

## Full-length article

**Anti-implantation effect of droloxifene in rats and its relationship with anti-estrogenic activity<sup>1</sup>**Yong HUANG, Yu SHEN, Ying FENG, Lin CAO, Ying LENG<sup>2</sup>*Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Shanghai 201203, China***Key words**

3-hydroxytamoxifen; implantation; estrogen; progesterone; rats

<sup>1</sup>Project supported by the National Natural Science Foundation of China (No 30000210).<sup>2</sup>Correspondence to Dr Ying LENG.

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Received 2005-06-17

Accepted 2005-06-24

doi: 10.1111/j.1745-7254.2005.00191.x

**Abstract**

**Aim:** To investigate the anti-implantation effect of droloxifene and study the possible relationship between the anti-estrogenic activity of droloxifene and its anti-implantation effect. **Methods:** Pregnant rats were treated orally with droloxifene at 10:00 AM on d 2 at doses of 1.25–20 mg/kg to observe anti-implantation effects, and then doses of 14 mg/kg or 2.5 mg/kg were given at different time on d 2 to d 5 to determine the optimal administration time for anti-implantation effects. Pregnant rats were treated with a combination of droloxifene (2.5 mg/kg, ig) and E2 (0.5–8.0 µg/kg, sc) on the optimal administration time to observe the antagonistic effect of external estrogen on the anti-implantation effect of droloxifene. Serum estrogen and progesterone levels were measured by carrying out radioimmunoassays on d 1 to d 6 in droloxifene-treated and control rats to determine the surge time for nidatory estrogen and the effect of droloxifene on ovary function. **Results:** Droloxifene has anti-implantation effects in rats. The optimal oral administration time was at 22:00 PM on d 4, which was after the surge time for nidatory estrogen (on d 4 at 10:00 AM). This suggests that the anti-implantation effect of droloxifene is not attributable to antagonism of the surge in secretion of nidatory estrogen. External estrogen did not antagonize the anti-implantation effect of droloxifene. Droloxifene had no effect on the serum levels of estrogen and progesterone on d 5 or d 6 when administered on d 4 at 22:00 PM. **Conclusion:** Droloxifene has an anti-implantation effect in rats, and the effect appears to be not completely due to its anti-estrogenic activity.

**Introduction**

Droloxifene, a derivative of the triphenylethylene drug tamoxifen, is a novel selective estrogen receptor modulator (SERM)<sup>[1]</sup>. Its higher affinity to the estrogen receptor, higher anti-estrogenic to estrogenic ratio, more effective inhibition of cell growth and division in estrogen receptor-positive cell lines, and lower toxicity give it theoretical advantages over tamoxifen in the treatment of human breast cancer<sup>[2]</sup>. Droloxifene may also be a potentially useful agent for the treatment of postmenopausal osteoporosis because it can prevent estrogen deficiency-induced bone loss without causing uterine hypertrophy<sup>[3]</sup>. Droloxifene may have an effect on bone and breast tissue because it induces apoptosis<sup>[4]</sup>.

The corpus luteum is an ovarian tissue that synthesizes and secretes progesterone, which plays a key role in the establishment and maintenance of pregnancy in mammals. Abnormal regression of the corpus luteum will disturb or even terminate both the implantation process and early pregnancy. Apoptosis is involved in the regression of the corpus luteum in many species<sup>[5]</sup>. Therefore, better understanding the compounds that induce the apoptosis of luteal cells may contribute to the development of new anti-implantation agents. Our laboratory was the first to report that droloxifene induced the apoptosis of rat luteal cells *in vitro* and the pre-implantation luteal cells in pregnant rats<sup>[6–8]</sup>. Moreover, droloxifene facilitates the apoptosis of luteal cells and short-

ens the period of pseudopregnancy in pseudopregnant rats<sup>[9]</sup>. These results suggest that droloxifene induces the regression of the corpus luteum and has potential anti-implantation effects. Exact equilibrium of estrogen and progesterone is essential for implantation, and any disturbance in the effects of these hormones can cause infertility<sup>[10]</sup>. As a novel selective estrogen receptor modulator with greater anti-estrogenic effects, droloxifene seems to interfere with the effect of estrogen and cause anti-implantation effects. However, the anti-implantation effect of droloxifene has not been evaluated and reported on. Therefore, in the present study, the anti-implantation effect of droloxifene was evaluated and the relationship between the anti-estrogenic activity of droloxifene and its anti-implantation effect was analyzed in rats.

## Materials and methods

**Drugs and reagents** The droloxifene was synthesized by Prof Peng XIA (Department of Organic Chemistry, College of Pharmacy, Fudan University, Shanghai) and was suspended in 1% sodium carboxymethylcellulose (CMC). Estradiol ( $E_2$ ) was purchased from the Shanghai 9th Pharmaceutical Factory (Shanghai, China) and was suspended in corn oil. Serum estrogen and progesterone radioimmunoassay (RIA) kits were obtained from DEPU Ltd (Tianjin, China).

**Animals and treatment** Sprague-Dawley rats (body weight: female, 220–250 g and male 300–350 g, SIPPR/BK Ltd Shanghai) were kept in a temperature-controlled (24–26 °C) and light-regulated (12 h light, 12 h dark) room, and were given *ad libitum* access to standard chow and water. The female animals were cohabited with male animals at a ratio of 2:1. The day that sperm were found in the vaginal smear was designated as d 1 of pregnancy.

**Evaluation of anti-implantation efficacy** Confirmed pregnant female rats were randomly assigned into different groups and were treated with droloxifene (ig), estradiol ( $E_2$ , sc), or 1% CMC (as a control, ig). The doses and treatment times of the different experiments are shown in the results section. At autopsy on d 9, the number of animals with implantation sites in each group was recorded. The Bliss method was used to calculate the  $ED_{95}$ ,  $ED_{90}$ , and  $ED_{50}$  of the anti-implantation effect of droloxifene.

**Assay of the serum levels of estrogen and progesterone** The pregnant rats were treated orally with 2.5 mg/kg droloxifene or 1% CMC at 22:00 PM on d 4. Blood samples of 0.5 mL were obtained from the tail veins of pregnant rats at 10:00 AM on d 1, d 2, d 3, d 4, d 5 and d 6, and at 22:00 PM on d 4. The serum levels of estrogen and progesterone were measured by RIA according to the manufacturer's inst-

ructions.

**Statistical analysis** Differences in pregnancy rates between the groups were tested by using the  $\chi^2$  test. Serum levels of estrogen and progesterone are expressed as mean $\pm$ SD and Student's *t*-test was used to calculate significance.

## Results

**Optimal administration time for the anti-implantation effect of droloxifene** Pregnant rats were treated orally with droloxifene with doses ranging from 1.25 to 20.0 mg/kg at 10:00 AM on d 2. Within the treatment time, droloxifene had an anti-implantation effect in rats ( $ED_{95}$ =17.62 mg/kg and  $ED_{50}$ =5.34 mg/kg; Table 1). To determine the optimal administration time for the anti-implantation effect of droloxifene, pregnant rats were treated orally with droloxifene at either a high dose (14 mg/kg) or a low dose (2.5 mg/kg) at 10:00 AM on d 2, d 3, d 4, or d 5, or at 22:00 PM on d 4. Although the differences in the anti-implantation rates in different groups were not significant for the 14 mg/kg groups, the anti-implantation rates of rats treated with droloxifene at 22:00 PM on d 4 were the highest in the two dose groups (Table 2). These results suggest that droloxifene has anti-implantation effects in rats, and that the optimal administration time is at 22:00 PM on d 4.

**Table 1.** Anti-implantation effects of droloxifene in rats treated in at 10:00 AM on d 2. *n*=10. Mean $\pm$ SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

| Droloxifene (mg/kg) | Pregnant rats  | Rate of anti-implantation (%) | $ED_{50}$ (mg/kg) | $ED_{95}$ (mg/kg) |
|---------------------|----------------|-------------------------------|-------------------|-------------------|
| 20                  | 0 <sup>c</sup> | 100                           |                   |                   |
| 10                  | 3 <sup>c</sup> | 70                            |                   |                   |
| 5                   | 5 <sup>b</sup> | 50                            | 5.34              | 17.62             |
| 2.5                 | 8              | 20                            |                   |                   |
| 1.25                | 10             | 0                             |                   |                   |
| 0                   | 10             | 0                             |                   |                   |

**$ED_{95}$ ,  $ED_{90}$ , and  $ED_{50}$  for the anti-implantation effect of droloxifene** Pregnant rats were treated orally with droloxifene at various doses (10, 5.0, 2.5, 1.25, 0.62, 0.31, or 0.15 mg/kg) at 22:00 PM on d 4. The anti-implantation rates of the droloxifene groups (0.62–10 mg/kg) were higher than that observed in the control group (*P*<0.01; Table 3). There was a dose-dependent relationship between the anti-implantation rates and droloxifene doses from 0.15 mg/kg to 5.0 mg/kg. The values of  $ED_{95}$ ,  $ED_{90}$  and  $ED_{50}$  were 3.70 mg/kg, 2.63

**Table 2.** Anti-implantation effects of droloxifene on rats treated at various times. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control. <sup>f</sup>*P*<0.01 vs droloxifene (at 22:00 PM, on d 4).

| Droloxifene (mg/kg) | <i>n</i> | Time of treatment | Pregnant rats    | Anti-implantation rates (%) |
|---------------------|----------|-------------------|------------------|-----------------------------|
| 14                  | 12       | 10:00 AM, d 2     | 3 <sup>c</sup>   | 75.0                        |
| 14                  | 12       | 10:00 AM, d 3     | 3 <sup>c</sup>   | 75.0                        |
| 14                  | 12       | 10:00 AM, d 4     | 2 <sup>c</sup>   | 83.3                        |
| 14                  | 12       | 22:00 PM, d 4     | 1 <sup>c</sup>   | 91.7                        |
| 14                  | 12       | 10:00 AM, d 5     | 10               | 16.7                        |
| 2.5                 | 10       | 10:00 AM, d 2     | 10 <sup>f</sup>  | 0                           |
| 2.5                 | 10       | 10:00 AM, d 3     | 8 <sup>b,f</sup> | 20                          |
| 2.5                 | 10       | 10:00 AM, d 4     | 4 <sup>b</sup>   | 60                          |
| 2.5                 | 10       | 22:00 PM, d 4     | 1 <sup>b</sup>   | 90                          |
| 2.5                 | 10       | 10:00 AM, d 5     | 10 <sup>f</sup>  | 0                           |
| 0                   | 12       | 22:00 PM, d 4     | 12               | 0                           |

**Table 3.** Anti-implantation effects of various doses of droloxifene in rats treated at 22:00 PM on d 4. *n*=10. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

| Droloxifene (mg/kg) | Number of pregnant rats | Rates of anti-implantation (%) | ED <sub>50</sub> mg/kg | ED <sub>90</sub> mg/kg | ED <sub>95</sub> mg/kg |
|---------------------|-------------------------|--------------------------------|------------------------|------------------------|------------------------|
| 10                  | 0 <sup>c</sup>          | 100                            |                        |                        |                        |
| 5                   | 0 <sup>c</sup>          | 100                            |                        |                        |                        |
| 2.5                 | 1 <sup>c</sup>          | 90                             | 0.79                   | 2.63                   | 3.70                   |
| 1.25                | 4 <sup>c</sup>          | 60                             |                        |                        |                        |
| 0.625               | 6 <sup>b</sup>          | 40                             |                        |                        |                        |
| 0.312               | 8                       | 20                             |                        |                        |                        |
| 0.15                | 10                      | 0                              |                        |                        |                        |
| 0                   | 10                      | 0                              |                        |                        |                        |

mg/kg and 0.79 mg/kg, respectively.

**Antagonistic effect of external E<sub>2</sub> on the anti-implantation effect of droloxifene** To investigate the antagonistic effect of external E<sub>2</sub> on the anti-implantation effect of droloxifene, the anti-implantation effect of external E<sub>2</sub> only was first evaluated. When rats were treated at 22:00 PM on d 4 with external E<sub>2</sub> at doses of 2.0 μg/kg or 8.0 μg/kg (sc), significant anti-implantation effects were observed (*P*<0.05), whereas at doses of 0.5 μg/kg or 1.0 μg/kg, there was no anti-implantation effect. For rats treated at 22:00 PM on d 4 with 2.5 mg/kg droloxifene alone or 2.5 mg/kg droloxifene com-

bined with various doses of E<sub>2</sub>, there was no difference in implantation rates, although E<sub>2</sub> at higher doses (2.0 or 8.0 μg/kg) reduced the anti-implantation rates (Table 4).

**Table 4.** Antagonistic effects of E<sub>2</sub> on the anti-implantation effect of droloxifene (2.5 mg/kg) in rats. Pregnant rats were treated with only E<sub>2</sub> (sc) or with a combination of droloxifene (ig) and E<sub>2</sub> (sc) at 22:00 PM on d 4. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs vehicle control group. <sup>d</sup>*P*>0.05 vs droloxifene (2.5 mg/kg) group.

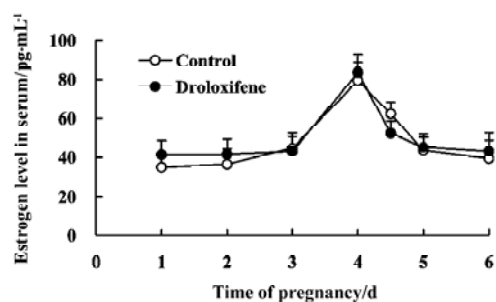
| Droloxifene (mg/kg) | E <sub>2</sub> (μg/kg) | Number of treated rats | Number of pregnant rats | Rate of anti-implantation (%) |
|---------------------|------------------------|------------------------|-------------------------|-------------------------------|
| 0.0                 | 0.5                    | 8                      | 8                       | 0                             |
| 0.0                 | 1.0                    | 8                      | 7                       | 12.5                          |
| 0.0                 | 2.0                    | 7                      | 4 <sup>b</sup>          | 42.9                          |
| 0.0                 | 4.0                    | 8                      | 2 <sup>c</sup>          | 75.0                          |
| 0.0                 | 8.0                    | 7                      | 1 <sup>c</sup>          | 85.7                          |
| 2.5                 | 0.5                    | 8                      | 0 <sup>b,d</sup>        | 100.0                         |
| 2.5                 | 1.0                    | 8                      | 1 <sup>c,d</sup>        | 87.5                          |
| 2.5                 | 2.0                    | 8                      | 0 <sup>c,d</sup>        | 100.0                         |
| 2.5                 | 4.0                    | 10                     | 4 <sup>c,d</sup>        | 60.0                          |
| 2.5                 | 8.0                    | 9                      | 4 <sup>c,d</sup>        | 55.6                          |
| 2.5                 | 0.0                    | 9                      | 1 <sup>c</sup>          | 88.9                          |
| 0.0                 | 0.0                    | 9                      | 9                       | 0                             |

**Effect of droloxifene on the serum level of estrogen during early pregnancy**

Pregnant rats were treated orally with 2.5 mg/kg droloxifene or 1% CMC at 22:00 PM on d 4. The rate of implantation was 100% and 0% in the control and droloxifene groups, respectively. In the control group, the serum level of estrogen remained at low levels from d 1 to d 3, began to rise on d 3, reached the maximum at 10:00 AM on d 4, then declined sharply, such that the levels on d 5 and d 6 were similar to those on d 3. The serum estrogen levels in the droloxifene group between d 1 and d 6 were not significantly different from those in the control group (Figure 1). These results indicate that there was a surge of estrogen in the pregnant rats at 10:00 AM on d 4, and that treatment with droloxifene at 22:00 AM on d 4 had no effect on the level of estrogen; however, a significant anti-implantation effect was induced. Therefore, the anti-implantation effect of droloxifene in rats appears not to be due to antagonism of a surge in the secretion of nidatory estrogen.

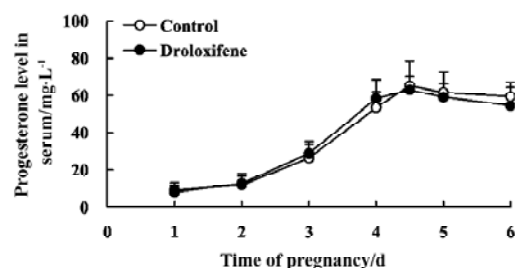
**Effect of droloxifene on serum levels of progesterone during early pregnancy in rats**

Pregnant rats were treated orally with 2.5 mg/kg droloxifene or 1% CMC at 22:00 PM on d 4. The rates of implantation were 100% and 0% in the control and droloxifene groups, respectively. In the control



**Figure 1.** Nidatory estrogen surge and effect of droloxifene on serum levels of estrogen during early pregnancy in rats.  $n=4$ . Mean $\pm$ SD.

groups, the serum levels of progesterone rose from 10:00 AM on d1 to 22:00 PM on d 4 and remained at high levels until d 6. In groups treated with droloxifene at 22:00 PM on d 4, the levels of progesterone were similar to that of controls (Figure 2). These results indicate that treatment with droloxifene at 22:00 PM on d 4 had no effect on the level of progesterone in early pregnancy.



**Figure 2.** Effect of droloxifene on the serum levels of progesterone during early pregnancy in rats.  $n=4$ . Mean $\pm$ SD.

## Discussion

It is well established that an exact equilibrium of estrogen and progesterone is indispensable for implantation in rats<sup>[10]</sup>. A nidatory surge that occurs on d 4 is essential for the sensitization of the uterus to induce decidual cell reaction, the most specific function of the progestational endometrium<sup>[11,12]</sup>. As a novel selective estrogen receptor modulator with considerable anti-estrogenic effects, droloxifene might disturb the hormonal effects and cause an anti-implantation effect. The present study found that droloxifene had anti-implantation effects in rats (Tables 1–3) and that 22:00 PM on d 4 was the optimal oral administration time. At this time there was a good dose-effect relationship between the anti-implantation rates and droloxifene doses from 0.31 mg/kg to 5.0 mg/kg. The ED<sub>95</sub>, ED<sub>90</sub> and ED<sub>50</sub> of droloxifene were 3.70 mg/kg, 2.63 mg/kg, and 0.79 mg/kg, respectively.

We found that the serum levels of estrogen in pregnant

rats reached a peak at 10:00 AM on d 4, which indicates that the nidatory estrogen surge before implantation occurs at approximately this time. However, the optimal oral administration time of droloxifene for anti-implantation effects was at 22:00 PM on d 4, 12 h later than the nidatory estrogen surge. Therefore, we propose that the anti-implantation effect of droloxifene is not caused by its interfering with the nidatory estrogen surge via its anti-estrogenic effect. The effects of droloxifene are different from those of tamoxifen, a triphenylethyl compound, which antagonizes the nidatory estrogen surge<sup>[13,14]</sup>.

In order to further clarify the relationship between the anti-implantation effect and the anti-estrogenic activity of droloxifene, the antagonistic effect of external E<sub>2</sub> on the anti-implantation effect of droloxifene was observed in rats. At first, the anti-implantation effect of external estrogen (0.5–8.0  $\mu$ g/kg, sc) was examined after administration at 22:00 PM on d 4. We found that E<sub>2</sub> at doses of 0.5–1.0  $\mu$ g/kg produced no anti-implantation effect (Table 4), and had no antagonistic effect on the anti-implantation effect of droloxifene ( $P > 0.05$ ; Table 4). When droloxifene was combined with E<sub>2</sub> at higher doses (4.0 or 8.0  $\mu$ g/kg), the anti-implantation effect of droloxifene was reduced, but the difference was not significant according to the  $\chi^2$  test. Therefore, it seems that the anti-implantation effect of droloxifene may be not related to its anti-estrogenic activity, especially at physiological doses.

Because an exact equilibrium of estrogen and progesterone is essential for implantation, and any disturbance in the effects of these hormones can cause infertility, we investigated whether droloxifene inhibited implantation by affecting the serum levels of estrogen and progesterone. We found that droloxifene had no effect on the serum estrogen and progesterone levels in early pregnancy when treated at 22:00 PM on d 4. However, in our previous study, we found that apoptosis of luteal cells and decreases in serum progesterone levels were induced by treatment with droloxifene at a dose of 20 mg/kg on d 2 in pregnant rats<sup>[8]</sup>. The differences between the two experiments can be explained by the different doses and administration times. In addition, the period of observation was too short in the present study.

In conclusion, droloxifene can inhibit implantation in rats and the optimal oral administration time is 22:00 PM on d 4. ED<sub>90</sub> was 2.63 mg/kg. The anti-implantation effect of droloxifene is not related to its antiestrogenic activity, or an antagonistic effect on the nidatory estrogen surge. The direct inhibition of endometrial receptivity to blastocyst signal (s) and the apoptosis of luteal cells might be involved in the anti-implantation mechanism of droloxifene. This characteristic may make droloxifene useful in developing new

contraceptives.

## Acknowledgment

We are grateful to Prof Zhi-ping GU for valuable discussions throughout this study and helpful comments on the manuscript.

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