Chlorpromazine inhibits the mitotic index, cell number, and formation of mouse blastocysts, and delays implantation of CBA mouse embryos

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Summary. Chlorpromazine, administered to pregnant CBA mice 56 h after copulation in single doses of 10 or 15 mg/kg bodyweight, inhibited the compaction of embryos, formation of blastocysts, and reduced the mitotic index and cell number of embryos 86 h after copulation but did not adversely influence their viability or induce structural chromosomal aberrations. Blastocyst formation was more severely affected than embryo compaction. When 86-h embryos were treated with chlorpromazine (10 or 15 mg/kg) and subsequently cultured for 120 h, there was delayed hatching from the zona pellucida, delayed attachment to the culture dish, outgrowth of the trophoblast and expansion of the inner cell mass. Mice treated identically and evaluated on the 18th day of gestation had fewer implanted embryos than did controls, and the fetuses weighed less. No resorptions, malformations or significant differences in intrauterine deaths were found. Chlorpromazine given in the same manner but at 0.5 mg/kg did not affect any of the aforementioned criteria. When 56 h embryos were cultured in vitro in the presence of 50 μM-chlorpromazine for a further 40 h, embryo compaction, blastocyst formation, the mitotic index and the total cell number were significantly reduced compared with controls. Blastocyst formation was again more severely affected than embryo compaction. The inhibition of embryo compaction, blastocyst formation, and reduction in mitotic index and cell number associated in this study with chlorpromazine in vivo and in vitro indicate that the drug inhibits the development of cleavage-stage embryos in the mouse. These effects might be mediated by antagonistic effects of calmodulin.

Introduction

Chlorpromazine, a phenothiazine tranquillizer and antiemetic, is an antagonist of the calcium-binding protein, calmodulin, which serves as an important mediator of calcium-dependent regulatory processes in eukaryotes. The drug has an IC50 value (concentration of drug needed to inhibit the calmodulin-induced activation of phosphodiesterase by 50%) of 40 μM (Prozialeck & Weiss, 1982). Chlorpromazine also has membrane-stabilizing properties (Dybing, 1973) and, like other phenothiazines, it inhibits the active transport of potassium across cytoplasmic membranes (Judah & Ahmed, 1964) and the activity of Na/K-activated ATPase in vitro (Jarnefelt, 1962).

Whilst chlorpromazine has been reported to be teratogenic in mice and rats by certain workers (Ullberg, Lindquist & Sjorstrand, 1970; Walker & Patterson, 1974) others have failed to demonstrate teratogenic effects (Murphree, Monroe & Seager, 1962; Ordy, Latanick, Johnson & Massopust, 1963).

There has been little work documented on the effects of chlorpromazine on preimplantation murine embryos. In rats in early pregnancy chlorpromazine arrested implantation, presumably by
inhibition of hypothalamic function (Psychoyos, 1968). The duration of the preimplantation period was extended by repeated administration of chlorpromazine or trifluoperazine (IC$_{50}$ 17 µm) until oestrogen was administered or the drug had been withdrawn for a number of days (Chambon, 1958; Psychoyos, 1963). Borland & Tasca (1975) reported that exposure in vitro of mouse blastocysts to chlorpromazine inhibited the uptake and incorporation of L-methionine, an amino acid regarded as essential for the hatching of embryos from the zona pellucida (Spindle & Pedersen, 1973).

In a culture system in which 2-cell embryos developed to the blastocyst stage in the presence of 500 µm-chlorpromazine or 250 µm-trifluoperazine, mouse blastocyst formation was not significantly affected (Lenz, Hart, Ax & Cormier, 1984). These findings differed from those which showed that blastocyst formation was inhibited when 4–8-cell embryos were cultured for 48 h in the presence of 0.5 µm-trifluoperazine (1/500 the dose used in the aforementioned study) (Pakrasi & Dey, 1984).

In this paper we present the effects of chlorpromazine administered 56 h after copulation on the formation, viability, mitotic index, cell number, chromosome structure and peri- and post-implantational development in vitro of mouse blastocysts. The study was conducted with the purpose of examining possible embryotoxic effects of the drug and elucidating the mechanisms by which chlorpromazine might inhibit early embryonic development.

**Materials and Methods**

*Experimental mice.* Inbred CBA mice were housed under conditions of controlled temperature, and alternating 12 h light and dark cycles, and were fed on standard laboratory mice cubes (Epol, Cape Town) with ad-libitum access to water. Naturally ovulating female mice, aged 6–8 weeks, were harem-mated with males (3:1) for 4 h. A vaginal plug 2 h after the end of the mating period indicated that copulation had occurred and this was taken as being the first day of gestation. The midpoint of the mating period was arbitrarily considered as being the time of copulation.

*Drug administration and retrieval of embryos.* Chlorpromazine hydrochloride (100% purity; Maybaker, Port Elizabeth, South Africa) dissolved in sterile water for injection was administered s.c. in single doses of 0.5, 10 or 15 mg/kg bodyweight to the animals 56 h after copulation. Controls were given s.c. injections with sterile water. At 86 h after copulation, the mice were killed by cervical dislocation and the embryos were flushed from the uteri using phosphate-buffered saline (PBS, Gibco, Grand Island, U.S.A.).

The embryos retrieved in this manner were assessed microscopically for embryo compaction and blastocyst formation. Those embryos that had not blastulated were cultured for 6 h in groups of 6–10 in 4 ml Whitten's Medium (Whitten, 1971) at 37°C in humidified 5% CO$_2$ in air. They were then re-evaluated for signs of compaction and blastocyst formation.

*Peri- and postimplantational development.* Embryos from the control and treated mice obtained 86 h after copulation were cultured in groups of 6–10 in 4 ml NCTC-135 medium (Flow Labs, Ayrshire, U.K.) on sterile glass coverslips in plastic culture dishes (NUNC, Nulcon, Kampstrup, Denmark). The medium was supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum, penicillin (10 000 i.u./100 ml) and streptomycin (5 mg/100 ml). The cultures were maintained at 37°C in humidified 5% CO$_2$ in air for 120 h. Embryos that had formed blastocysts were cultured separately from those that had not blastulated. The cultures were examined microscopically under phase contrast 6 and 12 h after the start of the culture to assess whether further embryos had blastulated. The cultures were examined every 24 h for evidence of the following distinct stages in development: (i) hatching from the zona pellucida; (ii) attachment to the glass coverslips; (iii) outgrowth of the trophoblast; (iv) expansion of the inner cell mass; and (v) differentiation of the inner cell mass into endoderm and ectoderm (Spielmann & Eibs, 1978).
**Cell number, mitotic index and chromosome structure.** The method used was based on that described by Tarkowski (1966). Test and control mice were treated as described above. In addition, the mice were injected intraperitoneally with colchicine (2 mg/kg body weight) 2 h before death, 84 h after copulation. The mice were killed and the embryos were retrieved in the usual manner. The embryos were kept in hypotonic solution (1% sodium citrate (Merck, Darmstadt, Germany)) for 10–20 min at room temperature and then fixed onto glass slides with a mixture of methanol: glacial acetic acid (3:1, v/v). The slides were air-dried and stained with 10% Giemsa (Merck) for 7 min. The cell number was counted, the mitotic index (number of cells in mitosis/total number of cells) was determined, and the chromosomes were scored for structural aberrations.

**Assessment of viability.** Embryo viability was assessed using a modification of the technique described by Mohr & Trounson (1980) (see also Whittingham, 1981). The embryos were incubated in fluorescein diacetate solution (2.5 µg/ml Medium PB1: Whittingham, 1971) for 1 min at room temperature. The basis of the method is that the fluorescein diacetate, being non-polar, crosses cell membranes quite readily and is hydrolysed by esterases within the cell to fluorescein, which fluoresces. The fluorescein is polar and accumulates in the cell if the membrane is intact. After incubation in fluorescein diacetate the embryos were washed in Medium PB1 for 5 min. The embryos were then examined under a fluorescence microscope (Wild & Leitz, magnification × 240). Fluorescence was measured using a centre-field light sample which was fed into a photomultiplier tube. When a predetermined amount of light was received the camera mechanism was triggered. The time taken to this point was determined using a specially designed digital timer (accurate to 0.1 sec) attached to the camera mechanism. The timer was automatically activated when light was fed into the photomultiplier and shut off when the camera mechanism had been triggered. An embryo was arbitrarily regarded as non-viable if it took longer than 13.0 sec to trigger the camera mechanism at an ASA speed of 400. We have found that viability determined by this means correlated well ($r = 0.863; P < 0.0001$) with subsequent in-vitro culture (I. Kola & P. I. Folb, unpublished data).

**Evaluation at term.** In a separate experiment, control and test mice treated in the same manner were killed by cervical dislocation on the 18th day of gestation and the fetuses were retrieved, weighed and examined for malformations. The uteri were inspected for resorptions and the numbers of implantation sites in the uteri and corpora lutea in the ovaries were determined. The number of preimplantation embryos that did not implant was calculated from the difference between the corpora lutea and implantation sites (Kirk & Lyon, 1984).

**In-vitro treatment of embryos.** Embryos obtained 56 h after copulation were flushed from the oviducts with PBS. They were cultured in Whitten's medium in the presence of 50 µM-chlorpromazine-HCl at 37°C in 5% humidified CO₂ in air for 38 h. The embryos were then evaluated for embryo compaction and blastocyst formation, and cultured for a further 2 h in the presence of colcemid (0.2 µg/ml medium). They were again examined for compaction, blastocyst formation, cell number, mitotic index and chromosome structure, and compared with controls.

**Statistical analysis.** The treated groups were compared with controls by the $\chi^2$ test, except when indicated otherwise. The Mann-Whitney U two-tailed test was used to compare groups for cell number and fetal weight.

**Results**

**Blastocyst formation**

In our mice, 80% (61 of 76) of the embryos retrieved 56 h after copulation had not yet compacted and were at the 4- to 8-cell stage of development, whereas the remaining 20% (15 of 76) were compacted 8-cell embryos.
Table 1. The effects of chlorpromazine on compaction of embryos and blastocyst formation in mice

<table>
<thead>
<tr>
<th>Chlorpromazine dose/conc.</th>
<th>No. of embryos</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Evaluated†</td>
<td>Compacted</td>
<td>Blastulated‡</td>
</tr>
<tr>
<td><strong>In vivo</strong> (mg/kg body weight)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>94 (12)</td>
<td>94 (100%)</td>
<td>82 (87%)</td>
</tr>
<tr>
<td>0.5</td>
<td>99 (12)</td>
<td>99 (100%)</td>
<td>77 (78%)</td>
</tr>
<tr>
<td>10</td>
<td>107 (12)</td>
<td>92* (86%)</td>
<td>30* (28%)</td>
</tr>
<tr>
<td>15</td>
<td>90 (12)</td>
<td>70* (78%)</td>
<td>19* (21%)</td>
</tr>
<tr>
<td><strong>In vitro (µM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>40 (5)</td>
<td>40 (100%)</td>
<td>34 (85%)</td>
</tr>
<tr>
<td>50</td>
<td>36 (5)</td>
<td>28* (78%)</td>
<td>12* (33%)</td>
</tr>
</tbody>
</table>

* P < 0.001, compared with controls (χ² test).
† Number of replicates in parentheses.
‡ For statistical analysis, the numbers of compacted embryos that formed blastocysts in control and treated groups were compared.

Maternal administration of chlorpromazine, 10 and 15 mg/kg, 56 h after copulation, significantly inhibited the compaction of embryos and the formation of blastocysts (P < 0.001 in each case) (Table 1). The formation of blastocysts was much more severely affected than embryo compaction in that only 78, 33 and 27% of the embryos that had compacted formed blastocysts in the groups treated with 0.5, 10 and 15 mg/kg, respectively (Table 1). That blastocyst formation was inhibited rather than delayed was shown by examination of embryos 6 and 12 h after the initiation of culture and every 24 h thereafter: those embryos that had not formed blastocysts by 92 h after copulation did not do so subsequently and remained unhatched even at the end of the 120-h culture period.

Culturing 56-h embryos for 40 h in the presence of 50 µM-chlorpromazine significantly interfered with the ability of these embryos to compact and form blastocysts (P < 0.001) (Table 1). Once again, the formation of blastocysts was more severely affected than was embryo compaction as only 43% of the embryos that had compacted formed blastocysts (Table 1). (At the start of culture 8 of 36 embryos exposed to chlorpromazine were compacted 8-cell embryos compared with 7 of 40 of the controls.) These findings provide evidence that chlorpromazine exposure in vivo and in vitro inhibits blastocyst formation.

Peri- and post-implantational development of blastocysts

This experiment was conducted to examine the effects of chlorpromazine on the development and differentiation of embryos that had already formed blastocysts. The results of various doses of chlorpromazine administered to pregnant CBA mice at 56 h after copulation on the subsequent differentiation in vitro of blastocysts retrieved at 86 h and cultured for a further 120 h are presented in Table 2.

Compaction of embryos and blastocyst formation were again inhibited. In the controls, 100% of embryos retrieved had compacted and 88% had formed blastocysts. In the groups treated with 0.5, 10 and 15 mg chlorpromazine/kg, 96, 87 and 81% of embryos respectively had undergone compaction, and 80, 36 and 24% respectively had formed blastocysts. Chlorpromazine in doses of 10 and 15 mg/kg, but not 0.5 mg/kg, significantly delayed hatching of the embryos from the zona pellucida, their attachment to the glass coverslip surface, trophoblast outgrowth, and expansion of the inner cell mass (Table 2; Figs 1 and 2). Although this indicates that chlorpromazine delays
attachment of blastocysts in vitro, it is also possible that this delay may be due to a perturbation of earlier events.

Cell number, mitotic index and chromosomal aberrations

These results are given in Table 3. The mitotic index and cell number of embryos 86 h after copulation were significantly reduced by chlorpromazine at doses of 10 and 15 mg/kg, but not by 0.5 mg/kg (Figs 3 & 4). Similarly, 50 μM-chlorpromazine significantly reduced the mitotic index and cell number of embryos in vitro. At each of the doses tested, in vivo and in vitro, chlorpromazine did
not exert a significant clastogenic effect (Table 3; Fig. 5). These results suggest that the reduced cell number of drug-treated embryos is likely to be a consequence of inhibition of cell proliferation rather than a cytotoxic effect.

**Viability of embryos**

Chlorpromazine administered to pregnant mice 56 h after copulation had no effect on the viability of the embryos at 86 h (Table 4; Figs 6 and 7).

**Evaluation at term**

The effects of administration *in vivo* of chlorpromazine at 10 and 15 mg/kg, 56 h after copulation, are presented in Table 5. The finding that fewer embryos in the chlorpromazine-treated animals underwent implantation is consistent with our observation that those embryos that did not form blastocysts remained unhatched and unattached during the entire 120-h culture period. It may be that chlorpromazine inhibition of blastocyst formation accounts for the implantation of fewer embryos.

**Table 4.** The effects of chlorpromazine administered to pregnant CBA mice 56 h after copulation on the viability of 86-h embryos

<table>
<thead>
<tr>
<th>Chlorpromazine dose (mg/kg body weight)</th>
<th>No. of embryos evaluated†</th>
<th>No. of viable embryos (%)</th>
<th>No. of non-viable embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>45 (6)</td>
<td>41 (91.1)</td>
<td>4 (8.9)</td>
</tr>
<tr>
<td>0.5</td>
<td>47 (6)</td>
<td>40 (85.1)</td>
<td>7 (14.9)</td>
</tr>
<tr>
<td>10</td>
<td>50 (6)</td>
<td>40 (80.0)</td>
<td>10 (20.0)</td>
</tr>
<tr>
<td>15</td>
<td>41 (6)</td>
<td>33 (80.5)</td>
<td>8 (19.5)</td>
</tr>
</tbody>
</table>

† Number of replicates in parentheses.

**Fig. 1.** Photomicrograph of an untreated blastocyst cultured for 72 h (× 192). The trophoblast (T) and inner cell mass (ICM) are shown.

**Fig. 2.** Photomicrograph of an untreated blastocyst cultured for 120 h (× 120). The inner cell mass has differentiated into the endoderm (END) and ectoderm (ECT).

**Fig. 3.** Photomicrograph of an embryo (× 100) 86 h after copulation from a mouse treated with 15 mg chlorpromazine/kg body weight. The embryo has 14 cells, none of which are in mitosis.

**Fig. 4.** Photomicrograph of an untreated blastocyst showing 22 of the 34 cells of the blastocyst (× 100). Two cells (arrowed) are in mitosis.

**Fig. 5.** Photomicrograph of an embryo (× 600) from a mouse treated with 15 mg chlorpromazine/kg body weight. The arrow indicates an aberrant chromosome that has undergone a chromosomal rearrangement.

**Fig. 6.** Fluorescent photomicrograph of an untreated 86-h blastocyst (× 240). The blastocoelic cavity (indicated by an arrow) has a very pale fluorescence.

**Fig. 7.** Fluorescent photomicrograph of an 86-h embryo (× 240) from a mouse treated with 10 mg chlorpromazine/kg body weight. This embryo failed to compact although all the blastomereres fluoresce brightly.
Table 5. The effects of chlorpromazine treatment of pregnant CBA mice 56 h after copulation on Day-18 fetuses

<table>
<thead>
<tr>
<th>Chlorpromazine dose (mg/kg body weight)</th>
<th>No. of corpora lutea†</th>
<th>No. of implantations‡ (mean no. of implantations/mouse in parentheses)</th>
<th>No. of unimplanted embryos‡ (%)</th>
<th>No. of resorptions, intrauterine deaths and malformations</th>
<th>No. of viable fetuses</th>
<th>Wt of fetuses (g) (mean ± s.e.m.)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>57 (8)</td>
<td>52 (6:5)</td>
<td>5 (9)</td>
<td>0</td>
<td>52</td>
<td>0·689 ± 0·012</td>
</tr>
<tr>
<td>0·5</td>
<td>35 (5)</td>
<td>31 (6:2)</td>
<td>4 (11)</td>
<td>0</td>
<td>31</td>
<td>0·651 ± 0·013</td>
</tr>
<tr>
<td>10</td>
<td>49 (7)</td>
<td>*27 (3·9)</td>
<td>*22 (45)</td>
<td>0</td>
<td>27</td>
<td>*0·574 ± 0·014</td>
</tr>
<tr>
<td>15</td>
<td>37 (5)</td>
<td>*16 (3·2)</td>
<td>*21 (58)</td>
<td>1¶</td>
<td>15</td>
<td>*0·516 ± 0·015</td>
</tr>
</tbody>
</table>

* P < 0·001.
† Number of replicates in parentheses.
‡ Compared with controls by χ² tests.
§ Compared with controls by two-tailed Mann-Whitney U test.
¶ Intrauterine death.

The lower fetal weight in the groups treated with 10 and 15 mg chlorpromazine/kg may be attributable to delayed implantation of embryos that do blastulate. There were no malformations, resorptions or significant differences in intrauterine deaths in the treated and control groups.

Discussion

Our findings that chlorpromazine inhibited embryo compaction, blastocyst formation, mitotic index and cell number of embryos 86 h after copulation in a dose-related manner may have bearing on the mechanisms of inhibition of blastocyst formation. Pakrasi & Dey (1984) showed that 0·5 μM-trifluoperazine inhibited blastocyst formation of mouse embryos whilst 1·2 μM-trifluoperazine sulfoxide (an inactive form of trifluoperazine and a very weak calmodulin antagonist) did not. They deduced from this that inhibition of blastocyst formation by trifluoperazine might be due to antagonism of calmodulin. On the other hand, Lenz et al. (1984) found that 250 μM-trifluoperazine and 500 μM-chlorpromazine did not inhibit blastocyst formation of 2-cell embryos whereas various sulphoxide derivatives at concentrations of 50 μM did. It is possible that the inhibition of blastocyst formation both in vivo and in vitro that we have shown could be related to chlorpromazine antagonism of calmodulin.

The process of compaction, which begins at the 8-cell stage in the mouse, is a prerequisite for morula to blastocyst transformation (Ducibella & Anderson, 1975; Surani, Kimber & Handyside, 1981; Pratt, Chakraborty & Surani, 1981). It has been demonstrated that extracellular calcium is essential for the compaction process (Ducibella & Anderson, 1975, 1979) and that intracellular calcium, which is modulated by calmodulin (Cheung, 1984), is associated with the induction and the initial phase of the compaction process (Bilozur & Powers, 1982; Pakrasi & Dey, 1984). Hence, inhibition of compaction shown in this study could be responsible, at least partly, for the reduced blastocyst formation. Our results in this study are consistent with those of Bilozur & Powers (1982) and Pakrasi & Dey (1984). Calmodulin antagonism may also account for our findings of a reduction of the mitotic index and cell number of in-vivo and in-vitro chlorpromazine-treated embryos. Calmodulin has an important role in the S- through to M-phases of the cell cycle, the initiation of DNA synthesis and mitosis, and the re-entry into the cell cycle of growth-arrested cells (Whitfield, Boynton, MacMarus, Sikorska & Tsang, 1979; Sasaki & Hidaka, 1982; Chafouleas, Lagage, Bolton, Boyd & Means, 1984). Calmodulin antagonists inhibit proliferation in vitro of Chinese...
hamster ovary cells (Sasaki & Hidaka, 1982) and glioblasts (Okumura-Noji, Kato & Tanaka, 1983). It may be that the inhibition of cell proliferation by calmodulin antagonism itself retards blastocyst formation. There is some evidence that the process of blastocyst formation is influenced by the number of nuclear divisions or DNA or chromosome replications that have occurred since fertilization (Smith & McLaren, 1977; Petzoldt, Burki, Illmensee & Illmensee, 1983).

The activity of Na/K (activated)-ATPase could be involved in the localization of organelles to opposed cell borders, the production of nascent blastocele fluid, and cavitation during murine blastocyst formation (Wiley, 1984). Chlorpromazine, being an inhibitor of Na/K-ATPase in common with other phenothiazines (Jarnefelt, 1962; Judah & Ahmed, 1964) might adversely affect blastocyst formation. In our study the chlorpromazine-treated embryos that had not blastulated were nevertheless viable and did not show a significant increase in structural chromosomal aberrations. Our own observations suggest that blastulation was not inhibited as a result of non-viability of the embryos or clastogenic effects of the drug, although non-specific cytotoxic effects of the drug, whilst unlikely, cannot be ruled out.

The delay in attachment in vitro caused by chlorpromazine in this study is consistent with a direct effect of the drug on the embryos (Borland & Tasca, 1975). Psychoyos (1963) found that a dose of 40 mg/kg body weight was necessary to cause implantational delay compared with 10 mg/kg body weight in this study. The differences in dose might be due to the different animal species used in the studies; certain species are more sensitive than others to drugs and chemicals administered during pregnancy (Tuchmann-Duplessis & Mercier-Parot, 1964).

In conclusion, this study has shown that chlorpromazine inhibits the formation of blastocysts, reduces the mitotic index and the cell number of embryos in vivo and in vitro. These effects could be mediated via the antagonism of the drug to calmodulin which may be involved in cell proliferation and early embryonic development.

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