

Original article

**Evidence of Increased Genetic Instabilities in Couples
Undergoing Assisted Reproductive Techniques**

Dinesh Roy D¹, Alex K Ittyavirah², Laly Alex², Mohanachandran Nair P³, Subbiah A⁴, Stephen J²

1. Genetika, Centre for Advanced Genetic Studies, Trivandrum – 695 024

2. Ittyavirah Scan & Genetic Research, Trivandrum – 695 011

3. Dept. of Demography, University of Kerala, Trivandrum

4. Dept. of Population Studies, Annamalai University, Tamil Nadu

Summary

Assisted Reproductive Technique (ART) is the last option for couples with infertility who have tried and failed to conceive. ART procedures such as in vitro fertilization and intracytoplasmic sperm injection are generally considered to be safe, but recent studies suggest a small excess of birth defects and low-birth weight in ART children. The primary question regarding ART, especially ICSI is if the technique carries an increased risk of genetic aberrations and malformations in the children. Hence a study was designed on sixty six infertile couples, undergoing ART, for genetic abnormalities if

any, by karyotype analysis, to evaluate the DNA repair pro-efficiency by mutagen induced chromosome sensitivity analysis and to evaluate the extent of somatic DNA damages by Cytokinesis-block micronuclei assay and compare with twenty healthy couples who have live children. Three parallel cultures (A, B and C) were set up for each subject. Constitutional chromosomal anomalies detected in 12.88% study subjects. Infertile husbands showed a mean b/c value of $0.6917 \pm .1288$ (SD) and infertile wives showed $0.6825 \pm .1247$ (SD). 0.5822 and 0.5639 were the mean b/c values of control husbands and wives respectively. The study subjects showed a

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mean CBMN frequency of 13.99 and control subjects showed 9.9. The CBMN frequency of subjects with abnormal karyotype was 16.943 and study subjects with normal karyotype was 13.56. The high incidence of genetic instabilities in the infertile population clearly warrants genetic testing and counseling prior to ICSI/IVF. If pathology is discovered prenatal diagnosis should be performed.

Key words

Infertility; genetic instability; karyotyping; assisted reproductive techniques;

Introduction

Infertility in humans affects 10–15% of couples¹. In vitro fertilization is the last option for couples with infertility who have tried and failed to conceive using standard therapies such as surgery, fertility drugs and artificial insemination. It was successfully applied for the first time in 1978 to bypass complete occlusion of fallopian tubes. Since then, the indications for in vitro fertilization have expanded internationally to include other infertility conditions such as male infertility, endometriosis-associated infertility, immunological infertility and unexplained infertility. Fertility largely depends on the quality of sperm or ovum, which may be affected by environmental and genetic factors².

In recent years, the frequency of assisted reproductive technology (ART) births has increased rapidly to account for 1-2% of all births in many developed countries. ART procedures such as in vitro fertilization and intracytoplasmic sperm injection are generally considered to be safe, but recent studies suggest a small excess of birth defects and low-birth weight in ART children. In addition, several clinical studies have reported an increased frequency of ART conceptions among children with various genetic syndromes³.

ART is defined as those fertility treatments in which both egg and sperm are manipulated in the laboratory; i.e., in vitro fertilization [IVF] and related procedures⁴. Although the use of ART has become a widely accepted and implemented therapy for some forms of infertility, there have always been concerns about the long-term safety of removing and handling the germ line. In a recent large series with a control group from the same population, Hansen et al⁵ compared 1,138 offspring of ART in Australia and also found LBW, as well as a twofold increase in major birth defects. It is impossible to clearly stratify the offspring with birth defects, given that the authors were restricted by confidentiality rules and could not indicate the specific mode of conception for or the number of birth defects in a given child.

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The genome instability, its causes and the possible consequences, especially about fertility status to be understood. This instability can be observed not only on chromosome structure but also on genes. Different chromosomes rearrangements involved in infertility including translocations and Y chromosome deletions are described. The Y chromosome is a model of instability, and this instability is the source of its evolution. The knowledge of all these instability mechanisms is essential to appreciate the risk for the offspring after intracytoplasmic sperm injection. Indeed we go round physiological barriers without a complete understanding of the mechanisms involved. Thus, this is an important challenge for research teams but also for all assisted reproduction centers, dealing with ART. Genome is unstable - the very basis of its evolution. But this is also the cause of mistakes with pathological consequences like infertility and mental retardation⁶.

Sperm DNA integrity is essential for the accurate transmission of genetic information. It has a highly compact and complex structure and is capable of decondensation—features that must be present in order for a sperm to be considered fertile. Any form of sperm chromatin abnormalities or DNA damage may result in male infertility. It was reported that in-vivo fecundity decreases

progressively when >30% of the spermatozoa are identified as having DNA damage⁷.

Mutagen sensitivity, a measure of an individual's intrinsic DNA repair capacity against free radical damage, is a risk factor for various disorders. It is suggested that normal persons are more resistant to mutagens compared to patients with various chromosome instability syndromes and cancer. Through cytogenetic analysis, variation in susceptibility to mutagen induced genomic damage can be quantitated. A positive correlation between chromosome sensitivity to mutagen and deficiency in DNA repair mechanism and individual variations in DNA repair capabilities was reported. Cytogenetic experiments with mutagen can provide an estimation of the individual's DNA repair capacity. The higher the number of individual breaks not repaired, the more deficient the repair mechanism⁸.

One of the most promising methods to assess DNA damage is the CBMN assay, which detects both chromosome and genome alterations in binucleated cells⁹. Owing to the direct correlation between MN production and genomic damage, the CBMN assay applied to human blood peripheral lymphocytes may be considered a reliable method to quantify genome-induced

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chromosome damage and/or genomic instability¹⁰.

The primary question regarding Assisted Reproductive Technique (ART), especially ICSI is, if the technique carries an increased risk of genetic aberrations and malformations in the children. There is also the added risk of mechanical injury to the spindle that could potentially lead to aneuploidy. The primary question regarding Assisted Reproductive Technique (ART), especially ICSI is if the technique carries an increased risk of genetic aberrations and malformations in the children. Moreover Sperm DNA integrity is essential for the accurate transmission of genetic information. By the introduction of various ART procedures, the potential risk of genetic defects, malformations and other adverse consequences for the children associated with the technique has been debated. Hence a study was designed on couples, who undergo ICSI

- 1) To investigate the infertile couples undergoing ART, for genetic abnormalities, if any, by karyotype analysis.
- 2) To evaluate the DNA repair pro-efficiency of subjects undergoing ART by mutagen induced chromosome sensitivity analysis.
- 3) To evaluate the extent of somatic DNA damages by Cytokinesis-block micronuclei (CBMN) assay in peripheral blood

lymphocyte of couple undergoing ART, who suffering with infertility.

Materials and Methods

Sixty six couples who, suffering with varying degrees of infertility, and planning to under go Assisted Reproductive Technology (ART) were selected. These couples were referred for genetic studies to Genetika, Center for Advanced Genetic Studies, Trivandrum and to Ittyavirah Scan and Genetic Research, Trivandrum. Detailed clinical history and other relevant informations were recorded using proforma. 5ml of venous blood was collected aseptically by venepuncture from all these study subjects (husband & wife). Peripheral blood samples were also collected from twenty healthy couples who had live children, as control subjects.

Three parallel cultures were set up for each sample. Culture A, B and C. The culture A was for detecting constitutional chromosome anomalies and culture B was for estimating the DNA repair pro-efficiency of the subjects. Culture C for quantitating the extent of somatic DNA damages by Cytokinesis block micronuclei (CBMN) assay. Lymphocyte microculture¹¹ was performed. For detecting constitutional chromosome anomalies, cell division was arrested at metaphase and Giemsa stained as well as GTG banded¹². GTG banded

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slides were observed under microscope and good quality metaphases were photographed. From the prints each chromosome was cut down and pasted according to the size, position of centromere and the banding pattern. Karyotypes were prepared according to ISCN (1995)¹³ pattern.

For detecting mutagen sensitivity analysis, the cultures were treated with bleomycin (0.03 unit/ml) at S phase¹⁴. At 70th hour all the cultures were treated by colchicine to arrest the cell division at metaphase. Giemsa stained and look for frank chromatid breaks. For mutagen sensitivity, the slides were stained with Geimsa. The frequency of chromatid breaks was considered as a measure of an individual DNA repair capacity. For chromosome sensitivity analysis the mean break/cell (b/c) was calculated. The frequency of breaks was expressed as b/c for comparison. Any individual expression mean b/c value <0.8 was considered as hyposensitive, >0.8 was considered sensitive and >1.0 was considered hypersensitive.

Cytokinesis block micronuclei (CBMN) assay was performed using Cytochalasin B. 0.3ml of whole blood was mixed with 4.7ml of RPMI 1640 medium and added with 10µgm/ml of Phytohaemagglutinin. Cultures were

incubated for 72 hours at 37⁰C. Cytochalasin B (6µgm/ml) was added 44 hour after culture initiation. Cells were then harvested and fixed. For each sample, 1000 binucleated cells were scored for micro nuclei analysis.

Results

Sixty six couples with severe infertility, who undergone ICSI or IVF technique were selected and analyzed. The observations and results were recorded. The age of male partners ranged from 26-45, with a mean age of 34.64 and the age of wives ranged from 19 -38, with a mean age of 28.66. The duration of married life ranged from 1 to 8 years with a mean duration of 3.11. For the comparison of the results 20 healthy fertile couples with husbands mean age of 34.9 and wives mean age of 29.9 and the mean duration of married life of 4.55. Chromosome abnormalities were observed in 6 out of 66 (9.09%) husbands and 11 out of 66 wives (16.66%). So the constitutional chromosomal anomalies detected in the present study were 12.88%. Among the Chromosomal abnormalities, 11 (8.33%) were numerical and 6 (4.5%) were structural. Numerical abnormalities include Klinefelter's syndrome, Turner's syndrome, etc.

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The incidence of chromosome abnormalities is higher in females than the males. The abnormalities associated with Y chromosome were higher among males. This indicates that a small but significant population of infertile male partners has Y chromosome abnormality. Mutagen (Bleomycin) induced mean b/c value of both study husbands and wives were noted. The mean b/c value of husbands was $0.6917 \pm .1288$ (SD) and the mean b/c value of wives was $0.6825 \pm .1247$ (SD). The control husbands showed a mean b/c value of 0.5822 and control wives showed a mean b/c value of 0.5639(**figure 1**)

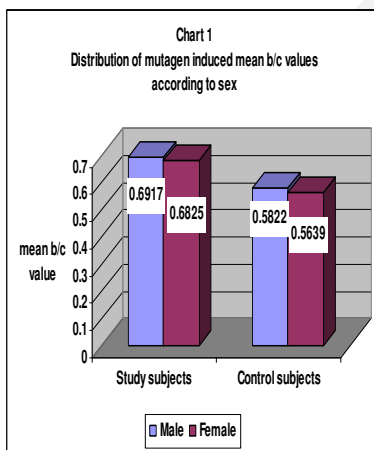


Figure 1
Distribution of mutagen induced mean b/c values according to sex.

25.75% of male subjects were sensitive to bleomycin and 22.72% of female subjects were also in sensitive group. These results strongly suggest that the DNA repair proefficiency of the infertile couples were weaker when compared to healthy fertile population. None of the control subjects were sensitive to bleomycin.

The study demonstrated a higher mean break per cell value among males (0.6917) than females (0.6825). However the incidences of chromosome anomalies were higher among females than males. Hence the potential role of passing these genetic defects to offspring is real and should be considered when infertile couples are counseled about this procedure.

Spontaneous or baseline MN frequencies in cultured human lymphocytes and exfoliated cells provide an index of accumulated genetic damage occurring during the life span of these cells. There were significant differences in mean CBMN frequencies among the subjects. The mean CBMN frequency of study subjects were 13.99 and the mean CBMN frequency of control subjects was 9.9. However the study subjects with abnormal karyotype shows increased frequency of MN. The CBMN frequency of subjects with abnormal karyotype was 16.94, CBMN frequency of

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the study subjects with normal karyotype was 13.56. However the control subjects showed a mean MN frequency of 9.9.

The study demonstrated increased micronucleus frequencies in subjects suffering with infertility, who undergone ART than the fertile control subjects. The mutagen induced mean b/c value of subjects with abnormal karyotype was 0.8611 and the study subjects with normal karyotype showed a mean b/c value of 0.6608. The control subjects showed a mean b/c value of 0.5731 (**Table 1**). (See below references). It may be noted that at all the age group, irrespective of the sex, the b/c value of infertile subjects were always higher than that of the corresponding control group. This is true in the CBMN frequency also (**Table 2**). (See below references).

Discussion

Intracytoplasmic sperm injection (ICSI) has been in clinical use since 1991 and has revolutionized the treatment of severe male infertility. The primary question regarding ICSI is if the technique carries an increased risk of genetic malformations in the children. This has been a special concern since spermatozoa with impaired motility and morphology are often utilized and, further more, immature spermatozoa derived from the epididymis and the testis are also used in combination with this

technique and it may rather be the selection of patients that constitutes a risk. A study on 150 men from couples requesting Intra Cytoplasmic Sperm Injection were investigated for genetic abnormalities, such as constitutive chromosome abnormalities, microdeletions of the Y chromosome (AZf region)¹⁵. Genetic analysis identified 16/150(10.6%) abnormal karyotypes, 8/150 (5.3%) AZFc deletions. Genetic abnormality was identified in 36/150 (24%) men with extreme oligozoospermia and azoospermia. In the present study chromosomal abnormalities were present in 11.36%.

Application of ICSI in these couples can result in offspring with an enhanced risk of unbalanced chromosome complement and male infertility due to transmission of a Y-chromosomal microdeletion. In the present study Y chromosome anomalies were seen in 33.3% of the abnormal male karyotypes reported. Correlating this conclusion with the present study, infertile males may be associated with azoospermia and oligozoospermia due to chromosomal breakages caused by a defective DNA repair mechanism. In the present study mutagen induced mean b/c value of the male infertile subjects was 0.6917 and the mean b/c value of female was 0.6825 the mean b/c value of control subjects were 0.5822 and 0.5639 respectively. From this it is clear that the DNA repair pro-efficiency of

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the control subjects is efficient than infertile couples. Peschka (1999)¹⁶ performed cytogenetic investigations in 781 couples prior to Intra Cytoplasmic Sperm Injection [ICSI] because of severe male infertility or fertilization failures in previous in-vitro fertilization attempts. The present study can be used as a diagnostic tool for chromosomal analysis. The study observed 11.36% of chromosome anomalies in subjects undergoing ART. Moreover these study subjects demonstrated a defective DNA repair compared to control population.

A collaborative retrospective clinical and cytogenetic study was performed¹⁷, to assess the frequency of chromosomal aberrations in French candidates for Intra Cytoplasmic Sperm Injection [ICSI]. The karyotypes of 3208 patients [2196 men (68.4 %), 1012 (31.6 %) women] included in ICSI programs over a 3 year period in France were collected. A total of 183 aberrant karyotypes were diagnosed, corresponding to an abnormality frequency of 6.1% (134/2196) for men and 4.85% (49/1012) for women. The following frequencies of abnormalities were observed respectively for men and women: 1.23% (n=27) and 0.69% (n=7) for reciprocal translocations, 0.82% (n=3) and 0.69% (n=7) for inversions, 3.32% (n=73) and 2.77% (n=28) for numerical sex chromosomes aberrations, and 0.59%

(n=13) and 0% for other structural aberrations. Among the male patients of this later group, 0.40% (n=9) had a Y chromosome abnormality. The current study demonstrated 8.33% of numerical chromosomal abnormalities and 4.5% of structural chromosomal abnormalities. According to Dinesh et al, (2004)¹⁸ sex chromosomal abnormalities are the most commonly observed chromosomal abnormalities associated with abnormal sexual development, reproductive performance, infertility and recurrent spontaneous abortions.

The role of sex chromosome in the initiation and maintenance of the normal menstruation needs no emphasis. The integrity of a critical area in the X chromosome (q13q26) is essential for normal ovarian function. In the present study sex chromosomal abnormalities were observed in 11.36%. Among these sex chromosomal abnormalities, numerical abnormalities of the sex chromosome were observed in 6.8%. Non disjunction and anaphase lag during meiosis or mitosis thought to be responsible for most cases of aneuploidy. The chances of having such errors increases when there are structural defects of the sex chromosome.

DNA repair, a cellular mechanism to correct damage to DNA before it can become fixed as a mutation or chromosomal

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aberration, may lead to deleterious results such as cell death or tumourigenesis. A number of anti cancer drugs and radiation treatments are designed to induce DNA damage in tumour cells. In the present study, bleomycin is used as DNA cross linking agent. Through mutagen sensitivity analysis, the study subjects can be grouped in to hyposensitive, sensitive and hypersensitive to mutagens. Approximate 50% of the study subjects were sensitive to bleomycin where as none of the control subjects were sensitive. Hence along with Karyotypes, the chromosomal breaks per cell value are calculated for every patient by performing bleomycin induced chromosome sensitivity assay.

According to Dinesh et al (2003)¹⁹, children with chromosome anomalies were more frequent in parents with increased age and/or high parental birth order. The mutagen sensitivity study also reveals high b/c value in children born to aged and/or high birth order parents. The present study also revealed an increased mean breaks/cell value in infertile couples, compared with normal fertile couples. This shows that infertile couples are more prone to mutagenic agents. Dinesh et al¹⁹ reported that, parents of children with chromosomal anomalies showed a mean b/c value of 0.6892, where as the normal control parents showed a mean b/c value of 0.5274. The

present study also observed a mean b/c value of 0.6871 among infertile couples and the control showed a mean b/c value of 0.5731. Spontaneous or baseline MN frequencies in cultured human lymphocytes provide an index of accumulated genetic damage occurring during the lifespan of circulating lymphocytes. The half-life and mean lifespan of long-lived T-lymphocytes has been estimated to be 3 years and 4 years, respectively. The observed genetic instability may also reflect accumulated mutations in the stem cell lineage from which the mature lymphocytes originate.

Reliable literature data showed that numerous factors could influence the increase of spontaneous micronuclei (MN) frequency, as well as the frequency induced by different chemical, physical and biological agents. The MN are small extranuclear bodies that arise in dividing cells from acentric chromosome, chromatic fragments or whole chromosome or chromatids that lag in anaphase and are not induced in the daughter nuclei in telophase. Determination of MN in blood lymphocytes is well established as a standard method for evaluation of both genomic instability and genotoxic exposure in human bio-monitoring studies²⁰.

These findings suggest that there is strong evidence for increased genetic instabilities among subjects suffering with

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infertility than the control population and provided important insight into the pathogenesis of infertility. The high incidence of genetic instabilities in the infertile population clearly warrants genetic testing and counseling prior to ICSI/IVF. If

pathology is discovered prenatal diagnosis should be performed. The current study demonstrated significant differences in the number of CBMN frequencies among the study subjects and the control subjects.

References

1. Huynh T, Mollard R and Trounson A Selected genetic factors associated with male infertility. *Hum Reprod Update* 8, 2002;183–198.
2. Jensen M, Leffers H, Petersen JH, Nyboe Andersen A, Jorgensen N, Carlsen E, et al, Frequent polymorphism of the mitochondrial DNA polymerase gamma gene (POLG) in patients with normal spermiograms and unexplained subfertility. *Hum Reprod.* 2004 Jan;19(1):65-70.
3. Maher ER. Imprinting and assisted reproductive technology. *Hum Mol Genet.* 2005 Apr 15;14 Spec No 1:R133-8.
4. Wright VC, Schieve LA, Reynolds MA, Jeng G Assisted reproductive technology surveillance—United States 2003, *MMWR Surveill Summ* 52:1–16.
5. Hansen M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *N Engl J Med* 2002; 346:725–730.
6. Vialard F, Benahmed M, Lombroso R, Selva J. Genomic instability and male infertility. *Gynecol Obstet Fertil.* 2004;32(12): 1013-22.
7. Agarwal A. and Tamer M. Said. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Human Reproduction Update, Vol.9, No.4 pp.331-345, 2003.*
8. Schantz SP, Zhang ZF, Spitz MS, Sun M, Hsu TC. Genetic susceptibility to head and neck cancer: interaction between nutrition and mutagen sensitivity. *Laryngoscope.* 1997 Jun;107(6):765-81.
9. Fenech M. The cytokinesis-block micronucleus technique: A detailed description of the method and its application to genotoxicity studies in human population. *Mutat. Res.* 1993;285: 35-44.
10. Kirsch-Volders M, Sofuni T, Aardema M, et al. Report from the in vitro micronucleus assay working group. *Mutat Res.* 2003 Oct 7;540(2):153-63.

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11. Moorehead P S, Nowell P C, Wellmann W.J. Chromosome preparation of leukocytes. Cultured from Human Peripheral Blood, *Exp. Cell. Res;* 1960, Vol. 20, pp 613-617.
12. Seabright M. A rapid banding technique for Human Chromosome *Lancet* 1971, pp 971-972.
13. ISCN, International System for Human Cytogenetic Nomenclature, Mittelman F, Karger S, Basal, 1995.
14. Hsu, TC, Cherry, LM,. And Saman, NAO,. Differential Mutagen sensitivity in cultured lymphocytes of normal individuals and cancer patients; *CancerGenet. Cytogenet*, 1985, vol.17, pp.307-313.
15. Dohle GR, Halley DJ, Van Hemel JO, van den Ouwel AM, Pieters MH, et al. Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. *Hum Reprod.* 2002 Jan;17(1):13-6.
16. Peschka B, Leygraaf J, Van der Ven K, Montag M, Schartmann B, et al. Type and frequency of chromosome aberrations in 781 couples undergoing intracytoplasmic sperm injection. *Hum Reprod.* 1999 Sep;14(9):2257-63.
17. Gekas J, Thepot F, Turleau C, Siffroi JP, Dadoune JP, et al. Chromosomal factors of infertility in candidate couples for ICSI: an equal risk of constitutional aberrations in women and men. *Hum Reprod.* 2001 Jan;16(1):82-90.
18. Dinesh Roy D, Alex K Ittyavirah, Laly Alex and Stephen J.. Sex chromosome abnormalities associated with Infertility and recurrent spontaneous abortions. *Proc. of the 29th annual conference of Indian society of human genetics*, 2004; pp.74.
19. Dinesh RD, K Pavithran, PY Henry, KE Elizabeth, P Sindhu and T Vijayakumar. Correlation of age and birth order of parents with chromosome anomalies in children. *Russian journal of Genetics* 2003.Jun;39:834-9.
20. Fenech M. The Advantages and disadvantages of the cytokinesis-block micronucleus method. *Mutat. Res.* 1997, 392. 11-18.

Corresponding author:

Dinesh Roy D
Genetika, Centre for Advanced Genetic Studies
TC 30/563, MMRA-128, Pettah P.O.
Trivandrum – 695 024, Kerala, India
Email: dineshroyd@rediffmail.com

Table 1 Distribution of CBMN frequencies and mean b/c values according to karyotype					
Subjects	Number	CBMN frequency		Mean b/c value	
Infertile subjects with abnormal Karyotype	17 (12.88%)	16.94	13.99	0.8611	0.6871
		13.56		0.6608	
Infertile subjects with normal Karyotype	115 (87.12%)				
Control subjects	40	9.9		0.5731	

Table 2 Distribution of mean b/c values and CBMN frequencies according to age range					
Subjects	Age range	mean b/c value		CBMN frequency	
		Husbands	Wives	Husbands	Wives
Study	<35	0.6755	0.6764	13.88	12.84
	≥35	0.7061	0.6878	14.19	14.94
Control	<35	0.5450	0.5475	9.77	8.77
	≥35	0.6125	0.5773	10.82	10