertility are important issues in pregnancy wastage especially as about one-fifth of all conceptuses abort spontaneously with chromosomal anomalies noticeably accounting for the early fetal loss. In the present study chorionic villi samples obtained from spontaneous abortuses were directly processed for cytogenetic analysis. Though no numerical or structural chromosomal aberrations were observed; a few samples evinced the phenomenon of premature centromere division/centromere separation. A male female sex ratio favouring female fetuses was observed. The average gestational age of the fetus was 8.5wks, with male fetuses aborting in older mothers and female fetuses in younger mothers. The mean maternal age was 27. 55yrs. The method can be used for rapid analysis of the cytogenetic causes of spontaneous abortions.

Key Words: Chorionic Villi, Sex ratio, Abortuses, Chromosomal aberrations.

Though on one hand there is an urgent need for population control encompassing a comprehensive multidisciplinary approach integrating various components of reproductive health (1), yet the issues of infertility and recurrent birth loss are a matter of concern from the point of view of pregnancy wastage. Disorders of fertility in couples have been reported in the order of 5-25% (2, 3) while in India alone, about 15% of the couples are infertile (4). The diminished rate of fecund ability is accountable by pregnancy wastage either before or during the process of implantation. A further loss of 15-20% of conceptuses as spontaneous abortions has been reported in clinically recognized pregnancies in the general population (5). The Shah Committee in India has suggested that for every 73 live births, there were 25 abortions including 10 spontaneous abortions and has further estimated that of 11.2 million abortions in 1991, 4.5 million were spontaneous (6).

The possible etiologic factors resulting in spontaneous abortions include genetic, hormonal and anatomic causes. In fact, about 50% of early fetal loss is because of fetal chromosome abnormalities (7), primarily polyploidy, trisomy and monosomy (5). Cytogenetic analysis of abortuses hence gains importance for clinical, therapeutic and prognostic reasons as it can contribute to prenatal diagnosis in subsequent pregnancies. Karyotyping spontaneous abortuses in different populations can further provide information on the prevalent frequency and types of chromosome anomalies in various ethnic groups, on their etiology, recurrence risk and the suspected activity of mutagens in a population (8,9,10).

Conventional tissue culture methods being laborious expensive and time consuming earlier hampered a routine cytogenetic analysis of abortions. However a method for obtaining metaphase spreads in 5hrs. from uncultured villi, biopsied at 8-12 weeks of gestation (11), has paved the way for directly analysing the chromosomes of chorionic villi both, for prenatal diagnostics and for investigating spontaneous abortions (7,12,13,14,15). Chorionic villi (CY) are coral-like projections surrounding the embryonic sac. They have the same genetic characteristics as the fetus as they originate from the trophoblast (16). The method of direct chromosomal preparations from CY samples has been reported to have a satisfactory karyotyping success rate and minimal maternal cell contamination (13). Of the three cell types present in the chorionic villi, the cytotrophoblasts show rapid (1 in 400 cells) cell division (17) and so can be exploited for direct chromosomal preparations without long-term cuhuring (18).

This communication reports direct chromosomal analysis of spontaneous abortions from chorionic villi samples using a slightly modified version of the earlier methods (11,19).

MATERIALS AND METHODS: Chorionic villi samples from mothers undergoing spontaneous abortions were obtained from the local Government Hospital, Family Planning Centres and Nursing and Maternity Homes in 1997-98. About 10 mg of villi is sufficient to facilitate both, direct and long term cultures (20). Therefore, 5-10 mg sample sizes between 8-12 weeks of gestation were collected by the Dilation and Curettage (D&C) method under aseptic conditions by the attending gynaecologists. These were kept in containers having heparinized Hank's Balanced Salt Solution (HBSS, Hi-Media, India) and were transported to the laboratory on ice for processing on the same day for cytogenetic analysis. Data with respect to personal, medical, occupational and genetic histories as well as dietary habits and life-styles were collected for each mother on pre-prepared questionnaires (21). Detailed pedigrees were also recorded to get information on their family histories. The gestational age of the aborting fetus was calculated from the first day of LMP (Last Menstrual Period) to the day of abortion in the mother.

The salient features in the processing of samples in HBSS included removal of the maternal decidua, blood and mucus using a forceps and needle under a dissection microscope (lOX). Further steps involved maceration of the tissue into manageable clumps and its incubation for 24hrs at 37°C in RPMI 1640 (5ml; Hi-Media, India) with 5% FCS (0.25ml; CSIR, India). A pre-treatment with colcemid (0. 06~g/ml; Sigma, USA) for 2hrs at 37°C arrested the chromosomes at the metaphase plate. Hypotonic treatment (1% Sodium Citrate for 30-35 min) enabled the swelling of cells for the spread of chromosomes. These was replaced by chilled fixative (3 methanol: 1 glacial acetic acid) for an overnight treatment at 4°C. Successive washings in fixative (twice), absolute methanol (70%, 50%, 20%) and in distilled water were further given. A 60% acetic acid treatment for 10 min. facilitated the dissociation of cells from the tiny clumps of tissue. Using a self-fabricated bent-pipette, the cell suspension was evenly distributed. across a pre-warmed coded slide (on 40°C set hot plate). The preparations were allowed to air- dry and staining was carried out in 5% Giemsa stain for 30min. The slides were then washed in tap water and allowed to air-dry. Well-

spread and well-stained metaphase plates were scanned at 40X initially, followed by scoring for chromosomal aberrations at 100X. For each sample, 15-25 spreads were screened for numerical (aneuploidy, polyploidy), structural (gaps, breaks, a centric fragments) and other chromosomal anomalies, so as to also rule out any low-grade mosaicism. A record of the total chromosomal count and the number of G-group chromosomes was also made.

RESULTS AND DISCUSSION: In the gradual process of standardization through experimentation and observation 23 CY samples were initially processed. Thereafter, another 18 samples were analysed for causes of pregnancy wastage. Table 1 depicts the general information regarding the patients and their spouses. The age of the patients ranged from 16-39yrs, and while some had been married for as less as 6 months others had a married life of up to 17yrs. Of the 18 cases, 5 had normal health status, 3 were healthy and the rest had a weak body constitution, probably a reflection of their obstetric histories/medical reasons. Table 2 presents data on the reproductive histories of these patients. Except for the young, the newly married women and one older mother (34yrs), all had live children. Induced abortions were rare while spontaneous abortions ranged from 1-3. The gestational age for the latter was on an average 8.5wks (7-10wks).

Table I : General Information on Undergoing Spontaneous Abortion

Patients code	Age in years	Health status	Spouse's			
			Occupation	Exposure History	Smoking Habits	Alcohol intake Per week c
001	19	Healthy	Shopkeeper	Dyes	-	++++
002	17	Weak	Hawker	-	-	+++
003	18	Weak	Labourer	-	+[a]	-
004	20	Normal	Office worker	-	-	++
005	16	Weak	Field worker	Fertilizer Pesticide	+	-
006	19	Weak	Rikshawpuller	-	+	-
007	17	Weak	Rikshawpuller	-	+[b]	-
008	28	Healthy	Labourer	Dyes Solvents	-	-
009	35	Normal	Office worker	-	-	+
010	27	Weak	Hawker	-	-	-
011	32	Weak	Plumber	Pesticides	-	-
012	32	Normal	Labourer	-	-	-
013	35	Normal	Labourer	-	-	+
014	39	Weak	Carpenter	Sawdust	-	+++
015	37	Weak	Mason	Cement	+	-
016	34	Normal	Rickshawpuller	-	+	+++
017	36	Healthy	Teacher	Pesticides	-	-
018	35	Weak	Painter	Solvents	-	-

[a] *patient earlier smoked bidis* [b] *smoked bidis chewed betel leaf with chuna daily*

[c] *++++ 2500- 3500ml +++ 1250- 2000 ml ++750- 2000 ml +250- 1000 ml*

Table 2: Reproductive Histories of Patients Undergoing Spontaneous Abortions

Patients Code	Age Yrs	Marriage Yrs	No of live Children		No of Abortions		Time of abortion weeks
			Male	Female	Spontaneous	Induced	
001	19	2 ½	1	-	2	-	9
002	17	2	-	-	1	-	8
003	18	2 ½	-	1	1	-	8
004	20	3 ½	1	1	1	-	8
005	16	6 mths	-	-	1	-	9
006	19	11mths	-	-	2	-	8
007	17	10mths	-	-	2	-	8
008	28	9	1	1	1	-	8
009	35	10	1	1	1	-	9
010	27	7	1	1	1	1	10
011	32	12	2	1	1	-	9
012	32	13	1	2	1	1	7
013	35	12	-	2	1	-	9
014	39	15	-	1	1	2	8
015	37	17	1	2	2	-	10
016	34	10	-	-	3	-	8
017	36	11	1	-	1	-	8
018	35	13	1	1	1	-	9

Table 3: Chromosomal Analysis from Chorionic Villi Samples Processed from Spontaneously Aborting Fetuses

Patients Code	Metaphases Scanned	Total chromosomal count	No of G group chromosomes	Chromosomal sex of the fetus	Chromosomal abnormalities
001	20	46	4	XX	-
002	20	46	5	XY	-
003	20	46	4	XX	-
004	20	46	4	XX	-
005	17	46	5	XY	-
006	20	46	4	XX	-
007	25	46	4	XX	-
008	20	46	4	XX	-
009	15	46	5	XY	-
010	25	46	4	XX	+
011	15	46	4	XX	+
012	20	46	5	XY	-
013	25	46	4	XX	-
014	15	46	5	XY	+
015	20	46	5	XY	-
016	15	46	4	XX	+
017	15	46	5	XY	-
018	18	46	4	XX	-

Table 4: Chromosomal Anomaly Details in Spontaneous Abortuses

Patients Code	Maternal Age yrs	Live Child-ren (no)	Sponta-neous Abor-tion (no)	Gesta-tional Age wks	Total Metap-hases Scored	Chro-Mosomal Sex of fetus	Metapha-ses with Centr-omere Separation No. (%)	Chromo-Somes with Centr-omere Separation (no)
010	27	2	1	10	25	XX	1 (4.0)	19
011	32	3	1	9	15	XX	2(13.3)	16 & 8
014	39	1	1	8	15	XY	4 (26.6)	Almost 46 in each
016	34	-	3	8	15	XY	2 (13.3)	20 & 9

Table5: Mother's Age, Gestational Age & Sex Ratio of Spontaneously Aborting Fetuses

Maternal Age in yrs	*Spontaneously aborting fetuses*						*Sex ratio (M/F)*
Range	**Average**	**Male**			**Female**		
		No.	**AGA#**	**AMA^**	**No.**	**AGA#**	**AMA^**	
16-20	18.00	2	8.50	16.50	5	8.20	18.60	0.40
27-34	**30.60**	**1**	**7.00**	**32.00**	**4**	**8.75**	**30.25**	**0.25**
35-39	36.17	4	8.75	36.75	2	9.00	35.00	2.00
Overall	**27.55**	**7**	**8.43**	**30.29**	**11**	**8.54**	**25.82**	**0.64**

 # AGA- average gestational age in weeks^ AMA- average maternal age in years

Cytogenetic analysis (Tables 3 and 4) revealed no numerical or structural aberrations on scoring the solid- stained preparations. The chromosomal count was normal (2n = 46) while the G-group count showed a preponderance of females (n = 11) as compared to male fetuses (n = 7). In 4 mothers, centromere separation phenomenon was recorded--while a 27yrs old mother had only I cell out of 25 metaphases with 19 chromosomes showing centromere separation, older mothers (32-39yrs) had 2-4 cells out of 15-20 metaphases with 9-46 chromosomes showing this phenomenon (also called premature centromere division). PCD has earlier been reported to lead to aneuploidy via non-disjunction with an increased frequency in older women (22). It also has been found to be associated with chronic myelogenous leukemia (23), Fanconi's anemia, ataxia telangiectasia (24) as well as chromosomal loss through anaphase lag during ageing (25). The association of PCD with aneuploidy (26) and recurrent spontaneous abortions (27) have also been reported though the sparse number of metaphases with PCD in the present sample cannot be cleat cut indications for the pregnancy wastage. Other genetic (parental ffi, A sharing (28). mutated early developmental genes (29) as well as non-genetic factors cannot be ruled out.

The overall male: female sex ratio for the abortuses (Table 5) was 0.64, which] however, varied at different maternal ages. The average gestational age of the fetuses was 8.5 weeks while the average maternal age for male and female fetuses was rather variant (30.29 and 25.82yrs, respectively), indicating that male fetuses aborted in old mothers and female fetuses in younger mothers. In the newborn population (30) the sex ratio reported was 1.06-1.07; in normal pregnancies monitored by CVS, it was 1.17 (31), being 1.16 in induced abortions (32). Sex ratios favouring females have also been reported: 0.71(7), 0.76 (33) and 0.77 (31). Among biological causes hypothesized for altered sex ratio is that failures during X-chromosome in-or re-activation could generate abnormal functional X and/or autosomal genes at early embryonic stages, leading on to an abortive pregnancy (31), i.e. a kind of female-specific developmental disadvantage at early stages of gestation (7).

In conclusions, the direct chromosomal preparation method using chorionic villi samples is less laborious and can help in providing rapid cytogenetic diagnosis. Its routine application could contribute to the identification of mutated early developmental genes (29) causing pregnancy wastage of chromosomally normal conceptuses. In the present study also, though no chromosomal abnormalities have been observed, it has a prognostic application because after abortion with normal karyotypes, in most cases normal karyotypes occur again (34,35,36). The study further diverts our attention to other genetic and non-genetic causes of spontaneous abortions.

REFERENCES:

1. ICMR. India's needs and priorities in fertility regulation research. ICMR Bulletin 1999,29 (2) : 17-27.

2. Dash RJ .The sunset of infertility .The Tribune, 1996, April 17. 3. Howkins J, Bourne G. In: Shaw's book of Gynaecology. Eds. Padubidri VG, Daftary SN. BI Publications, Mumbai 1998: pp 208-223.

4. Dutta DC. Textbook of Gynaecology. New Central Book Agency (P) Ltd., Calcutta 1994.

5. Wentz AC, Cartwright PS. Recurrent and spontaneous abortions. In: Novak's Textbook of Gynaecology. Eds. Jones II HW, Wentz AC, Burnett. LS. Williams and Wilkins, Baltimore, 1988, pp. 101-139.

6. Chhabra R. Abortions in India: An overview. Demogr. India 1996,25 : 83. 7. Eiben B, Bartels I, Bahr-Porsch S, Borgmann S, Gatz G, Gillert G, Goebel R, Hansmann W, Hentemann M, Osmers R, Rosekolp R, Hansmann I. Cytogenetic analysis of 750 spontaneous abortions with the direct preparation method of chorionic villi and its implications for studying genetic causes of pregnancy wastage. Am J Hum Genet 1990, 47 : 656 - 663.

8. Hassold T, Chen N, Funkhouser J, Jooss T, Manuel B, Matsuura J, Matsuyama A, et al. A cytogenetic study of 1000 abortions. Ann Hum Genet 1980,44 : 151-164.

9. Yamamoto M, Fujimori R, Ito T, Watanabe M, and Watanabe G. Causes of chromosome anomalies suggested by cytogenetic epidemiology of induced abortions. Hum Genet 1982, 60 : 360 -364.

10. Hansmann I, Beermann F, Hummler E, Pabst B, Theuring F. Mechanisms of non-disjunction and implications for testing aneugens. BioI Zentralbl 1989,108 : 323-331.

11. Simoni G, Brambati B, Danesino C, Rosella F, Terzoli GL, Ferrari M, Fraccaro M. Efficient direct chromosome analysis and enzyme detemlination from CVS in the first trimester of pregnancy. Hum Genet 1983, 63: 349- 357.

12. Ohno M, Maeda T Matsunobu A. A cytogenetic study of spontaneous abortions with direct analysis of chorionic villi. Obstet Gynecol 1991. 77 : 394 -398.

13. Blakemore KJ, Watson MS, Samuelson J, Breg WR, Mahoney MJ. A method for processing first trimester chorionic villous biopsies for cytogenetic analysis. Am J Hum Genet 1984.36 : 1386- 1393.

14. Miny P, Holzgreve W, Horst J, et al. Limb abnormalities and chorionic villus sampling. Lancet 1991,337 : 1423.

15. Ledbetter DH, Martin AO, Verlinsky Y, et al. Cytogenetic results from the US collaborative study ofCVS. Prenat Diag 1992. 12: 317-346.

16. Brambati B, Oldrini A. CVS for first trimester fetal diagnosis. Contemp Obstet Gynecol1985, 25 : 94-104.

17. Gelehrter TD, Collins FS. Principles of Medical Genetics. Williams and Wilkins. Baltimore. U.S.A. 1990, pp 278-280.

18. Smidt-Iensen S, Chistensen Band Lind AM. Prenat Diagn 1989,9 : 309. 19. Flora E. Nisand I, Flori I, Dellenbach P, Ruch IV. Direct fetal chromosome studies from chorionic villi. Prenat. Diagn. 1985,5 : 287-289.

20. Rooney DE, Czepulkowski BH. Prenatal diagnosis and tissue culture. In : Human cytogenetics- A practical approach. Eds Rooney DE, Czepulkowski BH. IRLPress, Oxford, New York, 1992, Vol. 1, pp. 55- 90.

21. Carrano A V, Natarajan AT. Minimal criteria for population monitoring using cytogenetic techniques. Handouts prepared for WHO and IARC sponsored. International course on "Health monitoring of Human Population exposed to Mutagens and Carcinogens" at Cancer Research Institute, Tata Memorial Centre, Bombay. Nov., 1986, pp 1-40.

22. Fitzgerald PH. A mechanism of X chromosome aneuploidy in lymphocytes of ageing women. Humangenetik 1975.28 (2) : 153 -158.

23. Vig BS. Centromere structure and function in neoplasia. Abstracts -Ist International symposium on Genetics, Health ~nd Disease, GNDU, Amritsar, Feb. 6-9, 1990.

24. Mehes K, Buhler EM. Premat:ure centromere division: a possible manifestation of chromosome instability. Am I Med Genet 1995, 56 (1) : 76- 79.

25. Stone IF, Sandberg AA. Sex chromosome aneuploidy and aging. Mutat Res 1995,338 : 107- 113.

26. Mehes K. Non-random centromere division: A mechanism of non- disjunction causing aneuploidy. Hum Hered 1978,28 : 255-260.

27. Fitzgerald PH, Pickering AF, Mercer IM, Miethke PM. Premature centromere division: a mechanism of non-disjunction causing X chromosome aneuploidy in somatic cells of man. Ann Hum Genet 1986, 38 : 417- 428.

28. Thomas ML, Harger IH, Wagener DK, Rabin BS, Gill TI. HLA sharing and spontaneous abortion in humans. Am I Obstet Gynaecol, 1985, 151 : 1053 -1058.

29. Rossant I, Ioyner AZ. Towards a molecular -genetic analysis of mammalian development. Trends Genet 1989, 82 : 377-385.

30. Visaria PM. Sex ratio at birth in territories with a relatively complete registration. Eugenics Q 1967, 14 : 132-142.

31. Bartels I, Hansmann I, Eiben B. Excess of females in chromosomally normal spontaneous abortuses. Am I Med Genet 1990,5 : 297-298.

32. Zhou X, Tong H, Wong S, Shen Q, Fu X, Cui Y. Chromosome abnormalities in early pregnancy analyzed by direct chromosome preparation of chorionic villi. Hum Genet 1989, 83 : 277-279.

33. Eiben B, Borgmann S, Schuble I, Hansmann I. A cytogenetic study directly from 140 spontaneous abortion. Hum Genet 1987,77 : 137-141.

34. Bou'e J, Bou'e A, Lazar P. Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous abortions. Teratology 1975, 12 : 11-26.

35. Lauritsen JG. Aetiology of spontaneous abortions: a cytogenetic and epidemiological study of 288 abortuses and their parents. Acta Obstet Gynaecol Scand (Suppl) 1976,52 : 1-29.

36. Warburton D, Kline J, Stein Z, Hutzler M, Chiu A, Hassold T. Does the karyotype of a spontaneous abortion predict the karyotype of a subsequent abortion ? -Evidence from 273 women with two karyotyped spontaneous abortions. Am J Hum Genet 1987,41 : 465- 483.