THE FREQUENCY OF ANEUPLOIDY IN THE SECONDARY SPERMATOCYTES OF NORMAL AND ROBERTSONIAN TRANSLOCATION-CARRYING RAMS

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Summary. A detailed analysis was made of the chromosomes in 1008 M II figures from three different types of heterozygous Robertsonian translocation-carrying rams (53,xy, t1; 53,xy,t2 and 53,xy,t3) and 225 M II figures from homozygous Robertsonian translocation-carrying rams (52,xy,t1,t1; 52,xy,t3,t3) and rams of normal karotype (54,xy). No hypermodal cells were recorded in either the normal or the homozygous rams, but from 4.5% to 9.2% of M II cells from the heterozygous rams were hypermodal. The heterozygous rams also produced a significantly higher level of hypomodal cells suggesting that, in addition to non-disjunction, lagging at anaphase I may have occurred. There were also distinct differences in M II aneuploid spermatocyte frequency between heterozygous versus normal and homozygous rams.

Fewer balanced translocation X-carrying M II cells were recorded than expected in three of the four 53,xy,t2 rams. This coincides with mating data which suggest that 26,x,t2 gametes may occur less frequently than expected.

Since ewes of normal karotype mated to 53,xy,t rams conceive to first service at a rate equal to or better than normal mating groups, and because no blastocysts with unbalanced karotypes associated with the t1 translocation have been recorded, it is suggested that only euploid spermatozoa are involved in fertilization. In the sheep, aneuploid spermatocytes probably degenerate before sperm maturation.

INTRODUCTION

There are now a number of conflicting reports on the effects of Robertsonian translocations on the fertility of man (Chandley et al., 1972; Fraccaro et al., 1973; Palmer et al., 1973) and domestic animals (Gustavsson, 1969; Pollock & Bowman, 1974; Bruère & Chapman, 1974; Bruère, 1974a, b, 1975). It has been assumed that non-disjunction of the homologues of the translocation chromosomes at anaphase I is a regular occurrence in translocation heterozygotes, and that a proportion of the unbalanced secondary spermatocytes become mature gametes. This occurs regularly in crosses of the tobacco mouse (Mus poschiavinus) and house mouse (M. musculus). In the latter, heterozy-
gotes aneuploid gametes actually produce a significant number of aneuploid zygotes which do not complete embryogenesis (Tettenborn & Gropp, 1970; Cattanach & Moseley, 1973; C.E. Ford & E.P. Evans, personal communication).

Breeding data from sheep carrying Robertsonian translocations in both the single heterozygous and double heterozygous state have yielded no evidence that either aneuploid gametes or aneuploid zygotes are formed (Bruère & Chapman, 1974; Bruère, 1974b, 1975). The fertility of such sheep is apparently normal and reduced fertility, if significant at all, occurs only under specific mating or possibly phenotypic conditions (Bruère & Mills, 1971; Bruère, 1974a).

The analysis of the chromosomes from secondary spermatocytes (M II) in both man and domestic animals has been limited both by the tedium and difficulty in obtaining cytologically unequivocal metaphase preparations for the accurate determination of chromosome number; for example see man (Luciani, 1970; Ferguson-Smith, 1972; Skakkebaek et al., 1973), bull (Gustavsson, 1969; Pollock & Bowman, 1974), goat (Datta, 1970) and sheep (Makino, 1943). The analysis of large numbers of M II cells in the mouse has been facilitated by the use of colchicine in the living animal (Tettenborn & Gropp, 1970; Döring et al., 1972; Cattanach & Moseley, 1973), a procedure which for humane reasons is not acceptable in domestic animals.

This report describes for the first time the levels of aneuploidy of M II figures from normal rams and from sheep with three different types of Robertsonian translocation.

MATERIALS AND METHODS

Terminology

The terminology used to describe the translocation chromosomes is the same as that used previously (Bruère, 1974b). The three translocations are referred to as either 53,xy,t1; 53,xy,t2; 53,xy,t3 or t1, t2 and t3 in the heterozygous state, or 52,xy,t1t1; 52,xy,t2t2; 52,xy,t3t3 in the homozygous state.

Animals

The animals used were all derived from the various mating experiments described previously (Bruère, 1974b).

Cytogenetic methods

The karyotypes of the rams were determined from the usual form of chromosome preparations made from whole blood leucocyte cultures.

The meiotic chromosome preparations were made from testicular tissue which was processed immediately following removal from the ram. The technique was developed for the ram (Chapman, 1974) and was a modification of the method of Evans et al. (1964). The meiotic preparations were stained with aceto-orcein before mounting and microscopic examination.

The C-bandung of chromosomes was done by the technique of Sumner (1972) as modified by Chandley & Fletcher (1973) with the [Ba(OH)2, 8H2O] incubation lasting for 10 min.
**Microscopic analysis of secondary spermatocytes**

Ambiguity can occur in counting M II chromosomes because of their contracted nature and the fact that not infrequently a single chromatid from a metacentric or acrocentric chromosome could be confused easily with the two chromatids of an acrocentric chromosome still held together at the centromere. For this reason, a system was used to count secondary spermatocytes similar to that of Tettenborn & Gropp (1970). This involved the counting of chromosome arms as well as the chromosome number. All acrocentric chromosomes were counted as having one chromosome arm. Metacentric and submetacentric chromosomes were counted as having two chromosome arms and the X and the Y chromosomes were both counted as having one chromosome arm, although the latter chromosome is submetacentric. In each cell the type of sex chromosome and the presence of a translocation chromosome was also noted.

The haploid chromosome complement for the sheep consists of 23 acrocentrics, 3 metacentrics and one sex chromosome, a total of 27 chromosomes or 30 chromosome arms.

The haploid number for the secondary spermatocyte of a normal ram was scored therefore as either \( \frac{27}{30}x \) or \( \frac{27}{30}y \); euploid secondary spermatocytes were NF/2 = 30, and aneuploid secondary spermatocytes had an NF/2 either > or < 30.

In a small number of metaphase cells the use of the C-banding technique greatly assisted in accurate chromosome counting by clearly defining the centromeres.

**Aneuploid frequencies**

Three estimates of aneuploid frequency were made, two being based on the method of Cattanach & Moseley (1973). The first estimate gave the total proportion of cells with either 29 or 31 chromosome arms, and the second was derived by doubling the frequency of cells with a chromosome arm count of 31. Because of the inevitable negative skew to hypomodal chromosome counts due to technical factors, the former frequency may be an overestimate of the frequency of aneuploid secondary spermatocytes, while the latter could be a slight underestimate.

A third estimate of aneuploid frequency was made by the method suggested to us by C. E. Ford & E. P. Evans (personal communication). This was an expression of the percentage of aneuploid cells derived by using the following formula \( \Sigma 29,31 \) classes \( \times 100/\Sigma 29,30,31 \) classes.

**RESULTS**

An analysis was carried out on a total of 87 secondary spermatocytes from normal rams, 1008 secondary spermatocytes from three different types of translocation heterozygote plus 138 secondary spermatocytes from two different types of translocation homozygote (Table 1).
Normal and translocation homozygotes

Because of the similarities between the data from the normal and translocation homozygous rams, and for ease of comparison with data from the heterozygous rams, the first two are considered together.

The analysis of the M II cells from ten normal rams and two homozygous rams, excluding the polyploid cells, is recorded in Table 2. In none of these three groups, 54,xy, 52,xy,t₁t₁, and 52,xy,t₃t₃ was any hypermodal cell recorded. In the 54,xy group, 16% of cells were hypomodal while 14.1% in the 52,xy,t₁t₁ and 8.7% in the 52,xy,t₃t₃ classes were also hypomodal (Text-fig. 1a). These percentages mainly represent chromosome loss during slide preparation. The numbers and percentages of polyploid cells are shown in Table 1. The percentages of polyploid cells were high in each respective group, but since in the 54,xy group the total cell counts from most rams were small and only one ram in each of the 52,xy,t₁t₁ and 52,xy,t₃t₃ groups was studied, it is difficult to draw conclusions.

<table>
<thead>
<tr>
<th>Karyotype of ram</th>
<th>No. of M II cells</th>
<th>Polyploid M II cells No. %</th>
<th>Total no. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>54,xy</td>
<td>62</td>
<td>25</td>
<td>28-73</td>
</tr>
<tr>
<td>52,xy,t₁t₁</td>
<td>99</td>
<td>11</td>
<td>10-60</td>
</tr>
<tr>
<td>52,xy,t₃t₃</td>
<td>23</td>
<td>5</td>
<td>17-85</td>
</tr>
<tr>
<td>Total of normal + homozygotes</td>
<td>104</td>
<td>41</td>
<td>18-22</td>
</tr>
<tr>
<td>53,xy,t₁</td>
<td>160</td>
<td>41</td>
<td>20-39</td>
</tr>
<tr>
<td>53,xy,t₂</td>
<td>331</td>
<td>53</td>
<td>13-80</td>
</tr>
<tr>
<td>53,xy,t₃</td>
<td>326</td>
<td>97</td>
<td>22-93</td>
</tr>
<tr>
<td>Total of heterozygotes</td>
<td>817</td>
<td>191</td>
<td>18-95</td>
</tr>
</tbody>
</table>

In the M II cells of the 52,xy,t₁t₁ ram the X chromosome was present in 52 cells and the Y chromosome in 46 cells, while nine X-bearing and thirteen Y-bearing cells were seen in the M II figures of the 52,xy,t₃t₃ ram. In neither case does this depart from the expected 1:1 ratio.

Translocation heterozygotes

Chromosome counts. The numerical and percentage distribution of euploid and aneuploid M II figures for the three different types of translocation heterozygote are shown in Table 2, and the distribution of euploid (NF/2 = 30) and aneuploid (NF/2<or>30) M II figures are shown in the frequency histograms (Text-fig. 1b). Photomicrographic examples of euploid, aneuploid and polyploid M II cells from normal and translocation-carrying rams are shown in Plates 1 and 2.

As expected, hypomodal cells were recorded in all three classes of transloca-
Fig. 1. Metaphase II cell, from a normal ram, with 30 chromosome arms and 27 chromosomes including three metacentrics and an X chromosome (X).

Fig. 2. Metaphase II cell, from a normal ram, with 30 chromosome arms, and 27 chromosomes and a Y chromosome (Y).

Fig. 3. Metaphase II cell from a 53,xy,t, ram. There are 26 chromosomes, including a translocation submetacentric (t) and a Y chromosome (Y).

Fig. 4. C-banded metaphase II cell from a 53,xy,t, ram. There are 27 chromosomes and 30 chromosome arms. Note absence of centrometric staining of the X chromosome (X).

(Facing p. 336)
Fig. 5. Polyploid metaphase II cell with two Y chromosomes (arrowed) from a 53,xy,t₂ ram.

Fig. 6. Metaphase II aneuploid cell from a 53,xy,t₂ ram. There are 31 chromosome arms and 27 chromosomes including a translocation metacentric (t) and an X chromosome. Note the position of the arms of the X chromosome.

Fig. 7. Balanced translocation-carrying metaphase II cell from a 53,xy,t₂ ram. There are 30 chromosome arms and 26 chromosomes including three metacentrics, a submetacentric (t) and a Y chromosome (Y).

Fig. 8. Metaphase II aneuploid cell from a 53,xy,t₃ ram. There are 29 chromosome arms and 25 chromosomes including three metacentrics, a translocation submetacentric (t) and a Y chromosome (Y).
Table 2. Distribution of euploid (NF/2 = 30) and aneuploid (NF/2 < or > 30) M II figures in normal rams and rams heterozygous and homozygous for a Robertsonian translocation (pooled data not including polyploid cells)

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>No. of rams</th>
<th>NF/2</th>
<th>&lt;28</th>
<th>28</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>&gt;31</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>53,xy,t1</td>
<td>6</td>
<td></td>
<td>5</td>
<td>3:13</td>
<td>6</td>
<td>3:75</td>
<td>33</td>
<td>20:63</td>
<td>107</td>
</tr>
<tr>
<td>53,xy,t2</td>
<td>4</td>
<td></td>
<td>10</td>
<td>3:02</td>
<td>42</td>
<td>12:69</td>
<td>84</td>
<td>25:38</td>
<td>180</td>
</tr>
<tr>
<td>52,xy,t1t1</td>
<td>1</td>
<td></td>
<td>6</td>
<td>6:06</td>
<td>2</td>
<td>2:02</td>
<td>6</td>
<td>6:06</td>
<td>85</td>
</tr>
<tr>
<td>52,xy,t3t3</td>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8:70</td>
<td>21</td>
</tr>
<tr>
<td>54,xy</td>
<td>10</td>
<td></td>
<td>6</td>
<td>9:68</td>
<td>1</td>
<td>1:61</td>
<td>3</td>
<td>4:84</td>
<td>52</td>
</tr>
</tbody>
</table>

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Text-fig. 1. Frequency histogram showing the distribution of euploid (NF/2 = 30) and aneuploid (NF/2 < or > 30) M II figures in (a) two homozygous rams and the pooled results from ten normal rams; and (b) rams heterozygous for t₁, t₂ and t₃ translocation chromosomes. (Results for each translocation type are pooled results from individual rams.)

In (a) there were 62 M II figures for 54,xy rams, 23 for 52,xy,t₁t₁ and 99 for 52,xy,t₁t₃ rams. In (b) there were 160 M II figures for 53,xy,t₁, 331 for 53,xy,t₂, and 326 for 53,xy, t₃ rams.

Aneuploid spermatocyte frequency in heterozygous rams. The aneuploid spermatocyte frequencies for those rams from which more than twenty M II cells were analysed are given in Table 3.

The frequencies of aneuploidy varied between rams and in each case the estimation of aneuploid frequency based on hypermodal cell counts was considerably smaller than the estimate based on the 29 and 31 chromosome arm counts. There were no significant differences between rams with regard to the distribution of euploid and aneuploid cells when these are considered as groups, although the individual frequencies for some rams were significantly higher than others. The aneuploid frequency 31 × 2(%) for the 53,xy,t₃ rams was considerably higher than those for either 53,xy,t₁ rams or 53,xy,t₂ rams.

Translocation chromosome-sex chromosome association. In the euploid data for t₁ rams, approximately equal numbers of balanced translocation cells and normal cells were recorded carrying the X and Y chromosomes respectively.
Aneuploidy in spermatocytes of translocation rams

However, when cells from both euploid and aneuploid classes were combined a departure from the expected 1:1:1:1 ratio of the +ty:+tx:−ty:−tx was observed ($\chi^2 = 10.52$, $P<0.05$). Fewer +tx cells and more +ty cells were present than expected.

Fewer balanced translocation-carrying M II cells than expected for $t_2$ rams were recorded from these data ($\chi^2 = 6.19$, $P<0.05$) (Table 4). This was attributed mainly to a deficiency of +tx cells ($\chi^2 = 9.05$, $P<0.05$). When both euploid and aneuploid data were pooled, there was a significant departure ($\chi^2 = 70.39$, $P<0.001$) from the expected equal numbers of translocation-carrying and non-translocation-carrying X and Y cells. More non-translocation-carrying cells than expected were found.

### Table 3. Aneuploid frequencies for $t_1$, $t_2$ and $t_3$ rams (heterozygotes) and normal and homozygous rams from those animals for which at least twenty M II cells were analysed

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Ram</th>
<th>29+31%</th>
<th>31×2%</th>
<th>$\Sigma 29,31$ Classes $\times 100%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>53,xy,$t_1$</td>
<td>B53/69</td>
<td>29-31</td>
<td>60-90</td>
<td>28-19*</td>
</tr>
<tr>
<td></td>
<td>A182</td>
<td>23-73</td>
<td>13-56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 8/72</td>
<td>19-05</td>
<td>0-00</td>
<td></td>
</tr>
<tr>
<td>53,xy,$t_2$</td>
<td>B25/72</td>
<td>24-59</td>
<td>13-11</td>
<td>35-48*</td>
</tr>
<tr>
<td></td>
<td>B87/72</td>
<td>31-79</td>
<td>10-60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B68/72</td>
<td>33-85</td>
<td>9-23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B66/72</td>
<td>25-93</td>
<td>0-00</td>
<td></td>
</tr>
<tr>
<td>53,xy,$t_3$</td>
<td>B13/72</td>
<td>29-61</td>
<td>16-50</td>
<td>38-68*</td>
</tr>
<tr>
<td></td>
<td>B76/72</td>
<td>47-47</td>
<td>22-20</td>
<td></td>
</tr>
<tr>
<td>52,xy,$t_1$t</td>
<td>B55/70</td>
<td>6-06</td>
<td>0-00</td>
<td>6-59</td>
</tr>
<tr>
<td>52,xy,$t_3$t</td>
<td>A134</td>
<td>8-70</td>
<td>0-00</td>
<td>8-70</td>
</tr>
<tr>
<td>54,xy</td>
<td>Grouped</td>
<td>4-84*</td>
<td>0-00*</td>
<td>5-45*</td>
</tr>
</tbody>
</table>

Analysis after the method of Cattanach & Moseley (1973) and C. E. Ford & E. P. Evans (personal communication).

* Calculation based on pooled data.

### Table 4. Association of translocation chromosomes with sex chromosomes (pooled data) in $t_1$, $t_2$ and $t_3$ rams

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>$NF/2 = 30$</th>
<th>$NF/2 &lt; or &gt; 30$</th>
<th>Euploid + aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$+ty$</td>
<td>$+tx$</td>
<td>$−ty$</td>
</tr>
<tr>
<td>53,xy,$t_1$</td>
<td>28</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td>53,xy,$t_2$</td>
<td>42</td>
<td>29</td>
<td>57</td>
</tr>
<tr>
<td>53,xy,$t_3$</td>
<td>29</td>
<td>43</td>
<td>48</td>
</tr>
</tbody>
</table>

The ratio of Y-carrying to X-carrying cells in $t_3$ rams differed ($\chi^2 = 7.37$, $P<0.01$) overall, there being an excess of X-carrying cells. Fewer +ty cells and more −tx cells were recorded than expected ($\chi^2 = 8.77$, $P<0.05$) when euploid data alone were considered. This trend was increased when euploid and aneuploid data were combined ($\chi^2 = 38.68$, $P<0.001$).
DISCUSSION

The data from Table 2 clearly show that the percentage distribution of euploid cells is lowest for the $t_1$, $t_2$ and $t_3$ rams. They also show that there were no hypermodal cells recorded in either the normal rams or in the homozygous rams ($t_1t_1$ and $t_2t_3$) and the number of cells with 29 chromosome arms is higher for the heterozygotes than for the homozygotes and normal rams. This is similar to the results described from similar studies on the tobacco mouse (Döring et al., 1972; Cattanach & Moseley, 1973).

This result is confirmed in another way by comparing the estimations of aneuploid frequency for the two different groups (Table 3) and in the frequency histograms (Text-figs 1a and 1b). In agreement with the tobacco mouse data of Cattanach & Moseley (1973), the ram data suggest that the first estimate of aneuploid frequency is too high while the second is too low. Further, for all three heterozygote groups ($t_1$, $t_2$ and $t_3$) there were significantly greater numbers of cells with 29 chromosome arms than in the complementary class with 31 chromosome arms. This suggests that some chromosome loss due to lagging at first anaphase probably occurred as well as non-disjunction.

The differences between the proportions of hypomodal cells and hypermodal cells in translocation heterozygotes varies between workers. Tettenborn & Gropp (1970), Döring et al. (1972) and C. E. Ford & E. P. Evans (personal communication) did not report significant differences between these two cell groups for the tobacco mouse, while the data from the sheep heterozygotes and $T_1Bnr$, $T_2Bnr$, $T_3Bnr$, $T_4Bnr$ and $T_5Bnr$ of the tobacco mouse heterozygotes studied by Cattanach & Moseley (1973), do show significant differences. One contributory factor to this difference could be the different techniques employed in making the chromosome preparations.

It is clear, however, that heterozygosity for a Robertsonian translocation in the sheep is a major factor leading to an increased frequency of aneuploid secondary spermatocytes and also the major factor associated with non-disjunction. This trend appears similar to that shown in the tobacco mouse hybrids (Cattanach & Moseley, 1973; C. E. Ford & E. P. Evans, personal communication).

It is interesting to note the variation in aneuploid M II frequency between individual rams (Table 3). Ram B76/72 (53,xy,t3), from which 99 cells were evaluated, had values of 47.5% to 22.2%, while B13/72 (53,xy,t3), from which 206 cells were counted, had values of 29.6% to 16.5% and B87/72 (53,xy,t3), from which 151 cells were evaluated, had counts of 31.8% to 10.6%. Differences of this nature have also been observed in mice heterozygous for any one metacentric chromosome (Evans et al., 1967; White & Tjio, 1967; Cattanach & Moseley, 1973; C. E. Ford & E. P. Evans, personal communication).

In the 53,xy,t2 rams, fewer balanced translocation-carrying female M II cells (26tx/30) were recorded than expected. A similar trend has been noted in the karyotype categories of the progeny of mating 53,xy,t2 rams to ewes of normal karyotype. From the mating data for 2 years, a deficiency of 53,xx,t2 has been recorded (Bruère, 1974b). This was not confirmed by additional mating data (Bruère, 1975). However these data could suggest a small selective
disadvantage against 53,xx,t.<sup>2</sup>. Non-translocation X- and Y-bearing cells predominated at M II in the three translocation types of this study and greater than expected numbers of X-bearing cells were found in both euploid and aneuploid cells in the 53,xy,t<sub>3</sub> groups (Table 4). Similar observations have been made on the mouse (White & Tjio, 1967; Evans <i>et al.</i>, 1967). At present more data are needed before a conclusion can be drawn, but significantly, apart from the 52,xy,t<sub>2</sub> data, no unequal number of progeny of any particular chromosome category has been recorded consistently in the various mating groups studied by Bruère (1974b, 1975).

Although a significant number of aneuploid M II cells are produced in rams heterozygous for each of the three Robertsonian translocations studied, there is no evidence from either blastocyst studies (Long, 1974) or from the mating data involving such rams (Bruère, 1974b, 1975; Bruère & Chapman, 1974) that aneuploid gametes are producing aneuploid zygotes. In the experiments of Bruère & Chapman (1974) with single and double translocation-heterozygous rams, the conception to first service rate was as good as that reported for non-translocation-carrying sheep, as were the numbers of non-producing ewes and the percentage of lambs born. This is in complete contrast to the tobacco mouse heterozygotes in which aneuploid zygotes are regularly formed from such matings with resultant embryonic loss (Döring <i>et al.</i>, 1972; Cattanach & Moseley, 1973; C. E. Ford & E. P. Evans, personal communication).

It would appear that in the sheep the aneuploid M II cells either degenerate during spermatogenesis (Roosen-Runge, 1973) or are selected against preferentially at fertilization. It is difficult to accept the latter concept. We believe that considerable variation between species exists for the fate of unbalanced M II cells, and that, in the ram, unbalanced M II cells degenerate during the later stages of spermatocyte maturation as suggested by Ortavant (1958) and Roosen-Runge (1973). This is one way in which unsuitable male cells are not disseminated as unsuitable gamete types.

The numbers of polyploid cells recorded from the M II cells is similar in all classes of rams (Table 1). Although the apparent level of polyploidy described in sheep is higher than reported in other species, insufficient data prevent a definite comparison being made. The cause of this polyploidy is uncertain. It may be artifactual, due to overlapping of cells as suggested by Hultén <i>et al.</i> (1970), Ford & Evans (1971) and Lin <i>et al.</i> (1971). Disturbances of cytokinesis in spermatogonial cells (Lin <i>et al.</i>, 1971; Pogosianz & Brujako, 1971), endomitosis and fusion of spermatogonial cells (Fechheimer, 1961; Pogosianz & Brujako, 1971) have all been suggested as causes of polyploidy. Probably the fate of these cells is similar to that of the aneuploid cells, since recent studies on human, mouse and rabbit spermatozoa indicate that there is little evidence for the existence of high numbers of polyploid spermatozoa (Carothers & Beatty, 1975).

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