Effects of clomiphene citrate on early pregnancy in guinea-pigs

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Summary. Clomiphene citrate (2 mg/kg body wt) given on the day of mating can block or interrupt pregnancy in guinea-pigs. Corpus luteum function, uterine histology, implantation and embryo development were studied in clomiphene-treated and control animals on Days 5, 9 and 20 of pregnancy. Following treatment, only 25% of the females were regularly pregnant, presenting large and healthy foetuses. The other females examined showed either pregnancy with embryos undergoing resorption or no sign of pregnancy. In these females, corpus luteum size was reduced, progesterone concentrations were very low and the endometrial glands and the epithelium were often altered. It is concluded that clomiphene causes a reduction in fertility by altering the uterus and, by directly or indirectly inducing luteolysis, causes later pregnancy loss.

Keywords: clomiphene; early pregnancy; guinea-pig; antifertility

Introduction

Clomiphene is a nonsteroidal, antioestrogen which also possesses weak oestrogenic properties (Dolginova et al., 1985). It is one of the most effective agents for the treatment of several human anovulatory disorders (Bishop, 1970; Kase, 1973; Natrajan et al., 1980) and inducing multiple follicular maturation for in-vitro fertilization and embryo transfer (Kerin et al., 1983). Even though ovulation rate is high with clomiphene, pregnancy incidence is relatively low and pregnancy wastage is high (Garcia et al., 1977; Toshinobu et al., 1979; Natrajan et al., 1980; Hammond et al., 1983). The latter effect may be due to a high number of chromosomal abnormalities (Boué et al., 1975), but the overall low pregnancy rate suggests that clomiphene given for ovulation induction may affect preimplantation embryo transport and viability, implantation and placental development due to uterine endometrial effects, or pregnancy maintenance due to direct or indirect hormonal actions (Prasad & Sarkaram, 1972). Clomiphene has been shown under certain conditions to interfere with ovarian steroidogenesis (Laufer et al., 1982; Sgarlata et al., 1984), to cause marked uterotrophic effects in ovariectomized (Roy et al., 1964) and immature (Lerner, 1964) animals and to alter the condition of the endometrium (Balash et al., 1983). For our studies on the effect of clomiphene on early pregnancy, the guinea-pig was chosen as the animal model because it perhaps most resembles man for early pregnancy studies (Blandau, 1972; Finn, 1983).

The experiments were designed to test the effects of clomiphene given to guinea-pigs at mating on pregnancy maintenance; specifically as related to effects on uterine histology, implantation, embryo development and function of the corpus luteum (CL). Observations were made on Days 5, 9 and 20 of pregnancy (in this species, pregnancy lasts an average of 63 days; Rowlands, 1949). By Day 5, luteinization is complete and a fully functional corpus luteum is present (Bland & Donovan, 1969; Challis et al., 1971; Hilliard, 1973) and implantation has not taken place (Blandau, 1949). Studies on clomiphene-treated animals at this time should give evidence of interference with the

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preimplantation uterus and normal CL formation and function. Studies on Day 9, when implantation has taken place (Days 6–7, Blandau, 1949) and implantation swellings are clear on the uterus, should give evidence for interference with implantation, decidualization and CL maintenance. Between Days 9 and 20, progesterone becomes essential for pregnancy maintenance (Days 11–14, Loeb & Hesselburg, 1917; Deanesly, 1960, 1963b; Csapo et al., 1981). The conceptus produces a luteotrophic substance (Days 12–25, Bland & Donovan, 1969; Blatchley et al., 1975; Antonini et al., 1976; Poyser & Maule Walker, 1979) and the switch of progesterone secretion from CL to placenta takes place from Day 15, (Deanesly, 1960; Heap & Deanesly, 1966). Hence, between Days 9 and 20 the possible importance of the effects of clomiphene on CL maintenance can be ascertained.

Materials and Methods

**Animals.** Adult albino Dunkin/Hartley guinea-pigs (A. Tuck and Sons Ltd, Battlebridge, Essex, UK) were kept in a controlled temperature (20°C) and photoperiod (14 h light from 06:00 to 20:00 h). Guinea-pig pellets (S.G.I., B.P. Nutrients Ltd, Northwich, UK) were given *ad libitum*, supplemented daily with fresh vegetables and hay. Vitamin C was supplied dissolved in the drinking water (0.7 g/l). Animals were mated to proven males after displaying two normal oestrous cycles. Mating occurred at night, and Day 1 of pregnancy was taken as the day a vaginal plug or spermatozoa were found in the vagina. Subsequently, vaginas were observed daily for evidence of vaginal opening and a return to cyclicity, indicating pregnancy failure.

**Treatments.** Clomiphene citrate (Sigma Chemical Company Ltd, Poole, Dorset, UK) (2 mg/kg body weight, dissolved in arachis oil:ethanol, 9:1 v/v) or vehicle (arachis oil:ethanol, 9:1) were given subcutaneously on the morning a vaginal plug or spermatozoa were found in the vagina (Day 1 of pregnancy). Animals from control and treatment groups were killed at random on Days 9, 5 and 20 of pregnancy. For controls, 4 females were used on Day 5, 3 on Day 9 and 5 on Day 20. For clomiphene treatment, 6 females were used on Day 5, 7 on Day 9 and 5 on Day 20. Blood samples, ovaries and reproductive tracts were collected at death. Earlier blood samples were taken by jugular vein puncture on Day 5 from animals killed on Day 9, and on Days 5 and 9 from animals killed on Day 20. Withdrawal of blood was carried out following sedation by Hypnorm (0·315 mg fentanyl citrate and 10 μg fluanisone/ml; Crown Chemical Ltd, Lamberhurst, UK) and diethyl ether anaesthesia. Serum was stored at –20°C until assay. Ovaries and reproductive tracts were fixed in formaldehyde/saline mixture and processed for light microscopy according to standard paraffin-wax-embedding procedures and haematoxylin–eosin staining.

**Assay.** Serum progesterone was assayed by a minor modification of a standard method (Orkzyk et al., 1979). Briefly, samples of serum (100 μl), extracted with 2 ml petroleum ether (40–60°C), [1,2,6,7,16,17-3H]-progesterone (Amersham International Ltd, Bucks, UK) and progesterone antiserum (with a cross-reaction of 3% with corticosterone, 8% with deoxycorticosterone and <1% with other steroids and clomiphene) were incubated overnight at 4°C. Separation of bound and free fractions was with charcoal. The sensitivity of the assay is 20 pg/tube and the intra- and interassay coefficients of variation are 12% and 18%, respectively.

**Morphological examination.** The uterus was examined for normality of epithelium and glands. In uteri of pregnant animals on Day 9, the implantation sites were visualized following a clearing technique (Orsini, 1962). Sites were dissected and embedded to examine the conditions of the decidua and of implanted blastocysts. To evaluate decidual development, the 3 largest perpendicularly orientated diameters of the decidual mass were measured and used to calculate the mean diameter. Uteri of pregnant animals at Day 20 were examined under a dissecting microscope. The uterine wall was opened and implantation chambers were examined. Embryo size, condition, number and implantation percentage (total number of embryos/total number of CL × 100) were recorded. Embryo size was measured from the top of the head to the point of maximum curvature of the back (crown–rump length). Embryo condition was evaluated from size, presence of blood in the implantation chamber and by examining serial sections for morphological abnormalities.

**Statistical analysis.** Serum progesterone and CL diameter data showed either heterogeneity of variance or a skewed distribution. When appropriate, therefore, the data were expressed as geometric means and ranges. Some differences in the values for individually treated animals from the control were assessed by showing that they differed by more than 2 standard deviations of the log mean of the controls. Differences in the percentage of embryos were calculated by $\chi^2$ (Campbell, 1974).

Results

Clomiphene treatment did not affect progesterone concentration or CL size by Day 5 (Table 1). The histology of the CL was also unaffected. The luminal epithelium of the uterus was usually
normal, but 2 of the 6 clomiphene-treated animals showed occasional dilated uterine glands with flattened epithelium (Fig. 1b) and, in one of these, small patches of hypertrophied cells (Fig. 2) were found on the epithelium. As in control females, the stroma in clomiphene-treated females was distinctly oedematous and many cells were undergoing mitosis. Decidual cells were occasionally observed. In all animals the vagina was closed by a membrane.

**Table 1.** Progesterone concentration, corpus luteum (CL) diameter and uterine histology of guinea-pigs sampled and killed on Day 5 of pregnancy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone conc. (ng/ml)</th>
<th>CL diam. (mm)</th>
<th>No. of animals with abnormal uterine histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.6 (3.9-9.3)</td>
<td>1.45 (1.42-1.48)</td>
<td>0</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Clomiphene</td>
<td>5.4 (2.5-7.0)</td>
<td>1.47 (1.33-1.67)</td>
<td>2</td>
</tr>
<tr>
<td>n</td>
<td>16*</td>
<td>27</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are geometric means and ranges, in parentheses, for the number of samples examined.

*Two samples lost during analysis.

Fig. 1. Effect of clomiphene citrate (2 mg/kg) given to guinea-pigs on the day of mating on the uterine endometrium: (a) normal endometrium, (Day 9, control), (b) dilated glands (Day 5, clomiphene treatment) (x 1000).
Control animals on Day 9 showed similar progesterone concentrations and CL sizes and morphology to Day 5 guinea-pigs (Tables 1 and 2). In the uterus, both the epithelium and the glands (Fig. 1a) appeared normal. The mean number of implantation sites/female was 2.7 ± 1.2. Dense masses of decidual cells (decidual reactions, 4.0–4.6 mm diam.) protruded into the lumen, frequently filling it completely. Implanted embryos were recognized. Normal uterine and CL histology (Fig. 3a) accompanied by increased progesterone concentration and increased CL and embryo size were observed on Day 20. All the animals showed 4 or 5 large uterine chambers; the implantation percentage (number of embryos/number of corpora lutea × 100) was 84 and embryo size ranged between 4-9 and 8-9 mm. In 3 of these control females, one of the chambers was filled with blood and contained partly resorbed embryos.

On the basis of the data obtained, the clomiphene-treated guinea-pigs were divided into three groups (a, b and c in Table 2). On Day 9, 5 of 12 animals were apparently normal (group a); progesterone concentrations were within 2 s.d. values of the normal log values and a normal number of implantation sites (implantation 77%) was observed. Two of these guinea-pigs were killed on Day 9. They had normal CL size and uterine histology. Eight large decidual reactions (4.0–4.8 mm diam.) and 10 CL were found. Under microscopic examination, the decidual cells appeared normal and many implanted embryos were recognized. Of the 3 remaining animals studied on Day 20, only one showed a normal pregnancy, with a progesterone concentration and CL size and morphology similar to the control group. The uterus contained 4 healthy foetuses which ranged from 4.1 to 8.3 mm, while another was already resorbed. The last 2 animals, though apparently normal on Day 9, had a perforate vagina on Day 17 and showed evidence of pregnancy loss by Day 20, by which time one had re-ovulated. Most of the foetuses were already resorbed and some blood was present in the uterine lumen and vaginal smear. The uterine endometrium showed normal glands and columnar epithelium. The ovaries contained small CL showing pale lutein cells with pycnotic nuclei (Fig. 3b). Pre-ovulatory follicles or 2-day-old CL were present in the 2 animals. Serum progesterone concentration was very low in both animals, but slightly higher in the female in the early post-ovulatory phase (Table 2).

Four animals on Day 9 (group b) showed lower than normal progesterone concentrations (all were less than 2 s.d. values of the log normal value). In addition, implantation in these females was 28%, which was significantly ($P < 0.001$) less than that of the control group. Two animals had regressed corpora lutea and all had abnormal uterine histology, with patches of hypertrophied cells and many enlarged glands. The decidual reactions appeared significantly ($P < 0.05$)
**Table 2.** Changes in pregnancy outcome, progesterone concentration, corpus luteum (CL) size, uterine histology and number of embryos in guinea-pigs sampled on Days 9 and 20 of pregnancy after control or clomiphene treatment at mating.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 9</th>
<th>Day 20</th>
<th>Implantation rate (%)†</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone conc. (ng/ml)</td>
<td>CL diam. (mm)</td>
<td>Abnormal uterine histology</td>
<td>Decidual diam. (mm)</td>
</tr>
<tr>
<td>Control*</td>
<td>5·8 ± 0·8 (4·6-6·8)</td>
<td>1·45 ± 0·1 (1·36-1·54)</td>
<td>None (4·0-4·6)</td>
<td>4·3 ± 0·3</td>
</tr>
<tr>
<td>No. of animals</td>
<td>8</td>
<td>12</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Clomiphene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Normally pregnant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>on Day 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6·6</td>
<td>8·1</td>
<td>1·53</td>
<td>No</td>
<td>4·7</td>
</tr>
<tr>
<td>10·0</td>
<td>6·3</td>
<td>1·54</td>
<td>No</td>
<td>4·6</td>
</tr>
<tr>
<td></td>
<td>4·1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5·1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(b) Pregnant abnormal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>on Day 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3·2</td>
<td>3·6</td>
<td>1·53</td>
<td>Yes</td>
<td>2·6</td>
</tr>
<tr>
<td></td>
<td>3·6</td>
<td>1·45</td>
<td>Yes</td>
<td>1·9</td>
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<tr>
<td></td>
<td>1·8</td>
<td>1·24</td>
<td>Yes</td>
<td>3·3</td>
</tr>
<tr>
<td></td>
<td>0·6</td>
<td>1·02</td>
<td>Yes</td>
<td>2·9</td>
</tr>
<tr>
<td>(c) No evidence of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pregnancy of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pregnancy</td>
<td>1·9</td>
<td>2·3</td>
<td>1·09</td>
<td>Yes</td>
</tr>
<tr>
<td>0·9</td>
<td>1·4</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td></td>
<td>0·9</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Values are geometric means and ranges in parentheses, for the number of samples indicated.
†Total number of embryos/total number of CL × 100.
‡Total number of embryos/total number of CL × 100.
§New ovulation after embryo loss.
$Significantly different from oil control, \( P < 0·001 \) by \( \chi^2 \) test.
Fig. 3. Effect of clomiphene citrate (2 mg/kg) given to guinea-pigs on the day of mating on corpus luteum histology: (a) normal luteal cells (Day 20, control), (b) pale luteal cells with pycnotic nuclei (arrows) (Day 20, clomiphene treatment, group a) (× 1000).

reduced in size (1.9 to 3.3 mm diam.) compared with the control, but did not appear altered histologically.

On Day 9, 3 of the clomiphene-treated guinea-pigs (group c) showed no signs of having been pregnant. They all had very low progesterone concentrations on Day 9 and the one killed on Day 9 had regressed CL and abnormal uterine histology. The glandular system appeared particularly affected, as almost all the glands were enlarged, though no hypertrophic luminal epithelium was found. The remaining 2 animals had a perforate vagina after 10–12 days, and had reovulated with new corpora lutea by Day 20.

**Discussion**

These results clearly show that clomiphene (2 mg/kg) has an antifertility effect when given to guinea-pigs as a single injection after mating. The effects were, however, variable, 3 animals failing to become pregnant, 6 displaying interrupted pregnancies and 3 remaining normally pregnant. This variable response suggests that the actions of clomiphene in affecting pregnancy may either be critical at the time and lead to pregnancy failure, or only become critical later in pregnancy. An affected uterus, for example, could lead to either implantation failure or abnormal implantation. The latter may not subsequently sustain the pregnancy. Alternatively, effects of clomiphene may be prolonged by either slow absorption from the injection site or by clomiphene adsorption to and later release from intraperitoneal fat. Information on blood concentrations of clomiphene after treatment and clomiphene half-life, which would help resolve these possibilities, is not available.
Possible modes of action have been considered in the present study, with particular emphasis on the processes involved in embryo implantation and CL function. The data of Staples (1966) suggests that clomiphene interrupts pregnancy in rats, not by blastotoxicity, but by an effect on the implantation process. Also, healthy preimplantation embryos have been commonly found in guinea-pigs given a dose of clomiphene that interfered with pregnancy (J.S.M. Hutchinson, unpublished observations). In guinea-pigs, if endometrial and embryo development are not compatible, attachment does not occur (Blandau, 1972) and blastocysts are lost (Deanesly, 1960). In the present experiment, 2 of 6 clomiphene-treated guinea-pigs killed on Day 5 (2 days before implantation; Blandau, 1949) and 5 of 7 animals killed on Day 9 showed endometrial abnormalities similar to those observed after oestrogen (Deanesly, 1963a; Gulino et al., 1984) or antioestrogen (Gulino & Pasqualini, 1980; Gulino et al., 1984) treatment. These abnormalities and the associated phenomena either impaired (group b, Table 2) or blocked (group c, Table 2) implantation and early embryonic development.

There is evidence that, in guinea-pigs, oestrogen given early in pregnancy or the oestrous cycle (Kelly, 1931; Rowlands, 1962; Spies et al., 1964; Choudary & Greenwald, 1968; Illingworth, 1969; Bland & Donovan, 1970) and clomiphene given early in the oestrous cycle (Meckley & Ginther, 1972; Westfahl, 1989) cause luteal regression, as measured directly or by observations of behavioural or vaginal oestrus 10–13 days after treatment. This latter period is similar to the shortening of the oestrous cycle obtained by Loeb (1911) and Dempsey (1937) after removal of the CL in the guinea-pig immediately after ovulation. In the present experiments, results obtained on Day 9 give evidence that clomiphene treatment can cause luteal regression similar to that found with oestrogen, with lowered progesterone concentration, smaller CL and premature vaginal opening in some cases. Direct effects on luteal cells and progesterone production have been previously suggested in mammals (Sgarlata et al., 1984).

At around Day 10–12, the conceptus starts to produce an antiluteolytic factor that, acting both locally and systemically (Heap et al., 1973), maintains pregnancy probably by inhibiting release of prostaglandin F-2a from the uterus (Bland & Donovan, 1969; Blatchley et al., 1975; Antonini et al., 1976; Poyser & Maule Walker, 1979). The reduced CL size observed in pregnant clomiphene-treated females on Day 20 suggests that these luteoprotective factor(s) were not released and/or that they were not effective probably because of the alterations already induced by effects on luteal cells. Regression of the CL, after normal formation on Day 5 in the present experiments, may therefore also involve clomiphene interference with the embryonic mechanisms maintaining CL function.

These data raise the possibility that luteolysis and reduced progesterone concentrations were responsible for pregnancy failure. Loeb & Hesselberg (1917), Deanesly (1960, 1963b) and Csapo et al. (1981) have shown that implantation can occur normally in the guinea-pig after ovariectomy on Days 3–5 after mating, and that development will continue up to Days 11–14 without exogenous progesterone. Regressing pregnancy (small decidua, resorbing embryos and reduced implantation percentage) on Day 9, therefore, cannot be due to the low progesterone concentrations. Further development, however, requires high progesterone concentrations (Deanesly, 1960); between Days 15 and 20, progesterone production changes markedly (Illingworth et al., 1970) and a progesterone-secreting placenta is gradually formed. The low progesterone values observed in pregnant, clomiphene-treated animals on Day 20 strongly suggest that the mechanisms leading to placental formation and function were negatively affected by the alterations induced in the endometrium by clomiphene treatment. Embryo loss, after initial implantation, in the present experiments could therefore depend on an abnormally developed placenta and on the consequently low progesterone concentration achieved.

The administration of exogenous progesterone to clomiphene-treated guinea-pigs (2 mg/kg) confirmed that the low progesterone concentrations were not directly responsible for pregnancy loss (C. M. Motta, unpublished observations). In 5 animals implanted with 25 mg progesterone, only one showed an apparently normal pregnancy with large decidual reactions and normal uterine
and CL histology. In the other animals, regressing CL and altered uterine endometrium were observed.

In conclusion, clomiphene given on the day of mating to guinea-pigs can cause pregnancy failure by interfering with the uterine endometrium. The alterations induced may lead to partial or complete implantation failure and to a subsequent pregnancy loss due, perhaps, to an irregularly secreting placenta. Direct effects on luteal cells and altered embryonic luteoprotective mechanisms also appear to be involved. Such a complex interplay of mechanisms may occur in women given clomiphene to induce ovulation and help to explain why pregnancy rate is low in some cases.

This work is from the M.Sc. thesis of C. M. Motta, who acknowledges receipt of a grant from the Italian Ministry of Education to work in Aberdeen, UK.

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Received 9 June 1990