Skewed sex ratio & low aneuploidy in recurrent early missed abortion

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Background & objectives: Conventional cytogenetic studies have revealed more number of females in spontaneous abortion and it has been assumed that a large proportion of those were resulted from maternal contamination and overgrowth of maternal decidua in long term culture. In this study we have attempted to overcome difficulties of conventional cytogenetics by using meticulous tissue dissection and molecular methods onto uncultured chorionic villous tissue thus bypassing long term culture to find out true sex ratio and frequency as well as type of common aneuploidy in early missed abortions.

Methods: Early missed abortion products (n=58) were collected from recurrent aborter in and around Lucknow, India, over a period of three years. All the cases were selected on the basis of ultrasonography diagnosis. Chorionic villous tissue was cleaned from maternal tissue and processed for conventional as well as molecular cytogenetic analysis.

Results: Conventional cytogenetics was successful in 15, of which 12 were females and 3 males. There were 3 cases of chromosomal abnormality, including one false. Interphase FISH with X, Y, 1, 9, 12, 16, 18 and 13/21 probes was carried out in all 58 cases. There were 43 females and 15 males. Four cases of chromosomal abnormality were detected by interphase FISH (6.9%). Comparative genomic hybridization was successful in 8 cases (6 females and 2 males). There was no aneuploidy; however, suspected gain and losses were seen in 4 cases.

Interpretation & conclusion: Our results suggested skewing of sex ratio (M : F, 1 : 2.9) and low aneuploidy rate, indicating that in early missed abortion from recurrent spontaneous abortion female outnumbers male. The various possibilities with literature support are presented that may serve as a template for future work.

Key words Aneuploidy - molecular cytogenetics - recurrent spontaneous missed abortions - sex ratio

Approximately 15-20 per cent of clinically recognized pregnancies are spontaneously aborted, mostly during first trimester. Most of these abortions are early missed abortions, defined as irregular pregnancy sac in which the disintegrating embryo has not developed beyond few weeks on two consecutive ultrasound examinations at an interval of one week. Several aetiologies such as
chromosomal abnormalities, hormonal imbalances, polycystic ovarian syndrome, immunological and abnormality of uterus have been attributed\(^2\). Cytogenetic studies of spontaneous abortions have revealed chromosomal abnormality as the major contributing cause in about 50-60 per cent cases\(^3\)-\(^6\). Chromosomal analysis of aborted foetus is important for prognostication\(^7\) because it gives information on frequency and type of chromosomal abnormality, on aetiology and risk assessment for future pregnancies\(^3,8\). Cytogenetic evaluation of chromosomally normal spontaneous abortions had given conflicting results about sex ratio\(^3,9\). However, most studies indicated greater number of female\(^10\)-\(^13\) particularly with alloimmune recurrent spontaneous abortion\(^14,15\) and complete hydatidiform mole\(^16\). Most significant factor influencing the sex ratio is the presence of different frequency of contamination and overgrowth of tissue cultures by maternal cells\(^3,17-20\).

In this study we tried to overcome difficulties of conventional cytogenetics viz., selected successful cases, clonal selection, restriction to dividing cell populations and maternal cell contaminations by selecting chorionic tissue under microscope by single trained person and using molecular methods, in particular interphase fluorescent in situ hybridization (FISH), and attempted to find out true sex ratio and frequency and types of common aneuploidy through interphase FISH onto uncultured chorionic tissue.

**Material & Methods**

Samples were obtained from women with recurrent spontaneous abortion (RSA) who had come for evaluation for known causes \(i.e.,\) hormonal, anatomic, immunologic, chromosomal (parental), etc. Idiopathic recurrent abortion cases having definitive chorionic tissue were selected for the study. A total of 58 such cases of early missed abortion were selected on the basis of ultrasonography (Figs 1A, 1B). Most terminations were carried out in different nursing homes/hospitals. Products of conception at termination were collected in culture medium/normal saline and transported to cytogenetic laboratory within 72 h, mostly by 24 h. Samples were processed in Medical Genetics Department of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, over a period of 3 yr (1999 to 2001). Samples were cleaned from maternal blood by repeated washing with medium and from maternal decidua by dissecting and selecting samples under dissecting inverted microscope. Each sample was divided into three parts, first for conventional cytogenetics, second for interphase FISH and third...
for DNA for comparative genomic hybridization (CGH) as well as for future quantitative fluorescent PCR.

Conventional cytogenetics was performed using amniomax medium (UK) in 25-ml plastic sterile culture flask. About 10-15 mg chorionic villous tissue was prepared by digestion with collagenase and cultured in duplicate according to standard protocol (supplied with amniomax medium). All cultures were long term culture (12 to 30 days, 15 days in most, before harvesting) from chorionic villous tissue. Harvesting was carried out through trypsin release, hypotonic treatment and fixation in methanol:acetic acid (3:1). Trypsin-geimsa banding and staining was carried out before karyotyping. Ten or more metaphases were examined unless numbers of metaphases were low.

Cell dissociation from product of conception (uncultured) for interphase FISH was done by collagenase (Sigma, USA; 2 mg/ml) treatment (45 min at 37°C) followed by hypotonic treatment (50 mM KCl for 20 min at 37°C) and methanol: acetic acid (3:1). Trypsin-geimsa banding and staining was carried out before karyotyping. Ten or more metaphases were examined unless numbers of metaphases were low.

Results

Conventional cytogenetic analysis was tried in 33 cases and culture was successful in 15 cases, overall culture success rate of 45.5 per cent. Twenty five cases were excluded from culture due to poor quality of tissue (advanced autolysis) and contamination. Ten or more metaphases were examined however in two cases analysis was limited to 5 metaphases due to paucity of metaphases. There were twelve females and three males with a sex ratio of 1M:4F. There were two cases of aneuploidy (Table I) and one case of polyploidy (tetraploidy) initially, which later on was found through FISH to be cultural artifact (Table II) hence a chromosome abnormality rate of 13.3 per cent. Both the aneuploidies were trisomy 18 female.

Interphase FISH was carried out with centromeric/heterochromatic regions specific probe for chromosome X, Y, 1, 9, 12, 16,18 and 13/21 on all 58 samples. There were 43 females and 15 males (Fig. 2A) with a sex ratio of 1M:2.9F (Table III). Interphase FISH with above 9 chromosome specific probes was able to detect four cases of aneuploidy i.e., an aneuploidy rate of 6.9 per cent (Fig. 2B, C; Table I).

CGH was carried out in ten cases, however, result were informative in eight. There were 6 females and 2 males. No chromosome abnormality (aneuploidy, gain, loses, etc.) was seen in 4 cases (Fig. 3A) and remaining 4 cases showed suspected areas of gains and losses (Fig. 3B; Table I), however, there was no case of aneuploidy.
Discussion

All the cases had previous history of similar abortions and were investigated in depth to rule out known causes viz., hormonal, anatomical, immunological (autoimmune) and chromosomal (of parents). Hence, our cases could be considered as cases of idiopathic recurrent missed abortions. However, controversial causes viz., alloimmune, have not been ruled out.

We had an overall culture success rate of 45.5 per cent in the study, which is much below the expected frequency. Literature review showed a success rate of culture in spontaneous abortion as 60-90 per cent and standard set by American College of Medical Genetics Committee on Laboratory Practice, 1999 was 75 per cent. Low success rate was due to poor quality of tissue and contamination. Most terminations were carried out in different nursing homes (mostly from Lucknow) and transported in saline/culture medium. Sometimes a sample was reached laboratory as long as 72 h after suction and evacuation of gestation sac and sterility was sub-optimal.

The percentage of chromosomal abnormality in our study was not as quoted in literature i.e., 50-60 per cent. Aneuploidy rate was much lower in our study: 13.3 per cent through conventional cytogenetics, 6.9 per cent through interphase FISH (for chromosomes

<table>
<thead>
<tr>
<th>Abnormal cases</th>
<th>Cytogenetics result</th>
<th>FISH (X, Y, 1, 9, 12, 16, 18 &amp; 13/21)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA25</td>
<td>Failed</td>
<td>XX</td>
<td>3 copy of 16</td>
</tr>
<tr>
<td>MA30</td>
<td>Failed</td>
<td>XXX</td>
<td>3 copy of 1, 18, 16, etc.</td>
</tr>
<tr>
<td>MA32</td>
<td>92,XXXX [13]/ 46,XX [7]</td>
<td>XX</td>
<td>2 copy of all tested chromosomes</td>
</tr>
<tr>
<td>MA38</td>
<td>47,XX,+E [4]/ 46,XX [4]</td>
<td>XX</td>
<td>2 copy of 18 (90%)3 copy of 18 (10%)</td>
</tr>
<tr>
<td>MA56</td>
<td>47,XX,+18</td>
<td>XX</td>
<td>2 copy of 18 (30%)3 copy of 18 (70%)</td>
</tr>
</tbody>
</table>

*In the case of trisomic zygote originated by paternal or maternal non-disjunction at the first or second meiotic cell division, mosaicism results from post-zygotic aneuploidy correction through mitotic error. This rescue of aneuploidy through the loss of a chromosome after fertilization during mitotic cell division in early embryogenesis may confer a selective survival advantage and result in mosaicism.

Table I. Details of conventional cytogenetics, fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH) findings

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cytogenetics</th>
<th>FISH (X, Y, 1, 9, 12, 16, 18 &amp; 13/21)</th>
<th>CGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of investigated samples</td>
<td>33</td>
<td>58</td>
<td>10</td>
</tr>
<tr>
<td>Total number of analyzed samples</td>
<td>15</td>
<td>58</td>
<td>8</td>
</tr>
<tr>
<td>Male/Female (M : F)</td>
<td>3/12 (1 : 4)</td>
<td>15/43 (1:.2.9)</td>
<td>2/6 (1 : 3)</td>
</tr>
<tr>
<td>Chromosomally normal</td>
<td>12 (80%)</td>
<td>54 (93%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Aneuploidy/polyplody</td>
<td>03 (20%)</td>
<td>04 (7%)</td>
<td>Nil</td>
</tr>
<tr>
<td>Tetraploidy</td>
<td>01</td>
<td>00</td>
<td>0</td>
</tr>
<tr>
<td>Triploidy</td>
<td>00</td>
<td>01</td>
<td>0</td>
</tr>
<tr>
<td>Trisomy pure</td>
<td>01 (Ch. 18)</td>
<td>01 (Ch. 16)</td>
<td>0</td>
</tr>
<tr>
<td>Trisomy mosaic</td>
<td>01 (Ch. 18)</td>
<td>02 (Ch. 18)</td>
<td>0</td>
</tr>
<tr>
<td>Other abnormality (gain/loss/structural etc.)</td>
<td>Nil</td>
<td>Not applicable</td>
<td>4 suspected*</td>
</tr>
</tbody>
</table>

*suspected partial gain/loss; need to be confirmed by FISH before accepting

Table II. Details of aneuploidy/polyplody cases detected through conventional cytogenetics and fluorescent in situ hybridization (FISH)
Fig. 2. Interphase XY (X as green and Y as red) dual color FISH (A and B) on chorionic tissue obtained from missed abortion cases. Note poor quality of DNA (moth eaten appearance of nuclei) in a male conceptus (A; XY) and XXY conceptus (B), which later proved XXY triploidy by chromosome 1 FISH. C. Interphase chromosome 18 (red) mono color FISH on trophoblastic nuclei confirming trisomy 18 (3 signals).
Fig. 3. CGH mean profile from chromosomally normal (A) and abnormal (B; suspected gain at 5q14-15, arrow) cases.
X, Y, 1, 9, 12, 16, 18 and 13/21) and 0 per cent through CGH. We had complete information on all chromosomes from 23 cases (15 through conventional cytogenetics and 8 through CGH) and 2 were aneuploid, hence aneuploidy rate of 8.7 per cent. Low aneuploidy rate may be due to small sample size and use of fewer FISH probes. However, it may be genuinely low because we have tested all chromosomes commonly involved in aneuploidy of early spontaneous abortion (excluding 22). Abnormal chromosome ploidy/numerical chromosome abnormality (aneuploidy and polyploidy) consists of more than 96 per cent of chromosomal abnormality in spontaneous abortion and X, Y, 13, 16, 18, 21 and 22 are frequently involved. However, maximum aneuploidy in early spontaneous abortion involves chromosome X and 16 (also 22) in addition to triploidy. Our FISH probe set covered all important chromosomes, however chromosome 22 was not included (information not available initially). All FISH probes were repeat satellite sequences/alphoid probes. Although these probes’ size was small (about 170 bp) target sequence was usually very large (1-2 Mb) due to tandem repeats of few hundred to few thousand times. This results in strong and large FISH signal even in poor quality of DNA/cells/tissue (due to small repetitive sequence, more chance of few DNA repeats and so hybridization). This provided results in all the cases including those where cytogenetic diagnosis failed despite the deterioration of the sample and partial autolysis. FISH probe for chromosome 1 was particularly chosen because of its rarity in pathogenesis in recognized conceptions. This helped in distinguishing triploidy from double trisomy. Further, we wanted to evaluate chromosome 1 in our samples as there was no recognizable foetal product and trisomy 1 has been reported with missed abortion. However, chromosome 1 aneuploidy was not found, chromosome 16 aneuploidy, which is the commonest in spontaneous abortion, was found only in one. Chromosome 18 aneuploidy was seen most frequently in our study. Low frequency of aneuploidy in idiopathic recurrent spontaneous missed abortions was similar to other reports which varies from 5-10 per cent (sporadic or unexplained abortions) to 18 per cent (RSA) to 26 per cent (alloimmune RSA).

Our interphase FISH study on uncultured chorionic villous cells showed a sex ratio of 1M to 2.9F. This is in contrast to most reports on recognized pregnancy (well-developed foetus) where an excess of male was seen including in our previous study on 154 unwanted terminations of pregnancy. A reversal of sex ratio was found consistently in pregnancy with unrecognized foetal development viz., early missed abortion, alloimmune RSA, anembryonic pregnancy or complete mole. Sex ratio in alloimmune RSA may be as high as 1M:13F, in early missed abortion/anembryonic pregnancy may be 1M to 3F and in molar pregnancy may be 1M:9F. Our result reaffirms a female specific developmental disadvantage in early stages of pregnancy with poor/no foetal development. This is supported by sex ratio at preimplantation and early post-implantation embryo which is about 1.06 M : 1 F in day 3 embryo to 1.34 M : 1 F in blastocyst stage embryo to 1.51 M : 1 F at 7-8 wk embryo indicating greater wastage of female conceptus before implantation and foetal recognition. Thereafter excess of male wastage was seen at any time between clinical recognition of fetus and birth (at 7-8 wk 151 males, at 11-12 wk 132 males and at birth 105-107 males for

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cytogenetics</th>
<th>FISH</th>
<th>CGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early missed abortions (&lt;12 wk)</td>
<td>(n=15)</td>
<td>(n=58)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Male</td>
<td>03</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>43</td>
<td>6</td>
</tr>
<tr>
<td>Sex ratio (male : female)</td>
<td>1 : 4</td>
<td>1 : ~2.9</td>
<td>1 : 3</td>
</tr>
<tr>
<td>Unwanted termination of pregnancy (&lt;12 wk)</td>
<td>ND</td>
<td>n=154</td>
<td>ND</td>
</tr>
<tr>
<td>Male</td>
<td>88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex ratio</td>
<td>~1.3 M : 1 F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X (missed abortion with unwanted pregnancy termination)</td>
<td>14.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FISH, fluorescent in situ hybridization; CGH, comparative genomic hybridization; ND, not done</td>
<td></td>
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</tr>
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</table>

**Table III.** Details of sex ratio in early missed abortions (present study) and unwanted termination of pregnancy (previous work) and 13/21) and 0 per cent through CGH. We had complete information on all chromosomes from 23 cases (15 through conventional cytogenetics and 8 through CGH) and 2 were aneuploid, hence aneuploidy rate of 8.7 per cent. Low aneuploidy rate may be due to small sample size and use of fewer FISH probes. However, it may be genuinely low because we have tested all chromosomes commonly involved in aneuploidy of early spontaneous abortion (excluding 22). Abnormal chromosome ploidy/numerical chromosome abnormality (aneuploidy and polyploidy) consists of more than 96 per cent of chromosomal abnormality in spontaneous abortion and X, Y, 13, 16, 18, 21 and 22 are frequently involved. However, maximum aneuploidy in early spontaneous abortion involves chromosome X and 16 (also 22) in addition to triploidy. Our FISH probe set covered all important chromosomes, however chromosome 22 was not included (information not available initially). All FISH probes were repeat satellite sequences/alphoid probes. Although these probes’ size was small (about 170 bp) target sequence was usually very large (1-2 Mb) due to tandem repeats of few hundred to few thousand times. This results in strong and large FISH signal even in poor quality of DNA/cells/tissue (due to small repetitive sequence, more chance of few DNA repeats and so hybridization). This provided results in all the cases including those where cytogenetic diagnosis failed despite the deterioration of the sample and partial autolysis. FISH probe for chromosome 1 was particularly chosen because of its rarity in pathogenesis in recognized conceptions. This helped in distinguishing triploidy from double trisomy. Further, we wanted to evaluate chromosome 1 in our samples as there was no recognizable foetal product and trisomy 1 has been reported with missed abortion. However, chromosome 1 aneuploidy was not found, chromosome 16 aneuploidy, which is the commonest in spontaneous abortion, was found only in one. Chromosome 18 aneuploidy was seen most frequently in our study. Low frequency of aneuploidy in idiopathic recurrent spontaneous missed abortions was similar to other reports which varies from 5-10 per cent (sporadic or unexplained abortions) to 18 per cent (RSA) to 26 per cent (alloimmune RSA).

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every 100 females\textsuperscript{32,37}). Hence, this excess of males in recognised pregnancy arises in embryogenesis by preferential loss of unrecognized female embryos before the foetal period\textsuperscript{38}.

Excess of female in early spontaneous abortions similar to our study has been reported earlier\textsuperscript{3,10-12,19,20,38,39}. It is generally thought that excess of female in spontaneous abortion was due to contamination by maternal decidua in long term culture of conventional cytogenetics\textsuperscript{3,10,11,17-20}. However, it was unlikely in our study because this is a problem of long-term culture and not of interphase FISH on uncultured villous tissue. In long-term culture of missed abortion maternal decidual tissue has an advantage over bad quality of foeto-placental tissue and hence the problem. Further, in our study villous tissue was selected after meticulous dissection from maternal tissue under dissecting inverted microscope and washed clean of blood before processing for interphase FISH. Our experience with interphase FISH on uncultured placental cells obtained from termination of pregnancy had shown maternal cell contamination rate of 1.8 per cent\textsuperscript{40} that did not affect gender interpretation.

The reason for skewed sex ratio in pre-foetal embryo \textit{i.e.}, mechanism by which female pre-foetal embryo exposed to higher risk of rejection is not known at present. On the basis of our findings and related studies three hypotheses could be proposed. The first hypothesis proposes that environmental pollutant, in particular, dioxin may have a negative effect on viability of early XY fertilized zygote leading to alteration in sex ratio\textsuperscript{41-43}. More extreme skewing of sex ratio (1 M : 4 F) was seen with neural tube defect (NTD) in same geographic area\textsuperscript{44} (around Lucknow) where both are prevalent. This supports environmental factors as cause.

Second possibility is uniparental diploidy or disomy or imprinting\textsuperscript{45}. Uniparental (paternal/androgenetic) diploidy \textit{e.g.}, complete hydatidiform mole results in placental development without any foetal development. Parthenogenetic blastocyst which lacks paternal genome shows very poor trophoblastic proliferation even in presence of normal inner cell mass\textsuperscript{46}. Sex difference in survival and growth during embryogenesis is dependent on epigenetic differences, such as genomic imprinting\textsuperscript{47} or X chromosome inactivation. Abnormal genomic imprinting normally imposed in oogenesis and growth retarded embryo. Normal paternal imprinting of X chromosome slows mammalian embryogenesis. Absence of normal maternal imprinting in oogenesis causes poor growth of the embryo proper and absence of normal paternal imprinting in spermatogenesis causes poor growth of extra-embryonic tissue\textsuperscript{48}. Thornhill and Burgoyne\textsuperscript{49} showed that a paternally imprinted X chromosome (possessed by all normal mammalian females and no normal males) retards early development in the mouse. Monosomy X is an example of excess loss of female embryos, which illustrates the importance of human embryogenesis and paternal epigenetic factors. At least 95 per cent of known lost 45,X conceptuses fail as embryo. Most live-born 45,X has a maternal X chromosome\textsuperscript{50}. High frequency of maternal X chromosome in live-born 45,X girl is due to preferential loss of embryos bearing paternally imprinted monosomic X chromosome. Evdokimova \textit{et al}\textsuperscript{12} observed a sex ratios of 0.77 for the most advanced embryos and 0.31 for anembryonic conceptuses \textit{i.e.}, earlier the failure, greater the fraction of female. These authors proposed that the expression of genes of the single, maternal (maternally imprinted) X chromosome in XY embryos support a more stable development during early embryogenesis as compared with XX embryos (with one paternally imprinted X chromosome). Over ninety per cent of complete molar pregnancy are 46,XX, and less than 10 per cent are 46,XY and hence a sex ratio of <1 M : 9 F\textsuperscript{16}. In complete mole all the chromosomes are of paternal origin (uniparental diploidy).

Third possibility is failure of X chromosome inactivation\textsuperscript{51} that arise either from mutation in Xic (X inactivation center) or some other means causing over dosage of X chromosome genes in female conceptions causing early lethality. Edward\textsuperscript{52} recently observed X inactivation failure in female preimplantation mouse embryo resulting complete developmental failure.

In conclusion, biological significance of skewed sex ratio is difficult to predict at present. However, studies in this area are important to understand reason
for skewed sex ratio in RSA. It seems worth exploring the possibility of preimplantation sexing and male embryo transfer in overcoming another abortion in allo-immune/idiopathic RSA or recurrent molar pregnancy as male foetuses are more likely to survive.

Acknowledgment

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