INTRODUCTION

Recurrent pregnancy loss (RPL), defined as the loss of three or more pregnancies, is experienced by approximately 1–2% of couples trying to conceive.[1] Several factors, such as genetic, anatomic, thrombophilic, endocrine, or immune, are known to contribute to RPL. Even after routine investigations, up to 50% of RPL cases remain unexplained.[2,3]

Skewed X chromosome inactivation in females[4,5] and Y chromosome microdeletions in their partners[6] have been suggested as a cause of miscarriage. Y chromosome microdeletion is known to cause spermatogenetic failure and male infertility.[7,8] Y chromosome microdeletions occur in at least three regions, called azoospermia factor (AZFa), AZFb, and AZFc. 10 recurrent deletions in the three AZF regions have been described in detail[9] which may lead to varying degrees of spermatogenic failure. AZF deletions have a negative impact on the sperm quality and abnormal spermatozoa that may be associated with RPL.[10] Based on previous studies, the complete deletion of the AZFb and AZFc may have a direct effect on early prophase and decrease the rate of normal pairing in pachytene stage of spermatocytes. AZFc region deletion shows variation in

ABSTRACT

CONTEXT: In majority of couples experiencing recurrent pregnancy loss (RPL), etiology is still unknown. Two genetic factors have been suggested to underlie miscarriage in a subset of patients, namely skewed X chromosome inactivation in females and Y chromosome microdeletions in their partners. In males, microdeletions of the Y chromosome are known to cause spermatogenetic failure and male infertility. AIMS: The aim of the study was to find out the role of Y chromosome microdeletion in male partners of couples experiencing RPL. SETTINGS AND DESIGN: University hospital and genetic laboratory. Prospective case–control study. SUBJECTS AND METHODS: 59 couples with a history of RPL and 20 fertile controls (FC) with no miscarriage were included in the study. The study subjects were divided into male partners of RPL couples with abnormal semen parameters (AS) (n = 8), and couples with normal semen parameters (NS) (n = 51). Fertile controls with normal semen parameters were (FC) (n = 20). Y chromosome microdeletion was performed on 40 male partners of RPL and 20 FC. STATISTICAL ANALYSIS USED: Chi-square test. P < 0.05 were considered statistically significant. RESULTS: 13 of the 40 RPL cases showed deletion in three azoospermia factor loci on the long arm of Y chromosome. The P value was significant with Y chromosome microdeletion in RPL cases as compared to 20 FC where no Y chromosome microdeletion was present. CONCLUSIONS: Y chromosome microdeletion may be an important hidden cause of recurrent pregnancy miscarriage and can be offered to couples with the undiagnosed cause of miscarriage.

KEY WORDS: Recurrent pregnancy miscarriage, semen analysis, Y chromosome microdeletion
clinical phenotype and testicular histology. Surprisingly, little is known about the function of the individual genes and transcription units in these regions.\textsuperscript{[8,11]} Y chromosome microdeletions are detected in approximately 7% of men with oligozoospermia.\textsuperscript{[9]} Dewan et al.\textsuperscript{[6]} conducted a study to determine if the Y chromosome microdeletions are associated with RPL and found a significant portion of male partners of women with RPL had Y-chromosomal microdeletions thereby suggesting it as an important cause of maintaining gestation.

Usually, semen analysis is the first diagnostic step in the evaluation of male factor as a cause of recurrent pregnancy miscarriage. Sperms play a critical role in early embryogenesis, and thus its role extends beyond fertilization in postimplantation events and embryogenesis.\textsuperscript{[12‑14]} It is possible that changes in molecular structure of the sperm may contribute to abortions, hence sperm DNA integrity is one of the important characteristic features.\textsuperscript{[15]} With the above-mentioned background in mind, this study was conducted to find out male factor as a cause of RPL (semen analysis, Y chromosome microdeletion) and to study the correlation of semen analysis with Y chromosome microdeletion, in cases of RPL versus fertile male as controls.

SUBJECTS AND METHODS

The study included 59 males, whose female partner was diagnosed with the history of three or more pregnancy miscarriages prior to 20 weeks of gestation. The subjects were randomly selected from the outpatient department over the span of 2 years. Detailed medical, lifestyle, and family history of both partners were recorded in a predesigned proforma. The study was approved by the Institute Ethical Committee of Institute of Medical Sciences, and informed consent was obtained from each couple. The female partners were evaluated for complete gynecological, clinical, and laboratory investigations to rule out endocrine disorders, anatomical defects, chromosomal, immunological disorder, acquired, and inherited thrombophilias. The male partners of RPL couples were classified into two groups viz. RPL men \((n = 8)\) with abnormal semen parameters (AS), RPL men \((n = 51)\) with normal semen parameters (NS), and the third group was assigned to fertile controls \((n = 20)\) with normal semen parameters (FC).

**Y chromosome microdeletion analysis**

In the Y chromosome microdeletion analysis, 54 blood samples were collected out of 59 males of couples with RPL (5 patients did not turn up for sampling) and they were screened for the presence of microdeletions using Y chromosome-specific sequence tagged site (STS) primer sets mentioned in Table 1. 14 blood samples from the collected 54 were rejected and could not be dissolved because of the delayed transportation and clotting. 11 STS loci spanning the long arm of Y chromosome (Yq) as shown in Figure 1 were analyzed. STS sY14 was used to test for the presence of the short arm of the Y chromosome. Initial polymerase chain reaction (PCR) amplification was done in 20 µl of master mix containing 200 ng of genomic DNA, 2 ml of

| Table 1: PCR primer sequences for Y chromosome microdeletion |
|------------------|------------------|------------------|------------------|
| Name         | Sequence          | Product size (bp) | Position on Y |
| SY83L        | CTGGAATCAAAGAAGGCCCT | 275             | AZFa            | 60°C               |
| SY83R        | CAATTGGTTTGGCTGACAT | 275             | AZFa            | 60°C               |
| SY69L        | GGAACAGCCTCTGCTGT  | 234             | AZFa            | 58°C               |
| SY69R        | ACTATGGGAGCAAGGCTTC | 234             | AZFa            | 58°C               |
| SY84L        | AGAAGGGTCTGAAGCGAGT | 326             | AZFa            | 58°C               |
| SY84R        | GCTCTACTCGAGGCTTC  | 326             | AZFa            | 58°C               |
| SY117L       | GTGGTTCCTGCTCACAT  | 262             | AZFb            | 58°C               |
| SY117R       | CAGGGAGAGCCCTTTTACC| 262             | AZFb            | 58°C               |
| SY127L       | GGCCTCAAAACGAAAAAGAA | 274           | AZFb            | 56°C               |
| SY127R       | CGGCCAGCGCTAAATAGGGA | 274           | AZFb            | 56°C               |
| SY131L       | ACATATCCCTTGCCTTCA | 143             | AZFb            | 56°C               |
| SY131R       | ACATATCCCTTGCCTTCA | 143             | AZFb            | 56°C               |
| SY152L       | AAGACAGTCTGCGATTTTCA | 125             | AZFc            | 58°C               |
| SY152R       | ACAGAGGGTACTGAGGCTT | 125             | AZFc            | 58°C               |
| SY254L       | GGGTTTACAGAAGGCAAA | 326             | AZFc            | 58°C               |
| SY254R       | GACCGTATCTACAAAGCACG | 326           | AZFc            | 58°C               |
| SY255L       | GTTACAGGAATCCGCGAT  | 126             | AZFc            | 58°C               |
| SY255R       | CTGTCTATCTGGCACCA  | 126             | AZFc            | 58°C               |
| SY157L       | CTTAGAAAAAGTGAGGCCG | 285             | AZFc            | 56°C               |
| SY157R       | CCTGCTGCTGCAAGATACA | 285             | AZFc            | 56°C               |
| SY158L       | CTCAAGAAGTCTCTACAAGT | 231           | AZFc            | 52°C               |
| SY158R       | ACAGTGTTTGTAGCCGGTA | 231             | AZFc            | 52°C               |

PCR= Polymerase chain reaction; AZF= Azoospermia factor
1X PCR buffer, 20 µM dNTPs, varying concentrations of forward and reverse primers, and 2.5 unit of Brit Taq DNA-polymerase (Applied Biosystems, USA) [Table 2]. Initial denaturation at 95°C for 10 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 45 s. A final extension was performed at 72°C for 10 min. The presence/absence of all the PCR fragments was tested in 2% agarose gels.

**RESULTS**

59 cases were enrolled in study and after doing the recommended tests in females, (cytogenetic, immunological, endocrinological, anatomical, and infectious); 62.7% (37/59) of cases remained inconclusive [Figure 2].

Semen analysis was abnormal in 8 out of 59 cases (13.5%) and was exclusive in 5 (8.4%) cases, that is, 5 out of the 59 cases were exclusively attributable to abnormal semen analysis without Y chromosome microdeletion and without any co-existing factor. All 8 cases had decreased motility with 4 of them showing decreased count. Only 1 of them had antisperm antibodies. None had abnormal sperm morphology as per WHO criteria 2010 (in the sperm morphology abnormal head, tail, the middle piece, etc., were seen). DFI and the hypo-osmotic swelling test could not be done due to nonavailability. Count, morphology, and motility were seen in RPL male patients and compared with fertile males.

Chi-square test was done to calculate the statistical significance where \( P < 0.05 \) were considered statistically significant.

**Semen analysis**

Semen samples from the male partners were collected through masturbation after 3–4 days of sexual abstinence according to the WHO protocol.\(^{16}\) The semen samples were allowed to liquefy at 37°C for 30 min. Physical examination such as liquefaction time, color, odor, and pH were recorded after 30 min. The basic microscopic examination was carried out to record the count, density, and motility of the sperm according to WHO protocol. DNA fragmentation index (DFI) and the hypotonic swelling test could not be done due to nonavailability. Count, morphology, and motility were seen in RPL male patients and compared with fertile males.

Table 2: Components of master mix (for 20 µl reaction volume)

| Components     | Concentration | Volume (µl) |
|----------------|---------------|-------------|
| Reaction buffer| 1X PCR buffer | 2 (ml)      |
| dNTPs (2.5 mM) | 20 µM         | 1.6         |
| Taq polymerase | 2.5 unit/µl  | 1           |
| Forward primer | 5 pM/µl       | 1           |
| Reverse primer | 5 pM/µl       | 1           |
| MgCl\(_2\) (25 mM) | 1.5 mM   | 2           |
| DNA (200 ng/µl) | 20 ng/µl   | 2           |
| MQ water       |               | 9.4         |

PCR= Polymerase chain reaction

In our study, Y chromosome microdeletion was present in 13 out of 40 (32.5%) cases. Among these, it was exclusively positive in 8 patients (13.5%) after excluding recommended tests for a female. AZFa deletion was seen in 4 patients, AZFb in 1 patient, AZFc in 8 patients. Figure 3a and b represent the multiplex PCR performed in patients and controls. The \( P \) value was significant, that is, 0.016 with microdeletion of
Y chromosome in RPM cases as compared to 20 FC where no Y chromosome microdeletion was present [Table 3]. Only 3 cases had abnormal semen analysis co-existing with Y chromosome microdeletion. Cases with Y chromosome microdeletion were categorized in [Table 4], showing the AZF location, corresponding normal or abnormal semen parameters and co-existing factors, if present. AZFa microdeletion was found in 4 out of 13, AZFb in 1, and AZFc in 8 out of the 13 positive cases.

**DISCUSSION**

Recurrent miscarriage is a distressing event for the couple who desire for pregnancy. Clinical investigation and treatment are usually done after three miscarriages but can also be considered in couples with two consecutive miscarriages if:

- Embryonic heart activity observed in earlier pregnancy loss
- Normal karyotype on products of conception
- Female partner age over 35 years
- Infertility.

In 40% cases, there is no identifiable cause and are attributed to unknown endometrial disorders leading to placental insufficiency. Approximately 70% of human conceptions fail to achieve viability with occult/preclinical/chemical loss as 50% (19% before implantation and 31% after implantation) and before 20 weeks (15%), respectively.

In our study, 59 couples with 3 or more miscarriage were recruited in the time span from August 2011 to June 2013. Couples were tested with recommended investigations of RPL. Additionally, Y -chromosome microdeletion and semen analysis was performed with a male partner as it contributes 50% to the conceptus but has been analyzed as a cause of RPL in limited studies. Three AZF regions on the long arm of Y chromosome are essential for normal spermatogenesis. AZF deletions have a negative impact on the sperm quality and abnormal spermatozoa that may be associated with RPL. The paternal genome provides the centrosome in the first mitotic division after fertilization. Sperm quality has been associated with the embryo’s ability to reach the blastocyst stage and progress to implantation. Paternally expressed genes modulate the proliferation and invasiveness of trophoblast cells and later placentral proliferation. Considering previous studies, the complete deletion of the AZFb and AZFc located on the Y chromosome may have a direct effect on early prophase

**Table 3: Distribution of RPL cases according to semen analysis and Yq deletion compared to fertile controls**

| Type of Y chromosome microdeletion | Abnormal semen analysis (n=8) | Normal semen analysis (n=51) | Normal semen analysis in fertile controls (n=20) | \( \chi^2 \) | \( P \) |
|-----------------------------------|-------------------------------|-------------------------------|-----------------------------------------------|---------|-------|
| Yq deletion                       | Yq deletion: 3/8              | Yq deletion: 10/51            | Yq deletion: 0/20                             | 6.885   | 0.016 |

RPL= Recurrent pregnancy loss

**Table 4: Distribution of Y chromosome microdeletion positive cases with respect to three AZF regions, semen analysis, and co-existing factors**

| Type of Y chromosome microdeletion | Total sperm count (millions/ml) | Percentage of motility (A + B) | Co-existing factor |
|-----------------------------------|---------------------------------|------------------------------|-------------------|
| AZFa                              | 25                             | 45                           | Immunological abnormality in female |
| AZFa                              | 32                             | 42                           | Absent |
| AZFa                              | 22                             | 55                           | Absent |
| AZFa                              | 27                             | 60                           | Cytogenetic abnormality in female |
| AZFc                              | 40                             | 45                           | Absent |
| AZFc                              | 36                             | 62                           | Absent |
| AZFc                              | 34                             | 60                           | Absent |
| AZFc                              | 37                             | 52                           | Absent |
| AZFc                              | 35                             | 64                           | Absent |
| AZFc                              | 42                             | 80                           | Absent |
| AZFc                              | 13                             | 28                           | Endocrine abnormality in female |
| AZFc                              | 10                             | 25                           | Endocrine abnormality in female |
| AZFc                              | 10                             | 25                           | Cytogenetic abnormality in female |

AZF= Azoospermia factor
of mitosis and decrease the rate of normal pairing in pachytene stage of spermatocytes. Thus, this pairing failure may increase chromosomal abnormalities and could be related to recurrent pregnancy miscarriages.[17,18] A study conducted on US population in 2006 found the frequency of 82% (14/17) in RPM cases, 20% (2/10) in infertile cases and none among 18 fertile cases.[24] The frequency of Y chromosome microdeletion in our study was 32.5% (13/40) and thus supports the findings of previous studies.

It is possible that the involvement of microdeletions in the etiology of RPL differs between populations. Different populations have different Y chromosome haplogroups, and it has been shown that the susceptibility to Y-chromosomal microdeletions varies between different haplogroups.[19] Studies done by Wettasinghe et al. in 2010 in Srilanka, Ghobrial et al. on Iranian population in 2012 and Venkatesh et al. in 2011 at AIIMS, New Delhi did not detect any microdeletion on Y chromosome in RPL cases suggesting variation between different haplogroups.[20-22] Our study is also comparable to study by Karaer et al. in Turkey[23] where 43 men from couples with recurrent pregnancy miscarriage and 43 men from couples with a live birth and no history of miscarriages were recruited and DNA was tested for the presence of 4 STSs spanning 4 AZF regions: DYS220 (AZFb), DYS235, DYS236, and DYS237. 16% (7/43) couples with recurrent pregnancy miscarriage had microdeletions in 1 or more of the 4 segments studied, whereas none of the fertile men had any microdeletions (P < 0.05). Their microdeletions were all found specifically at locus DYS 220 (AZFb). Despite the controversy over whether or not sperm quality is related to recurrent miscarriages, there are recent data which suggest that further studies into more specific sperm quality markers may be more revealing. The evidence is accumulating for paternal genome effect in early embryonic development as sperm integrity is vital for sperm–egg interactions, fertilization, and early embryonic development. Semen analysis in our study revealed abnormality in 13.6% (8/59) RPL cases as compared to the 20 fertile males (with no history of abortion) where none revealed any abnormality as per WHO criteria 2010. Although, the mean of count and motility was low in RPL cases, but P value was insignificant as compared to controls which supports the previous study.[24]

There were no significant changes in sperm morphology in the present study as per WHO criteria for semen analysis 2010 which supports the previous study by Sbracia et al.[25] In our study, microdeletion of Y chromosome was present in 13 cases and co-existed with abnormal semen analysis in only 3 cases that is 7.5% (3/40) whereas semen analysis was normal in 10 of the cases with Y chromosome microdeletion.

Since etiology of RPL is multifactorial, both genetics, as well as environmental factors, contribute to its etiopathogenesis. In the present study, a significant association was found between Y-chromosome microdeletion in RPL cases and fertile couples. So, we recommend Y-chromosome microdeletion in patients with unexplained cause of RPL. The present data are preliminary, and further work is required to consider it as a routine part of the investigation in RPL and we may learn in near future how to treat some of these disorders. Currently, preimplantation genetic diagnosis is a method to evaluate embryos for aneuploidy. In the future, we may find other treatments that are more effective in treating couples with recurrent pregnancy miscarriage due to male factor.

What we investigate is only tip of the iceberg regarding many things which remain unexplained starting from ovum, sperm, zygote, implantation and acceptance of alloimmune environment affecting implantation which remains an unresolved mystery but we should always try to find out the etiology of correction and management. There are many etiological factors, and so there is no single therapy which may be applicable to all cases. Tender love and care are perceived as an important part in management specially where etiology remains inconclusive.

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Conflicts of interest
There are no conflicts of interest.

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