Induction of triploidy in the mouse by cytochalasin B

By ANNA NIEMIERKO

From the Department of Embryology, Zoological Institute, University of Warsaw

SUMMARY

Mouse eggs fertilized in vivo were treated with cytochalasin B in vitro (5 μg/ml of culture medium) at the moment of extrusion of the second polar body (2-5, 3-0, 3.5 h after copulation). Cytochalasin B inhibits cytokinesis of the second maturation division, so that triploid di-gegenic eggs are formed in over 50% of treated eggs.

Triploid eggs were transplanted to the oviducts of recipients. On the 4th and 5th day of development 41-7% of transplanted eggs were recovered. All embryos recovered on the 4th day were morulae, while on the 5th day blastocysts predominated. Recovered embryos were studied for cell number and ploidy. Twenty-three of 27 embryos with analysable metaphase plates were triploid and four were diploid (the latter were found in females into which both triploid and control diploid eggs were transplanted).

Sex chromosome constitution was determined in seven cases: four triploids were XXY and three were XXX.

Preliminary observations showed that triploid embryos obtained by the use of cytochalasin B can implant and survive at least to the 9th day.

INTRODUCTION

Spontaneous suppression of the second maturation division of the oocyte is one mechanism leading to triploidy. In an attempt to induce or increase the frequency of this anomaly, the effect of various factors (heat-shock, colchicine, colcemid, pharmacological agents and delayed fertilization) on the second maturation division of mouse, rabbit and rat oocytes has been investigated (see Discussion).

In 1967 Carter found that cytochalasin B inhibits cytokinesis of cells without interfering with karyokinesis. Snow (1973) and Tarkowski, Witkowska & Opas (personal communication) induced tetraploidy in the mouse by suppressing the second cleavage division with cytochalasin B. Inhibition of the maturation divisions in the oocytes of the snail Spisula solidissima under the influence of cytochalasin B was described by Longo (1972). These three pieces of information suggested the use of cytochalasin B as a tool to induce triploidy in the mouse by suppressing second polar body formation.

1 Author’s address: Laboratory of Experimental Embryology, Institute of Obstetrics and Gynaecology, 00-315 Warsaw, Karowa 2, Poland.
MATERIAL AND METHODS

Spontaneously ovulating females of the inbred strain A, kept under a 16 h light/8 h dark cycle centred on midnight, were used throughout the experiment. Between 8.00 and 10.00 a.m. the females were paired with males and examined at 20 min intervals for the presence of a vaginal plug (delayed matings). The females were killed 2.5, 3.0, 3.5 h after copulation and the oocytes fertilized in vivo were cultured under paraffin oil in drops of Whitten’s medium (1971) containing 5 μg/ml of cytochalasin B for 5–7 h (usually 5 h). Cumulus cells were either removed with hyaluronidase before culture or left intact. After thorough washing the oocytes were cultured for 2 h and examined under an inverted microscope for the presence of the second polar body and for the number of pronuclei. Some eggs were fixed and mounted as permanent preparations; the others were transplanted into the oviducts of Swiss albino females on the first day of pseudopregnancy (Tarkowski, 1959). The transplanted embryos were examined on the 4th and 5th and 9–11th day of development. Chromosome preparations were made of morulae and blastocysts by the method of Tarkowski (1966).

RESULTS

When fertilized eggs are subjected to the action of cytochalasin B (CB) in vitro at the moment of extrusion of the second polar body (2 P.B.), cytokinesis of the second maturation division is suppressed so that triploid digynic eggs are formed (Fig. 1).

As shown in Table 1 the yield of triploid eggs varies according to the length of time between mating (fertilization) and treatment with CB. When oocytes were obtained 2.5 h after copulation and treated with hyaluronidase to remove the cumulus oophorus, only 39.0% were fertilized, but all of these were triploid.

In order to increase the number of fertilized eggs, the oocytes were subjected to CB at 3 and 3.5 h after copulation. Although the percentage of fertilized eggs increased to 68.1% and 80.0% respectively, the proportion of triploids rose only to 52.7% and 54.5%. The remaining fertilized eggs must have extruded 2 P.B. before treatment and were diploid.

The yield of triploid embryos obtained in 3 h (cumulus present) and 3.5 h (cumulus absent) series are similar, showing that penetration of CB into eggs is not impaired by the presence of the cumulus oophorus. The observation of cumulus-free oocytes in the process of extrusion of 2 P.B. showed that CB rapidly passes through the zona pellucida and that its action is very fast. The bulging of cytoplasm of 2 P.B. is retracted in about 2 min. When transferred from medium with CB to normal medium the eggs react by wrinkling of the surface which lasts for 0.5–1 h. No delayed extrusion of previously suppressed 2 P.B. was observed. However, in a certain number of cases the tri-nucleate eggs undergo fragmentation (Table 1).
Fig. 1. Triploid mouse egg. Long arrow: two female pronuclei. Short arrow: male pronucleus. × 830.

Fig. 2. Triploid metaphase plate of 3-5-day-old morula. × 1230.

Fig. 3. Triploid blastocysts 4-5 days old. × 480.

All triploid eggs transplanted to recipients originated from a 3 h series.

Preimplantation development of triploid embryos

Two hundred and forty-three eggs were transplanted to 32 recipients. This number included 223 triploid eggs (91.8% of transferred eggs) and 20 diploid eggs (8.2%). Thirteen females received both 3n and 2n eggs and 19 received 3n eggs only (Table 2). Twenty-five transplantations were successful. The recovered embryos were subjected to colchicine or colcemid (1 µg/ml of culture medium for 1–2 h) and examined in air-dried preparations for the number of cells and ploidy.

All embryos recovered on the 4th day (3-5 days old) were morulae, with not more than 20 cells (Fig. 4). Of 24 morulae recovered on the 4th day and cultured...
Table 1. Effect of cytochalasin B on suppression of 2 P.B. and on induction of triploidy

<table>
<thead>
<tr>
<th>Time after mating (h)</th>
<th>Cumulus oophorus</th>
<th>Total no. of eggs</th>
<th>Unfertilized eggs</th>
<th>Fragmenting eggs</th>
<th>Fertilized eggs</th>
<th>3n eggs</th>
<th>2n eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2½</td>
<td>Absent</td>
<td>41</td>
<td>22</td>
<td>3*</td>
<td>16</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100%</td>
<td>53.6%</td>
<td>7.4%</td>
<td>39.0%</td>
<td>39.0%</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Present</td>
<td>726</td>
<td>211</td>
<td>20†</td>
<td>495</td>
<td>383</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100%</td>
<td>29%</td>
<td>2.9%</td>
<td>68.1%</td>
<td>52.7%</td>
<td>15.4%</td>
</tr>
<tr>
<td>3½</td>
<td>Absent</td>
<td>55</td>
<td>11</td>
<td>—</td>
<td>44</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100%</td>
<td>20%</td>
<td>—</td>
<td>80%</td>
<td>54.5%</td>
<td>25.5%</td>
</tr>
</tbody>
</table>

* 3n embryos. † Includes eight 3n embryos.

in vitro for 24 h, 17 transformed into morphologically normal blastocysts (Table 2, 2nd row).

In embryos recovered on the 5th day the 2-h culture in CB-free medium was omitted. All but one were triploid. Twenty blastocysts and 13 morulae were recovered. Most of the 4-5-day morulae were similar in cell number to the 3-5-day ones (Fig. 4). The 4-5-day blastocysts exhibited wide variations in cell number (12–54); a third of them contained over 30 cells.

A total of 71 embryos were karyologically studied: 35 morulae and 36 blastocysts. Of the embryos examined, 38 had metaphase plates but ploidy could be determined in 27 embryos only. Twenty-three embryos were triploid (Fig. 2) and four were diploid (Table 3, Fig. 5). The sex chromosome complement of 3n embryos was determined in seven cases: three triploids were XXX and four XXY. The Y chromosome was identified according to the criteria of Ford (1966).

The karyologically identified triploids and diploids constituted 11.6% and 20% of the transplanted 3n and 2n eggs, respectively. On the unlikely assumption that all transplanted 2n eggs developed into morulae and/or blastocysts, and hence that 16 karyologically unidentified diploids are hidden among the 33 embryos lacking metaphase plates, then the remaining 17 embryos must be triploids. The number of recovered 3n embryos would then increase to 40, giving a minimum estimate of 20% development of the 3n eggs transferred in successful transplantations.

Karyologically identified triploid blastocysts attain on the 5th day of development a cell number similar to that found in four diploid-treated blastocysts of the same age (Fig. 5). This suggests that the slow rate of development of triploid
Table 2. Transplantation of eggs treated with cytochalasin B

<table>
<thead>
<tr>
<th>Age of embryos (days) and place of development</th>
<th>No. of transplantations</th>
<th>Transplanted eggs</th>
<th>Recovered embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>3n</td>
</tr>
<tr>
<td>3-5, in vivo</td>
<td>13</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>10*</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5, 3-5 days in vivo + 1 day in vitro</td>
<td>9</td>
<td></td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>8*</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5, in vivo</td>
<td>10</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>7*</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total, 4-5</td>
<td>19</td>
<td></td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>15*</td>
<td></td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grand total</td>
<td>32</td>
<td></td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>25*</td>
<td></td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Successful transplantations.
embryos results from the treatment applied in vitro to the newly fertilized eggs, rather than from the triploid condition per se.

Postimplantation development of triploid embryos

In a preliminary experiment, 40 tri-nucleate eggs were transplanted to six recipients which were killed on the 9th, 10th and 11th day of pregnancy. Seven implantations were found in two females. The implantations were opened and inspected under a dissecting microscope. One female killed on the 9th day had five implantations: four contained a normal egg-cylinder delayed by 1 day as compared with normal development, and one implantation contained no embryo. Another female killed on the 10th day had two failed implants. Although karyological studies have not been carried out on these embryos it seems probable that they were triploid.
Fig. 5. Number of cells in 3n morulae and blastocysts and 2n blastocysts.

Table 3. Karyology of 3-5- and 4-5-day embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Age of embryos (days)</th>
<th>Number of embryos</th>
<th>Ploidy</th>
<th>Sex chromosome complement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Examined</td>
<td>Without metaphase plates</td>
<td>With metaphase plates</td>
<td>With analysable metaphase plates</td>
</tr>
<tr>
<td>Morulae</td>
<td>3-5</td>
<td>17</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4-5</td>
<td>18</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Blastocysts</td>
<td>4-5</td>
<td>36</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>33</td>
<td>38</td>
<td>27</td>
</tr>
</tbody>
</table>

* Number of triploid embryos.
CB inhibits cytokinesis of the second maturation division of mouse oocytes and offers an effective method of obtaining triploid mouse embryos (Table 1). Its effectiveness in this study (over 50% of treated eggs) is limited only by the non-simultaneous penetration of spermatozoa into oocytes.

The first attempts to induce triploidy in mammals were performed on the mouse by Beatty & Fischberg (1949), Fischberg & Beatty (1951a) and Braden & Austin (1954), who by applying heat-shock at the time of fertilization obtained 25.7% and 15.3% triploid 3-5-day-old embryos and 17% tri-nucleate eggs respectively. Edwards (1954, 1958a, b; 1961), using colchicine and colcemid, obtained 19% and 50% of triploid one-cell eggs, and up to 10% of triploid embryos at 3-5 days. In similar studies McGaughey & Chang (1969) obtained 1.3–8% of triploid eggs at the first cleavage. A high incidence of triploid eggs and embryos was recently reported by Takagi (1970) in superovulated females of A/He strain. This is the only known case of increased frequency of triploidy in the mouse as a result of superovulation. Piko & Bomsel-Helmreich (1960) after colchicine treatment found 38% rat eggs with suppressed second polar body. The highest percentage of triploid eggs – up to 97% – was obtained by Bomsel-Helmreich (1965) by using colcemid on rabbit oocytes fertilized in vitro, from which 71% developed into blastocysts.

The efficiency of previous methods of inducing triploidy in the mouse was relatively low. Antimitotic agents like colchicine and colcemid, which have been successfully used to induce triploidy in the rabbit, in the mouse interfere with fertilization (McGaughey & Chang, 1969) and in a high proportion of eggs cause scattering of the chromosomes of the second meiotic metaphase into several groups and consequently the formation of many nuclei. Besides, these substances, especially colchicine, are toxic because they are bound to proteins of microtubular structures (Borisy & Taylor, 1967) and for this reason they may interfere with further development of the eggs.

CB does not interfere with the course of karyokinesis of the second meiotic division, so that two pronuclei are regularly formed. It also does not impair the further development of eggs treated during cleavage (Snow, 1973; Tarkowski, Witkowska & Opas, personal communication). In the concentration used in the present work, CB does not interfere with the fertilization of mouse oocytes in vitro and yields a high percentage of triploid eggs (Komar & Niemierko, unpublished observations).

Despite its high effectiveness in vitro, the attempt to suppress the formation of 2 P.B. with CB in vivo was unsuccessful. Injection of culture medium containing 5 μg/ml of CB into the oviduct 2-5 and 3 h after mating failed to induce triploidy (Niemierko, unpublished observations).

When triploidy is induced with CB in vitro and the treated eggs are transplanted to recipients, loss of a certain proportion of eggs is inevitable. In the
Triploidy in the mouse

Present study no eggs were recovered from seven females into which 44 triploid eggs were transplanted. In the successful transplantations developing embryos were found, representing 41.7% of the transplanted eggs, as well as 30 one-cell eggs and 42 fragmenting eggs, which may have included some CB-treated eggs in addition to native eggs of the recipients.

It seems that the slower rate of development of triploids resulted mainly from the fact that shortly after fertilization the eggs were subjected to stress (CB, culture in vitro, transplantation). Culture in vitro is probably responsible for the lower cell number in 4-5-day embryos which spend the last day in culture as compared to embryos developing 4-5 days in vivo (mean cell number 27 and 43-4 respectively). The present data do not permit the effect of triploidy itself on the survival and rate of preimplantation development to be analysed because very few control diploid eggs were submitted to the same treatment. However, there are reports that triploidy may affect the survival of embryos even before implantation. For instance, Braden (1957) observed that in 'silver' strain mice only half the triploid eggs of various origins (suppression of 1 P.B., suppression of 2 P.B., dispermy), which at the one-cell stage constituted 8.3%, reached the blastocyst stage. This author suggests, following Fischberg & Beatty (1952), 'lower survival rate during cleavage of the inbred triploid embryos resulting from intrastrain matings'.

The eggs used in the present study were obtained from delayed matings. According to some authors delayed fertilization increases the incidence of digynic and dispermic triploidy in laboratory animals and man (Schindler & Mikano, 1970). In the mouse Vickers (1969) noted in the PDE strain a ninefold increase of triploidy of unknown origin (2-9% versus 0-32%). Marston & Chang (1964) observed 4-2% of digyny and 3-3% of dispermy in eggs fertilized 12-13 h after ovulation. On the contrary, no increase in incidence of triploidy was observed by Gates & Beatty (1954) and Braden & Austin (1954). Litters of normal size were observed in spontaneously ovulating females mated in the early morning following ovulation (Krzanowska, 1964; Tarkowski, Witkowska & Nowicka, 1970), showing that by the time of day when the present experiments were carried out the oocytes were fully viable and that the incidence of abnormalities of oocyte maturation and fertilization had not significantly increased.

From earlier studies it is known that, notwithstanding the way in which it arises and the sex chromosome constitution, triploidy in laboratory animals is lethal around the middle of gestation (rabbit, Bomsel-Helmreich, 1965; rat, Piko & Bomsel-Helmreich, 1960; mouse, Fischberg & Beatty, 1951b; Vickers, 1969; Wróblewska, 1971). In man triploidy is usually lethal at 6-9 weeks (Carr, 1970, 1971a, b) but occasionally triploid foetuses can develop till birth (20 such cases have been reported). The 14 infants for which details are published exhibited a constellation of anomalies, with no clear syndrome (Walker, Andrews, Gregson & Gault, 1973).

Comparison of the descriptions of triploid mouse embryos given by various
authors suggests that the morphological expression of triploidy depends on the genetic background. Wróblewska (1971) observed a definite syndrome in CBA/CBA-T6T6 digynic embryos, expressing itself in the arrest of development of the embryonic part of the egg-cylinder. Morphologically normal triploid embryos were encountered by Fischberg & Beatty (1951b) at 9-5 days in ‘silver’ strain, by Vickers (1969) at 9-5–11-5 days in PDE strain and Takagi (1970) at 7-5 days in A/He strain mice. My preliminary investigations on the post-implantation development of triploid A-strain embryos, obtained by the use of CB, demonstrated that on the 9th day the embryos were represented by egg-cylinders normally developed but delayed by one day in comparison with control embryos. The further fate of these embryos is under investigation.

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REFERENCES


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