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# Assessment of chromosomal instabilities in the form of micronuclei in couples with recurrent pregnancy loss

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## Abstract

**Background**: The present study aims to assess chromosomal instabilities between fertile couples and couples with recurrent pregnancy loss (RPL) and the causative relation of chromosomal instabilities with RPL.
**Methods:** A case-control study was performed with a study sample of 27 couples with a history of RPL who attended the Department of Obstetrics and Gynecology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) and twenty-seven healthy fertile couples as controls. The procedure done was Cytokine Blocked Micronucleus Assay (CBMN). After obtaining consent and details of the couple, 3 ml of heparinized blood was collected from cases and controls. Cases comprising couples with RPL (gestational age ⩽ 24 weeks) were included, and couples with a history of diabetes mellitus, thyroid disorders, and hypertension were excluded. Among cases, women with a history of two or more two spontaneous abortions ⩽ 24 weeks of gestation were selected. Blood was collected and assessed from both male and female partners. The lymphocytes were cultured per the standard protocol and were screened as the number of micronuclei per 1000 binucleate cells under X 200 magnification in CBMN.
**Results:** Chromosomal instabilities in the form of micronuclei in cases were found to be 7.52±3.99 and 0.07±0.26 in controls ($p<0.05$). A statistically significant difference was revealed among those with and without chromosomal instability.
**Conclusion:** Chromosomal instability serves as a significant causative factor for those couples leading to pregnancy losses.

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## Highlights

Since cells of the fetus cross the placenta, the possibility of assessing MN in fetal cells and correlating that with MN in lymphocytes in parents could be explored, it may also help to know if the chromosomal instability is observed in the fetal cells.

## Introduction

Clinically recognized pregnancy loss is a common problem affecting 15-20 % of pregnancies. Recurrent pregnancy loss (RPL), based on the modern definition by the American Society for Reproductive Medicine (ASRM), is the "loss of two or more failed clinical pregnancies." Two losses are seen in 5% of couples and three or more in 1% of couples" (1).

According to World Health Organization (WHO), "Reproductive health is a state of complete mental, physical, and social well-being which is related to all stages of reproductive processes" (2).

The causes for RPL are genetics, reasons related to implantation, autoimmunity, endocrine abnormalities, anatomic uterine defects, paternal factors, alloimmunity, and the like. However, in half of the cases, the etiology of RPL cannot be determined (3). Few epidemiological studies have suggested that it could be multifactorial, involving the interaction of predisposing genetic and environmental factors in its pathogenesis (4).

According to previous studies, aborted specimens of fetuses were karyotyped, and the karyotype abnormalities in fetuses were high compared to those in parents with RPL. Karyotype abnormalities in couples with RPL were found to be 3-5% which is five times greater than in the common population. (4).

Micronuclei (MN) are "small, round nuclei clearly separated from the main cell nucleus which forms from acentric chromosome fragments or whole chromosome during cell division." The frequency of micronuclei has been used as an indicator of genomic damage and instability in various studies (5).

The present study has been undertaken to inquire into the nature of cytogenetic instabilities among couples with RPL and to compare it with fertile couples. In couples without cytogenetic abnormalities, chromosomal instability could contribute to RPL, which serves as the novelty of the present study.

Hence, the current study has been done to determine chromosomal instability in the form of micronuclei in couples with RPL compared to the control group. Cytokine Blocked Micronucleus Assay (CBMN) will assess chromosomal instability.

The principle of this test is that micronuclei are visible only after cell division. Then, cytokinesis is blocked by cytochalasin B, inhibiting actin filaments polymerization and the formation of contractile microfilaments. Thus, binucleated cells are formed with Micronuclei present in their cytoplasm.

About 3 ml of heparinized blood was collected from cases and controls. Among couples, women with a history of two or more spontaneous abortions ⩽ 24 weeks of gestation were included, and couples with a history of diabetes mellitus, thyroid disorders, and hypertension were excluded. The lymphocytes were cultured per the standard protocol and were screened as the number of micronuclei per 1000 binucleate cells under X 200 magnification in CBMN.

## Methods

This case-control study was conducted in the Department of Anatomy in collaboration with the Department of Obstetrics and Gynecology (OBGY), JIPMER, Puducherry. The approval of the Institute ethics committee (IEC) & Postgraduate Research Monitoring Committee (PGRMC) was obtained before the study. Convenient sampling selected subjects among couples attending outpatient departments, OBGY. Cases comprising couples with RPL were included, and couples with a history of diabetes mellitus, thyroid disorders, and hypertension were excluded. Among cases, women with a history of two or more two spontaneous abortions ⩽ 24 weeks of gestation were selected. Blood was collected and assessed from both male and female partners. Controls were healthy

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fertile couples. Couples who have proved their fertility by the birth of one or two children were selected as controls. The sample size was calculated as twenty-seven couples in each group. After informed written consent, the couple's demographic details and medical history were obtained in the predesigned data collection proforma. 3 ml of heparinized peripheral blood was withdrawn by venipuncture under aseptic precautions. Peripheral blood lymphocyte cell culture

Samples were processed on the same day of collection. Sterile culture tubes were kept in the incubator at 37ºC for 44 hrs. On day 3, 3 µl of Cytochalasin B (6µg/ml) was added and gently mixed at 44 hrs. It was again placed in the incubator for another 28 hrs at 37ºC. On day 4, culture harvesting, slide preparation, and staining were done. Slides were stained with Giemsa.

"Peripheral blood lymphocyte cell culture was carried out as per the protocol" (6).

### Scoring of micronuclei (7)

Lymphocytes were screened for the presence of micronuclei. They were noted as- the number of micronuclei per 1000 binucleated (BN) cells under X 200 magnification in Olympus BX53 binocular brightfield microscope using "CellSens Standard" software. The slide was scanned from one edge to another and manually scored for micronuclei. An assay sheet was used to note the findings, and scoring was done. In binucleate cells with intact cytoplasmic background and nuclear membrane, two nuclei should be the same size without overlapping, and the diameter of micronuclei should be shorter than one-third or one-sixteenth of the main nuclei. The micronuclei were found inside the binucleate cell "along with" principal nuclei without overlapping were considered micronuclei. About 1000 binucleated cells were scored for each slide; micronuclei frequency was reported as no micronuclei per 1000 binucleate cells.

The third nucleus in a binucleated cell which was comparatively larger than micronuclei but a few diameters less than main nuclei, bleb like an extension from primary nuclei, and granular appearance in the cytoplasm of a binucleated cell were not considered as micronuclei.

The mean rank of CBMN is the arithmetic average of micronuclei per 1000 binucleate cells.

### Statistical parameters & analysis

Statistical analysis was done using SPSS version 19. The number of micronuclei per 1000 binucleate cells was expressed as Mean ± SD. The chi-square and Mann-Whitney tests were used to compare chromosomal instability in cases and controls.

## Results

Findings showed that the frequency of micronuclei (MEAN±SD) in cases was 7.52±3.99, and in controls, it was found to be 0.07±0.26 with a p-value of 0.00. The frequency of micronuclei in cases was found to be higher than that in controls (Figures 1 & 2).

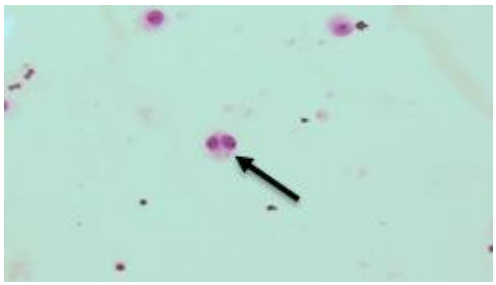

**Figure 1**. Binucleated cell with one micronuclei

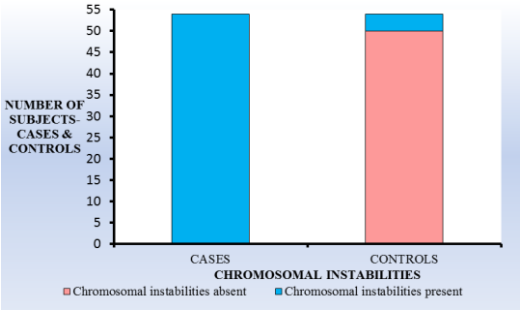


**Figure 2**. Chromosomal instabilities in cases and controls

Chromosomal instability was compared in cases and controls using the chi-square test, and a statistically significant difference was found with a p-value of 0.00. The odds of occurrence of pregnancy loss were 14 times higher in those with chromosomal instabilities than those without, with a 95% CI 5.63 to 37.33. The mean rank of CBMN among controls was 27.5, and among cases was 81.5. Mann-Whitney U test was done to test whether this difference in mean rank among cases and controls was statistically significant. Mann-Whitney U: 0.000 (p-value=0.000). It was found to be statistically significant.

The frequency of micronuclei among male partners of cases was 7.48±3.97, which was higher than controls (0.11±0.32). The mean MN among female cases was 7.56±4.08, and in controls, 0.04±0.19. Like males, female cases had a higher value than female controls (Table 1).

**Table 1.** Mean micronuclei frequency in cases and controls

| S.No. | Subjects | Cases (Mean±SD) | Controls (Mean±SD) |
|---|---|---|---|
| 1 | Males | 7.48±3.98 | 0.11±0.32 |
| 2 | Females | 7.56±4.08 | 0.04±0.19 |

The MN/1000 binucleated cells were marginally higher among females than males. However, this was not statistically significant.

As shown in Table 2, 202 micronuclei were scored altogether among fifty-four male cases, of which 194 had one micronuclei, seven had two micronuclei, and one had three micronuclei. In total, 204 micronuclei were scored among fifty-four female cases, of which 195 had one micronucleus, eight had two micronuclei, and one had three micronuclei. Their corresponding Mean ± standard deviation was also calculated and mentioned. Figure 3 shows an example of bi-nucleated cells with one, two, or three micronuclei.

**Table 2.** Micronuclei distribution among male and female cases

| Cases n=54 | No. of Micronuclei/BN cells | 1 MN | 2 MN | 3 MN |
|---|---|---|---|---|
| | | Males | | |
| Total | 202 | 194 | 7 | 1 |
| Mean ± SD | 7.48 ± 3.98 | 7.19 ± 3.74 | 0.26 ± 0.71 | 0.04 ± 0.19 |
| | | Females | | |
| Total | 204 | 195 | 8 | 1 |
| Mean ± SD | 7.56 ± 4.09 | 7.22 ± 3.77 | 0.29 ± 0.54 | 0.03 ± 0.19 |

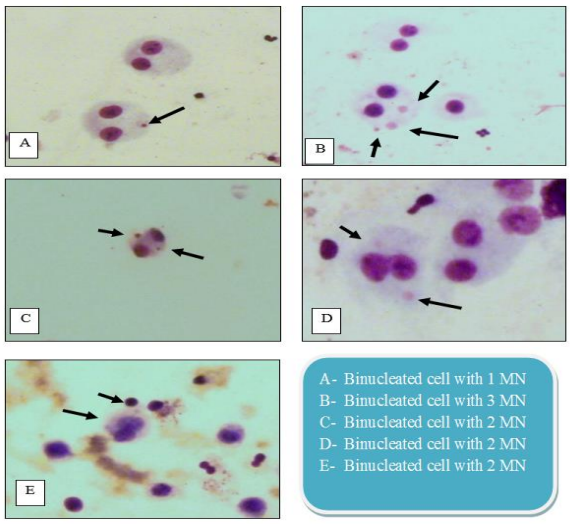


**Figure 3.** Binucleated cells with different numbers of micronuclei (The arrows show the micronuclei)

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**Table 3.** Comparison of studies done for chromosomal instabilities [(I) – Individuals]

| S. No | Author & year of study | Country | Gender | No. of Subjects (Couples) | MN per 1000 BN cells | Total No. of MN | 1 MN | 2 MN | 3 MN |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Present study Cases | India | M | 27 | 7.48±3.97 | 202 | 194 | 7 | 1 |
| | | | F | 27 | 7.56±4.08 | 204 | 195 | 8 | 1 |
| | Controls | | M | 27 | 0.11±0.32 | 3 | 3 | 0 | 0 |
| | | | F | 27 | 0.04±0.19 | 1 | 1 | 0 | 0 |
| 2. | Milosevic-Djordjevic O et al. (5) 2012 Cases | Kragujevac, Serbia | M | 18 | 9.22±4.70 | 152 | 140 | 10 | 2 |
| | | | F | 18 | 13.5±2.5 | 232 | 222 | 9 | 1 |
| | Controls | | M | 15 | 6.27±2.66 | 92 | 90 | 2 | 0 |
| | | | F | 15 | 6.8±2.98 | 102 | 102 | 0 | 0 |
| 3. | Moyet et al. (12) 2015 Cases | Baghdad, Iraq | F | 64 (I) | 8.66±1.74 | - | - | - | - |
| | Controls | | F | 41(21+20) (I) | | | | | |
| | Pregnant | | F | 21 | 3.83±0.74 | - | - | - | - |
| | Non-pregnant | | F | 20 | 3.61± 1.02 | - | - | - | - |
| 4. | Sumitha Prabu et al. (13) 2015 Cases | Kerala, India | M&F | 74 | 13.47 | - | - | - | - |
| | Controls | | M&F | 25 | 10.48 | - | - | - | - |
| 5. | Trkova et al. (14) 2000 Cases | Czech republic | M&F | 62 | 30.23±9.34 | - | - | - | - |
| | Controls | | M&F | 30 | 21.20 ±4.26 | - | - | - | - |
| 6. | Rajesh et al. (11) 2008 Cases | India | M | 72 | 9.1±3.6 | - | - | - | - |
| | | | F | 72 | 9.3±2.9 | - | - | - | - |

## Discussion

The principal purpose of the current study was to evaluate the instabilities in the chromosomes in cases and controls and to compare the outcome between the two groups. Chromosomal instabilities were notably higher among couples with RPL compared to fertile couples. The aforementioned agrees with the previous studies highlighting that RPL couples carry a greater risk for genomic irregularities than fertile couples (5,8,9,10).

In the present study, chromosomal instability is significantly increased in cases compared to the controls (in four out of fifty-four fertile individuals). In the present study, the mean micronuclei were higher among cases (7.52±3.99) than controls (0.07±0.26). Among the cases, females (7.56±4.08) had slightly higher micronuclei count compared to males (7.48±3.98). This study was concordant with the results of the study by Rajesh et al. (11). In the study among Baghdad women done by Moyet et al. involving only female cases, the mean micronuclei frequency was higher among cases (8.66+1.74) when compared to pregnant and non-pregnant women (3.83±0.74 and 3.61±1.02 respectively). This study also applied sister chromatid exchange (SCE) to recognize genomic impairments and concluded that both SCE and CBMN assay could detect genomic instability (12). The present study's mean was 7.56±4.08 vs. 0.04±0.19 among female cases and controls. As per the study carried out on thirty-six subjects with reproductive failure and thirty healthy controls in Serbia using CBMN assay- cases had higher MN frequency (males-9.22±4.70, females-13.5±2.5) than controls (males-6.27±2.66, females-6.8±2.98). As previously described, the micronuclei were higher in females than in males (5).

As depicted in Table 3, cases with one MN/1000 BN cell were higher than those with three MN/1000 BN cells (5).

The comet assay is a sensitive and reliable marker of DNA damage. The comet assay results among couples with early pregnancy losses showed elevated comet tail length, significantly correlated with elevated MN index. The study concluded that comet assay and MN index were sensitive indicators of genomic instability (11).

Mean micronuclei in controls were reported to be lower than the cases in other studies. However, in this study, it was significantly lower in controls (0.07±0.26). According to the statistical analysis, the probability of experiencing a pregnancy loss was 14 times higher in those with chromosomal instabilities.

Studies have shown that the elevated MN frequency in the peripheral blood reflects DNA damage in the sperm. This could lead to an unfavorable reproductive outcome. According to the WHO, 38% of adverse reproductive outcomes were attributed to maternal factors, 20% to paternal factors, and 27% to a combination of both. The cause of the remaining cases was unknown. (15).

Studies have described decreased micronuclei among smokers. As Milosevic et al. reported, this could be due to the defective binucleate formation in impaired cells of smokers, and thus they could not be scored (5).

In a study by Michael Fenech in 2020, all types of chromosomal instabilities such as structural/numerical chromosome aberrations and chromosome mal-segregation during mitosis expressed as Micronuclei, anaphase bridge formation expressed as nucleoplasmic bridges, and gene amplification or elimination of unresolved DNA complexes expressed as nuclear buds were assessed by CBMN cytome assay (16).

Wieland et al.'s review study stated that microsatellite instability could be a causative factor for recurrent miscarriages and suggested the evaluation of microsatellite instability in preimplantation embryos (17).

## Conclusion

Chromosomal instabilities in micronuclei frequency were elevated in couples with RPL than in fertile couples. A statistically significant difference was observed, suggesting that pregnancy losses were due to the genomic derangements detected in micronuclei. Though the genetic cause was among many factors leading to RPL, it could not be left without being assessed.

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## Ethical statement

This is to certify that the project JIP/IEC/SC/8/602, entitled "Assessment of chromosomal instabilities in couples with recurrent pregnancy loss," submitted by Dr. N. Sujithaa has been approved by JIPMER Institute Ethics Subcommittee (Human Studies).

## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

## Author contributions

SN conceived and designed the experimental design, contributed to analyzing, drafting and revising the article. SH & LC collected the data and drafted the manuscript. All authors read and approved the final manuscript.

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