

**Original Research Article**

# COMPARISON AND CORRELATION OF CHROMOSOMAL ABNORMALITIES AND CHROMOSOMAL INSTABILITIES IN COUPLES WITH RECURRENT PREGNANCY LOSS

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**Abstract:** Previous studies have demonstrated the type of chromosomal abnormalities found among couples with RPL. But a majority of the couples show a normal karyotype. The present study has been undertaken to inquire the nature of cytogenetic abnormalities and instabilities among couples with recurrent pregnancy loss and to compare it with fertile couples. Study objectives are to assess chromosomal abnormalities and instabilities between fertile couples and couples with recurrent pregnancy loss (RPL) and to study the causative relation of chromosomal abnormalities and instabilities with recurrent pregnancy loss. It is a Case-control study of study population 27 couples with the history of recurrent pregnancy loss who attended the Department of Obstetrics and Gynecology, JIPMER and 27 healthy fertile couples as controls. Karyotyping and Cytokine Blocked Micronucleus Assay (CBMN) was performed. In the present study, abnormal karyotype was found in 3 cases. All controls had a normal karyotype. Chromosomal instabilities in the form of micronuclei in cases were found to be  $7.52 \pm 3.99$  and in controls were found to be  $0.07 \pm 0.26$ , ( $p < 0.05$ ) and there was a statistically significant difference among those with and without chromosomal instability. Causative relation of chromosomal abnormalities with RPL could not be stated with fewer abnormal karyotypes. Chromosomal instability serves as a significant causative factor for those couples leading to pregnancy losses.

**Index Terms -** *Recurrent pregnancy loss, chromosomal abnormalities, Chromosomal instability, Karyotyping and Cytokine Blocked Micronucleus Assay (CBMN).*

## I. INTRODUCTION

Recurrent pregnancy loss (RPL) is termed as the “occurrence of three or more clinically recognized pregnancy losses before 20 weeks of the last menstrual period” [1]. “Recurrent spontaneous abortion”, “Recurrent miscarriage” are the alternative terms used for the same clinical condition. Fetal weight in these cases will be less than 500 gms [2].

WHO defined recurrent abortions as “loss of a fetus weighing less than or equal to 500 gms, which would normally be at 20-22 completed weeks of gestation” [3]. The majority of the recurrent pregnancy losses are early clinical pregnancy losses. Among these, embryonic losses are the most common. Sporadic pregnancy losses are different from recurrent pregnancy loss. The former are due to an abnormal embryo which has lost its potential to attain viability [2,4].

Chromosomal abnormalities or chromosomal aberrations are defined as “a clinical condition caused by the constitution of an abnormal chromosome, resulting in duplication, loss or rearrangement of chromosome material” [5]. It is categorized into defects in the arm and defects in the centromere.

Chromosomal instability is defined as “an increased rate of numerical and structural chromosomal changes during cell division” [6]. Chromosomal instability in lymphocytes is found in many clinical conditions for example polycystic ovarian syndrome, spontaneous abortions, and infertile cases.

Micronuclei are defined as “small, round nuclei clearly separated from the main cell nucleus which forms from acentric chromosome fragments or whole chromosome(s) during cell division” [6]. Micronucleus assay is a technique used in the assessment of cytogenetic damage. Studies have shown increased instability in the genome of females compared to males in couples with reproductive failure [6].

During anaphase I or II of meiosis, it was proposed that derangements of DNA and or spindle apparatus, preceded to “lagging whole chromosomes and acentric fragments that induced the formation of micronucleus in the germ line”

Numerous studies have demonstrated the type of chromosomal abnormalities found among couples with RPL. But a majority of the couples show a normal karyotype. The present study has been undertaken to inquire the nature of cytogenetic abnormalities and instabilities among couples with recurrent pregnancy loss and to compare it with fertile couples. In couples without cytogenetic abnormalities, chromosomal instability could be a contributing factor to recurrent pregnancy loss. Studies done to correlate karyotype with the extent of damaged DNA in the form of micronuclei assay are scanty

Hence, the present study has been done to determine chromosomal instability in the form of micronuclei and its the association with karyotype in couples with RPL compared to the control group.

## II. METHODOLOGY

After obtaining approval from Postgraduate research monitoring committee (PGRMC) on and Institute ethics committee (IEC) the study was conducted in the Department of Anatomy in collaboration with Department of Obstetrics and Gynecology, JIPMER.

**Study design:** Case – control type of Analytical study

**Study participants:** Human subjects

**Study groups:**

Two groups, the group, consist of couples. Both male and female partner were evaluated.

Cases: Couples who attended the Department of Obstetrics and Gynecology, JIPMER with the history of recurrent pregnancy loss.

Controls: Healthy fertile couples.

**Inclusion criteria**

Women with history of two or more than two spontaneous abortions  $\leq$  24 weeks of gestation

**Exclusion criteria**

1. Women with history of fetal anomalies or fetal demise
2. History of Cervical incompetence
3. Uterine factors
3. Intrauterine adhesions, Leiomyoma
4. Antiphospholipid antibody syndrome, Thrombophilias
5. Couples with History of Diabetes mellitus, Thyroid disorders, Hypertension

**Sample size calculation:**

The sample size was calculated as 27 couples in each group, 108 altogether, with the outcome measure of chromosomal instability expressed as the mean  $\pm$  standard deviation of micronuclei. The expected Mean difference between the groups of cases and controls as 2.95 with a standard deviation of 3.8 at 5% level of significance and 80% power using OPEN EPI software, Version 2, Open source calculator –SS MEAN.

**Sampling technique:** Convenient sampling

**Sample collection:**

After informed written consent, details of the couple were obtained in the predesigned data collection proforma. 3ml of heparinized peripheral blood was withdrawn for the following procedures.

1. Karyotyping
2. Cytokinesis blocked Micronucleus assay

Without delay, samples were processed on the day of collection of blood.

**Study procedure: Karyotyping – Standard operative protocol**

On day – 1, About 1 to 2 drops of heparin was taken in a syringe and 3ml of peripheral blood drawn in the same syringe and mixed gently to avoid lysis. Heparin added to prevent coagulation. The blood sample was labeled as T-1 M, C-2 F. And then Peripheral blood lymphocyte culture was setup with reagents such as Roswell Park Memorial Institute (RPMI)-1640 media, fetal bovine serum, Phytohemagglutinin (PHA), antibiotics- penicillin-streptomycin and about 8 to 9 drops of blood was added. The sterile tube was incubated for 69 hours at 37°C. On day-4 Culture harvesting was done by adding 60 $\mu$ l of colcemid at 68<sup>th</sup> hour, then incubated, centrifuged, hypotonic solution(kcl) was added and again centrifuged. Similar steps viz. centrifugation, clearing the supernatant, followed by fixative was repeated twice. Finally, cell pellets were collected and suspended in 0.5 to 1ml of fixative until a hazy or cloudy solution was obtained. Slide preparation was done. The cell suspension was kept ready. The slide immersed in methanol was taken out, wiped with a tissue paper, transferred to the next Coplin jar with methanol, swirled in icy cold water. The cell suspension was added with a pipette by dropping over the slide in contact with it. Then a layer of fixative was added and blown subsequently to provide humidity and also to prolong the drying time to some extent. The slide was placed on the slide warmer maintained at 60°C for few seconds and dried by blotting on a tissue paper. The time taken from adding the cell pellet to blotting should be 30 seconds. This time was adjusted in cold and warm situations accordingly. On day-5, Aging was done by keeping the slide inside hot air oven at 90°C for 20 minutes and banding of slides by trypsin. Then slides were stained with Giemsa stain solution for 2 minutes, washed with distilled water and blotted with lab tissue paper. Slides were analyzed under Trinocular Research Microscope Olympus BX51. *Standardization time for treatment with trypsin was done based on the banding pattern by trial and error.* Twenty metaphase spreads were captured and analyzed for each of the samples. Images were obtained using *automated karyotyping software, IKAROS*. Ikaros software is installed in a computer which is attached to a Trinocular microscope *Olympus BX51*. Chromosomal abnormalities and heterochromatin were detected. Wherever heterochromatin was present or in doubt, C-banding was done.

**Study procedure: Cytokinesis Blocked Micronuclei Assay (CBMN) [7] .**

Blood collection and Peripheral blood lymphocyte culture setup is similar to karyotyping. Sterile culture tubes were kept in the incubator at 37°C for 44 hrs. On Day-3, Culture tubes were taken out at 44 hrs, **3µl of Cytochalasin B** (6µg/ml) was added and gently mixed. It was again placed in the incubator for another 28 hrs at 37°C.

On day-4, Culture harvesting was done after 72 hours of incubation. Culture tubes were taken out and centrifuged at 1000 rpm for 10 minutes. After discarding the supernatant, 5ml of mild hypotonic solution (RPMI and MilliQ water in 1:1 ratio) were added after removal of supernatant.<sup>[37]</sup> Supernatant discarded and about 10ml of fixative was added and kept in the refrigerator for 20 min. After thawing to room temperature, centrifuged twice with 5ml of fixative (methanol and glacial acetic acid in the ratio of 3:1) to obtain a cell pellets. Slides were **prepared in a unique way for CBMN** and not in the identical method as we do for karyotyping. Slides dipped in methanol were used to drop the cell suspension using a pipette. Immediately they were dried on a warmer bench at 60°C for 15 to 20 seconds and then stained. Slides were stained with Giemsa solution for 10 min. On day-5, Scoring of micronuclei<sup>[38]</sup> was done and 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. An assay sheet was used, to note down the findings and scoring were done. About 1000 binucleated cells were scored for each slide; micronuclei frequency was reported as no of micronuclei per 1000 binucleate cells.

### Statistical parameters & Analysis

Categorical data like presence or absence of chromosomal abnormalities (translocations, inversions) and heterochromatic regions was described as proportions. Number of Micronuclei per 1000 binucleate cells was expressed as Mean ± SD. The difference in the proportion of chromosomal aberrations and heterochromatin in cases and controls was tested using Fisher's exact test. Tests used to compare chromosomal instability in cases and controls were chi-square and Mann-Whitney test.

Mann-Whitney test was done to assess the significant difference between Chromosomal abnormalities and instabilities among those with and without recurrent pregnancy loss.

### III. RESULTS

Detailed history was collected from couples with recurrent pregnancy loss as well as from fertile couples. The number of abortions served as a significant risk factor. Most of the cases (approximately 70%) had more than two abortions. Gravidity among cases is shown in figure-1. All the cases were above the third gravida. Two cases had gravidity of nine.

Figure – 2 shows a Male karyotype showing heterochromatin in chromosome 9 and 16 which was detected by C- banding technique. There were around 15 micronuclei scored per 1000 binucleate cells in the same patient. So male factor could also be a cause for recurrent pregnancy loss.

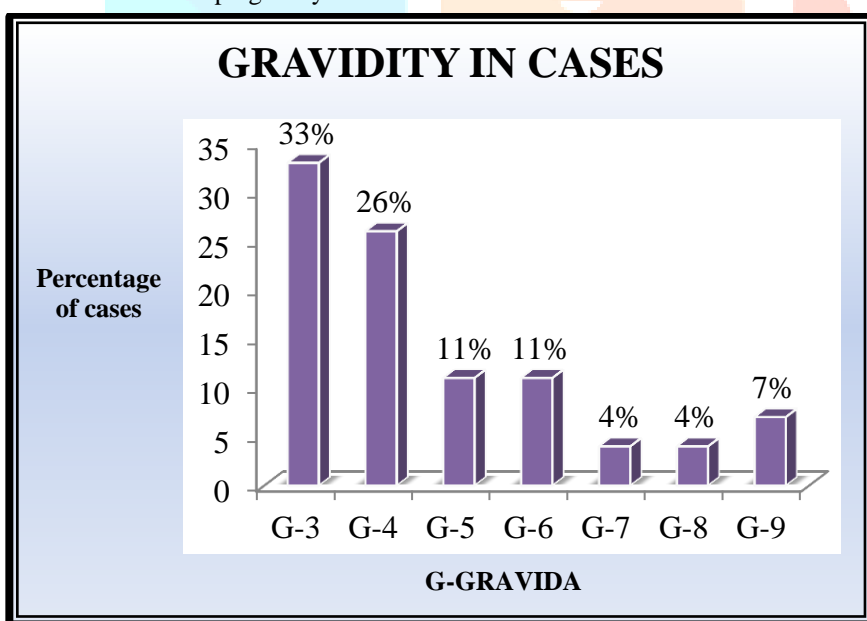
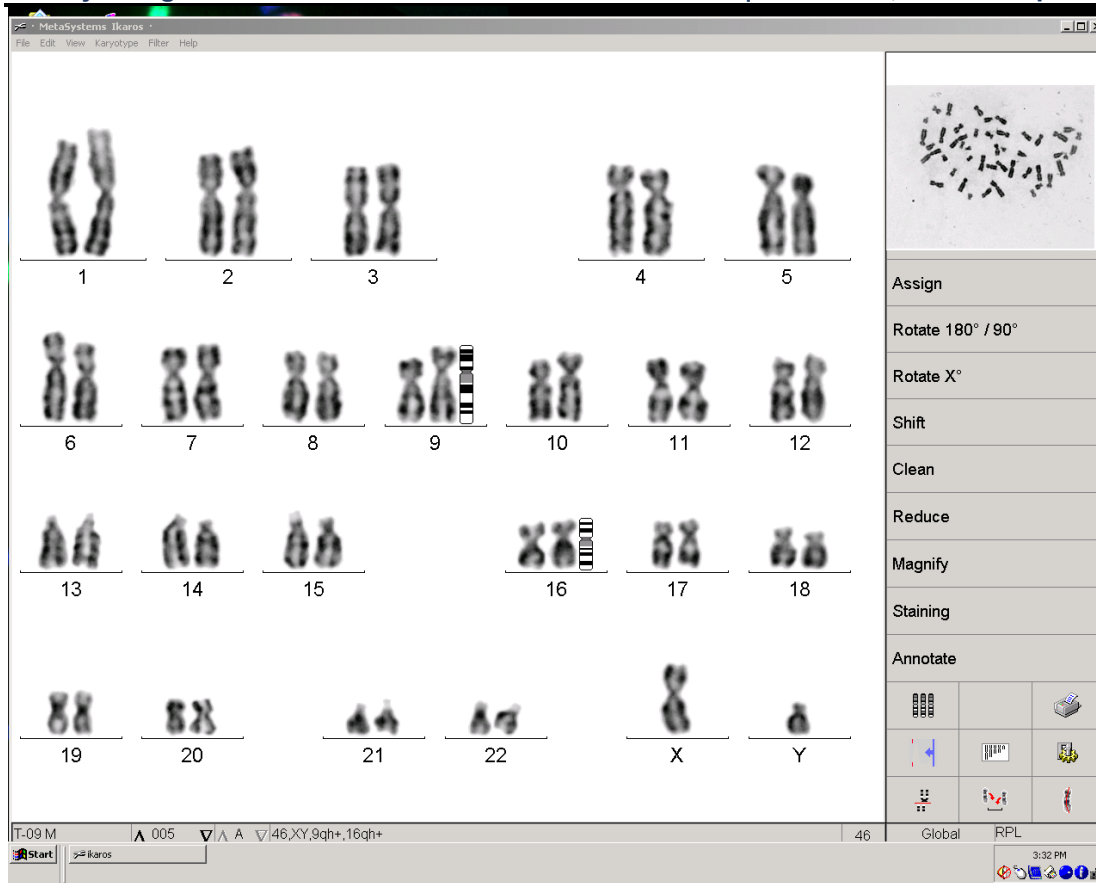


Figure-1: Gravidity in Cases

### Combining Chromosomal Abnormality and Instability (Table -1 & Figure-4)

Fisher's exact test was done to compare chromosomal abnormality and chromosomal instability among cases and controls. It was statistically insignificant with a p-value of 0.25 and with an odds ratio of 1.06 with 95% CI of 0.99 - 1.12. Mean ranks of CBMN among those with and without chromosomal abnormality was found to be significantly different using Mann-Whitney U test with a p-value of 0.016 and Mann-Whitney U: 35.5. When Mann-Whitney test was performed considering only cases, few positive cases of chromosomal abnormality was not adequate to comment on the significance (p=0.12).



**Figure – 2 : Male karyotype showing heterochromatin in chromosome 9 and 16 (46,XY,9qh+,16qh+)**

Following were considered as micronuclei

- ❖ Binucleate cells with intact cytoplasmic background and nuclear membrane.
- ❖ Two nuclei should be of the same size without overlapping.
- ❖ The diameter of micronuclei should be shorter than one-third or one-sixteenth of the main nuclei.
- ❖ Micronuclei were found inside the binucleate cell along with principal nuclei without overlapping.

Following were not considered as micronuclei

- ❖ Third nucleus in a binucleated cell which was comparatively larger than micronuclei but few diameters less than main nuclei.
- ❖ Bleb like an extension from main nuclei.
- ❖ Granular appearance in the cytoplasm of a binucleated cell.

About 1000 binucleated cells were scored for each slide; micronuclei frequency was reported as no of micronuclei per 1000 binucleate cells. Figure – 3 shows a picture of binucleated cell with 2 micronuclei.

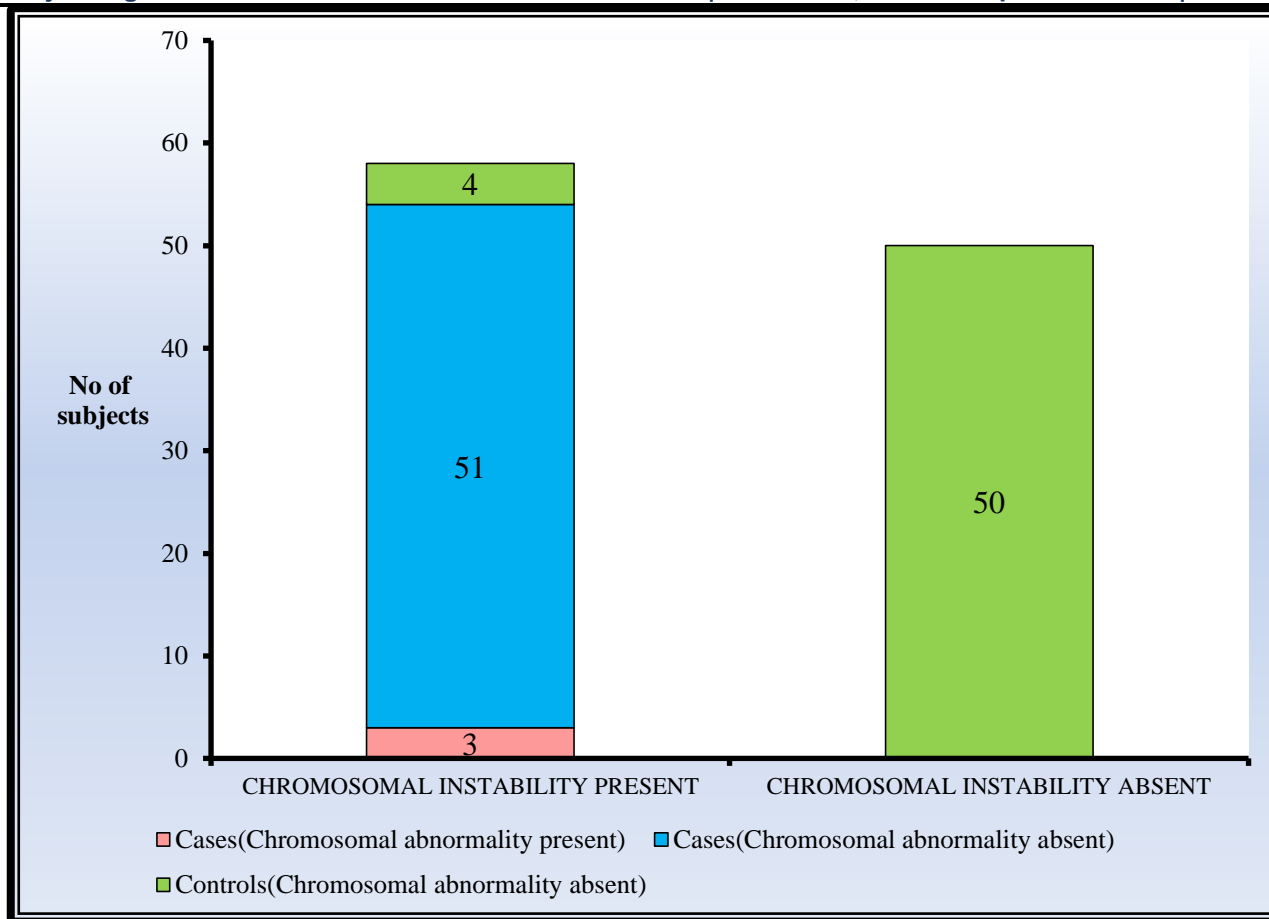


Figure-3 shows a binucleated cell with 2 micronuclei, arrows indicate micronuclei

Chromosomal abnormality as assessed by karyotyping had positive results among cases. Chromosomal instability as assessed by number of micronuclei per 1000 binucleated cells was scored and both cases and controls had micronuclei. Thus, as both chromosomal abnormality and chromosomal instability was correlated and compared in couples of recurrent pregnancy loss and fertile couples. Table-1 shows chromosomal instability in the form of the number of micronuclei present in 'chromosomal abnormality' positive cases.

Table – 1: Combining chromosomal abnormality and instability in Cases and Controls

| CASES | GENDER | KARYOTYPING                          | CBMN ASSAY                        |
|-------|--------|--------------------------------------|-----------------------------------|
| n=54  | M/F    | Structural Chromosomal Abnormalities | No. of Micronuclei /1000 BN cells |
| 1.    | M      | 46,XY,9qh+,16qh+[20]                 | 15                                |
| 2.    | M      | 46,XY,1qh+[33]                       | 10                                |
| 3.    | F      | 46,XX,del(22)(p11.1)[20]             | 8 (7+1)                           |



**Figure – 4: Combining Chromosomal abnormality and Chromosomal instability in Cases and Controls**

#### IV. DISCUSSION

Cytogenetic aberrations were assessed in couples with recurrent pregnancy loss and those couples with one or two living children. The primary aim of the current study was to determine the abnormalities and instabilities in the chromosomes in cases and controls and to compare the outcome between the two groups.

We found that the chromosomal instabilities were present in all the cases irrespective of the karyotype being normal or not.

In previous studies in Turkish population, abnormalities and heterochromatic variations were accounting to be around 70% [8]. In the studies in Indian population, abnormalities and heterochromatic variations were less than 20%, on a start of 7% among cases. In all the previous studies, cases had more chromosomal abnormalities compared to controls [9-12]. In our study chromosomal abnormalities was found to be 33.3% and heterochromatic variations to be 66.7% altogether 11% as total.

In a study by Milosevic-Djordjevic O et al.[6] in 2012 on 36 subjects with reproductive failure and 30 healthy controls in Serbian population using CBMN assay-micronuclei frequency was found to be  $9.22 \pm 4.70$  in male cases and  $13.5 \pm 2.5$  in female cases which was estimated to be more than in controls. In controls it was found to be  $6.27 \pm 2.66$  in male controls and  $6.8 \pm 2.98$  in female controls. In another study by Moyet et al. [13] in 2015 MN cells per 1000 binucleate cells was found to be  $8.66 \pm 1.74$  in individuals who are cases. In individuals who are controls, it was found to be  $3.83 \pm 0.74$ . In another study in Indian population, micronuclei frequency was calculated to  $9.1 \pm 3.6$  in males and  $9.3 \pm 2.9$  in females [12]. None of the previous studies compared and correlated chromosomal abnormality with chromosomal instability.

In the current study, the mean micronuclei were higher among cases ( $7.52 \pm 3.99$ ) compared to controls ( $0.07 \pm 0.26$ ). Among the cases, females ( $7.56 \pm 4.08$ ) had slightly higher micronuclei count compared to males ( $7.48 \pm 3.98$ ). In our study, the mean was  $7.56 \pm 4.08$  vs.  $0.04 \pm 0.19$  respectively among female cases and controls.

The case with 22 p deletion had seven binucleated cells with one MN. Our literature search could not find above type of deletion being described elsewhere. Cases with heteromorphisms in chromosome 9, 16 and 1, showed higher MN (15 and 10) compared to mean MN among male cases.

In the present study, the chromosomal abnormality was negligibly small compared to micronuclear index seen in cases. Thus, micronuclei could be used as a predictor of RPL. Therefore, it is hypothesized that the chromosomal instabilities could be one of the causative factors for RPL.

Elevated micronuclei frequency in peripheral blood reflects DNA damage in sperm which in turn leads to unfavourable reproductive outcome. As per WHO, the adverse reproductive outcome was due to maternal factors in 38%, paternal in 20% and both in 27% and unknown in remaining [11]. Elevated frequency of micronuclei in subjects with normal karyotype was also noted.

## V. CONCLUSION

Chromosomal instabilities were elevated in couples with RPL than fertile couples. Genomic instabilities are one of the causative factors for RPL as there was statistically significant difference in frequency of micronuclei among the two groups. Statistically significant difference was observed suggesting that pregnancy losses were due to the genomic derangements detected in the form of micronuclei. Though genetic cause was one among many of the factors leading to RPL, it could not be left without being assessed.

## VI. ACKNOWLEDGMENT

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