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Apoptosis induced by droloxifene and C-myc, Bax, Bcl-2 protein expression in corpus luteum of pregnant rats[1]

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KEY WORDS: droloxifene; corpus luteum; apoptosis; pregnancy; rats; proto-oncogene proteins c-*myc*; proto-oncogene proteins c-*bcl*-2; proto-oncogene proteins

ABSTRACT

AIM: To investigate the effects of droloxifene on apoptosis of luteal cells in pregnant rats, and analyze the possible relationships between the expression of C-myc, Bax, Bcl-2 protein in corpus luteum and apoptosis of luteal cells induced by droloxifene. **METHODS**: Pregnant rats were treated orally with droloxifene 20 mg·kg^{-1} on d 2. Ovaries were collected on d 4 or d 8 for detecting the apoptosis of luteal cells by hematoxylin-eosin staining and terminal deoxyribonucleotidyl tranferase-mediated deoxyuridine triphosphate-biotin nick end labelling (TUNEL), and observing the expression of C-myc, Bax, Bcl-2 protein in corpus luteum by immunohistochemistry. The ovarian fresh weight, protein contents, and serum progesterone levels were also determined on d 4 and d 8. **RESULTS**: Apoptotic luteal cells appeared on d 4 and more apoptotic cells could be observed on d 8 in droloxifene treated rats. The ovarian fresh weight, protein contents, and serum progesterone concentration were found to be decreased significantly on d 8 as compared to the control group. In corpus luteum of droloxifene treated rats, the increased expression of C-myc and Bax protein could be observed on d 4 and d 8, respectively, whereas no obvious changes could be found in the expression of Bcl-2 protein. **CONCLUSION**: Droloxifene could induce apoptosis of luteal cells of preimplantation in pregnant rats. An increased expression of C-myc protein and Bax/Bcl-2 ratio could be induced by droloxifene, which might be associated with the mechanisms of apoptosis of luteal cells induced by droloxifene.

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INTRODUCTION

Corpus luteum is one of the fastest growing tissues in the adult female and is one of the few mature tissues that exhibit periodic growth and regression[1,2]. Through its production and secretion of progesterone, the corpus luteum has a critical function for the establishment and maintenance of pregnancy in mammals[3]. If the luteal function is blocked or disturbed, the implantation process and early pregnancy may be interfered or even terminated.

Since the initial paper in 1972[4], apoptosis or programmed cell death has emerged as one of the dominant themes in biology[5,6]. As a kind of physiological cell death that plays a key role in the maintenance of homeostasis of various animals[7], apoptosis was found to be involved in the regression of corpus luteum in many species, such as rat[8], cattle[9,10], rabbit[11], ewe[12], non-human primate[13] and human being[14,15]. Apoptosis is recognized to be a gene-directed process, which is controlled by the expression of a number of regulatory genes including c-*myc*, p53, *bcl*-2, *bax* and APO1/*fas*[16]. In particular, it has been suggested that an interaction between the proto-oncogene c-*myc* and members of the *bcl*-2 family may be of importance in controlling the rate of apoptosis[17]. These genes are also associated with the regulation of apoptosis of luteal cells[18,19]. The localization of c-*myc* mRNA in rat ovaries has been demonstrated[20], and it might interact with other factors to induce apoptosis during structural luteal regression in the primate[18]. In the regressed corpus luteum of bovine, *bax* mRNA level was observed to be increased and might be associated with apoptosis of luteal cells[19]. In human, the expression of Bax and Bcl-2 protein has also been demonstrated in the corpus luteum, although its content remained unchanged throughout the luteal

phase[21, 22].

Study of the compounds that induce apoptosis of luteal cells may contribute to the development of new agents of implantation termination and early pregnancy. In our previous study, the effects of some agents on apoptosis of rat luteal cells were estimated *in vitro*. Droloxifene, an anti-estrogenic compound whose effect on the therapy of human breast cancer had been studied widely[23] was found to induce apoptosis of cultured luteal cells of rats[24]. The increase in c-*myc* mRNA expression might be one of the initiating factors and the elevated ratio of *bax/bcl-2* mRNA may also be involved in this[25]. It has been observed that droloxifene has an anti-implantation effect in rats (unpublished), and it can also facilitate apoptosis of luteal cells in pseudopregnant rats[26]. In the present study, the effects of droloxifene on apoptosis of luteal cells in pregnant rats were investigated, and the expression of C-myc, Bax, and Bcl-2 protein in corpus luteum was observed to analyze the possible relationships between these proteins and apoptosis induced by droloxifene.

MATERIALS AND METHODS

Drugs and reagents Droloxifene was synthesized by Prof XIA Peng (Department of Organic Chemistry, Shanghai Medical University). Proteinase K was obtained from MERCK (Merck kGaA, Darmstadt, Germany). Progesterone radioimmunoassay kits were purchased from Diagnostic System Laboratories, Inc. Programmed Cell Death Assay kits and 3′, 3-diaminobenzidine were purchased from Sino-American Biotechnology (Shanghai, China). Rabbit anti-mouse Bax polyclonal antibody (sc-526), rabbit anti-mouse Bcl-2 polyclonal antibody (sc-492), and mouse C-myc monoclonal antibody (sc-41) were purchased from Santa Cruz biotechnology (Santa Cruz, CA). Biotinylated goat anti-rabbit IgG, biotinlyated goat anti-mouse IgG, and ABC kit were obtained from VECTOR Laboratories (Peterborough, UK).

Animals and treatments Sprague Dawley-rats (♀, $n=80$, 220 – 250 g; ♂, $n=20$, 300 – 350 g), provided by the SIPPR/BK Ltd (Shanghai, China) were housed in an air-conditioned room with controlled lighting conditions (12 h light/dark) and allowed free access to water and rat chow. The female rats were mated with male rats in a 2:1 ratio. The morning of finding sperm in the vaginal smear was designated as d 1 of pregnancy. On d 2 of pregnancy, rats were administered orally droloxifene 20 $mg \cdot kg^{-1}$ or 1 % CMC (vehicle control). Six rats from each group were dissected on d 4, and the other eight rats from each group were dissected on d 8 of pregnancy. Blood samples were collected to determine serum progesterone. The ovaries of each rat were enucleated and weighed to measure protein contents, or fixed in 10 % neutral buffered formalin to undergo histological examination, apoptotic detection and immunohistochemistry analysis.

Protein assay The ovaries were weighed and homogenized in Tris-HCl buffer (Tris-HCl 25 $mmol \cdot L^{-1}$, edetic acid 0.1 $mmol \cdot L^{-1}$, pH 7.5) containing sucrose 0.25 $mol \cdot L^{-1}$ at a concentration of 30 mg wet weight/mL at 0 ℃. The homogenates were centrifuged at 10 000 × *g* for 15 min, and the supernatant fractions were collected. The Coomassie Brilliant Blue G assay was used for the protein determination with bovine serum albumin as the standard.

Progesterone determination Serum progesterone was measured using radioimmunoassay kits. The inter- and intra-assay coefficients of variation were 10.1 % and 4.5 %, respectively.

Histological examination and apoptosis detection *in situ* Ovaries were fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and mounted onto glass slides. For histological examination, sections were stained with hematoxylin and eosine according to standard procedures. Other sections were utilized to examine apoptosis of luteal cells in ovaries by terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kits. The detection was carried out according to the instructions of the supplier. Briefly, tissue sections were deparaffinized, hydrated, and treated for 15 min at 37 ℃ with proteinase K 20 $mg \cdot L^{-1}$ followed by 3 washes with distilled water. Endogenous peroxidase activity was blocked by incubation in 0.3 % hydrogen peroxide for 20 min. Then, the sections were incubated with labeling buffer containing terminal deoxynucleotidyl transferase and biotin-11-deoxyuridine triphosphate for 1h at 37 ℃ in a moisture chamber. After 3 washes with PBS (pH 7.4), the slides were treated with horseradish peroxidase-conjugated avidin for 30 min at 37 ℃ followed by 4 washes with PBS. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3′, 3-diaminobenzidine 0.5 $g \cdot L^{-1}$ for 10 min. At last, the sections were counterstained with hematoxylin and observed under light microscopy. The nuclei of apoptotic cells that contained DNA fragments were stained brown.

Immunohistochemistry of C-myc, Bax and Bcl-2 Tissue sections were deparaffinized and hydrated through graded alcohol to water. Endogenous peroxidase activity was blocked by incubation in 3 % hydrogen peroxide for 10 min. After 3 washes with TBS (Tris-HCl 0.05 $mol \cdot L^{-1}$, NaCl 0.9 %, pH 7.8), the sections were blocked with 1:50 normal horse serum (37 ℃, 30 min) to suppress nonspecific background staining. Then, the primary antibody, mouse C-myc monoclonal antibody, rabbit anti-mouse Bax polyclonal antibody, or rabbit anti-mouse Bcl-2 polyclonal antibody was applied at 1 : 50 dilution, respectively. For control sections, TBS was used in place of primary antibody. After incubation for 20 h at 4 ℃, the sections were further incubated with biotinlyated goat anti-mouse IgG (for C-myc) or biotinlyated goat anti-rabbit IgG (for Bax or Bcl-2) with a 1:50 dilution ratio at 37 ℃ for 30 min, followed by avidin-biotin-peroxidase complex (1:100, 37 ℃, 30 min) from a Vector ABC Kit. The sections were subsequently incubated with 3', 3-diaminobenzidine 0.5 $g \cdot L^{-1}$ (25 ℃, 10 min), counterstained in hematoxylin and observed under light microscopy.

Statistical analysis Data were expressed as $\bar{x} \pm s$. Statistical differences were evaluated by t test. Differences were considered to be significant if $P < 0.05$.

RESULTS

Ovarian weight, protein contents, and serum progesterone concentration When pregnant rats were treated orally by droloxifene 20 $mg \cdot kg^{-1}$ on d 2, the values of ovarian fresh weight, protein contents, and serum progesterone concentration were lower than the control group. However, the difference between treated and control groups had no statistical significance on d 4 ($P > 0.05$). On d 8 of pregnancy, the fresh weight and protein contents of ovary and serum progesterone concentration of droloxifene treated group was found to be decreased significantly with respect to the control rats ($P < 0.05$, Tab 1).

Tab 1. Effects of droloxifene on ovarian fresh weight, protein contents, and serum progesterone levels of pregnant rats. Pregnant rats were treated orally with droloxifene 20 $mg \cdot kg^{-1}$ or 1 % CMC on d 2. Ovarian fresh weight, protein contents, and serum progesterone concentration were measured on d 4 ($n=6$) and d 8 ($n=8$). $\bar{x} \pm s$. $^bP < 0.05$ vs control.

Group	Ovarian weight /mg		Protein contents /$mg \cdot g^{-1}$		Serum progesterone levels/$\mu g \cdot L^{-1}$	
	d 4	d 8	d 4	d 8	d 4	d 8
Control	78±14	75±9	84±10	87±6	62±8	66±8
Treatment	63±17	64 ± 8^b	73±8	79 ± 8^b	58±8	54 ± 9^b

Effect of droloxifene on apoptosis of luteal cells in pregnant rats Pregnant rats were treated by gavage of droloxifene 20 $mg \cdot kg^{-1}$ or 1 % CMC on d 2, the histological examination and apoptosis detection *in situ* was performed on d 4 and d 8, respectively. Hematoxylin-eosin stain showed that degenerating luteal cells with condensed nuclei appeared on d 4, and apoptotic bodies and enlarged intercellular space could be observed on d 8 in droloxifene treated group. In control rats, healthy luteal cells with abundant cytoplasm and regular, circular nuclei were apparent. By TUNEL detection, the existence of oligonucleosome formation in apoptotic luteal cells on d 4 and d 8 of droloxifene treated group was confirmed. It was found that apoptotic luteal cells with positively stained nuclei appeared on d 4, and more apoptotic luteal cells could be observed on d 8. However, the sections of ovaries of control rats exhibited no apoptotic cells in corpus luteum (Fig 1).

Expression of C-myc protein C-myc protein, which was stained brown can be found predominantly in the cytoplasm of luteal cells of rats. Negative control sections incubated with TBS in place of primary antibody were free of immunostaining. In corpus luteum of control rats, the majority of cells were found to be free from staining, except that scattered luteal cells with weak or moderate staining could be observed. When pregnant rats were treated orally with droloxifene 20 $mg \cdot kg^{-1}$ on d 2, the expression of C-myc protein in corpus luteum was found to be increased. On d 4 of pregnancy, an elevated intensity and localization of immunostaining for C-myc could be observed in corpus luteum of droloxifene treated group, and almost all of luteal cells exhibited moderate or intense staining of C-myc protein on d 8 (Fig 2).

Expression of Bax and Bcl-2 protein Photomicrographs of immunohistochemical localization of Bax and Bcl-2 protein in corpus luteum of rats and the control sections were shown in Fig 3. The negative controls for Bax and Bcl-2 were consistently free of staining.

The brown positive staining of Bax was found primarily in the cytoplasm of luteal cells in all of the sections examined. On d 4 of pregnancy, no obvious difference in the intensity or localization of immunostaining for Bax could be observed between the droloxifene treated rats and control. However, a marked increase in intensity

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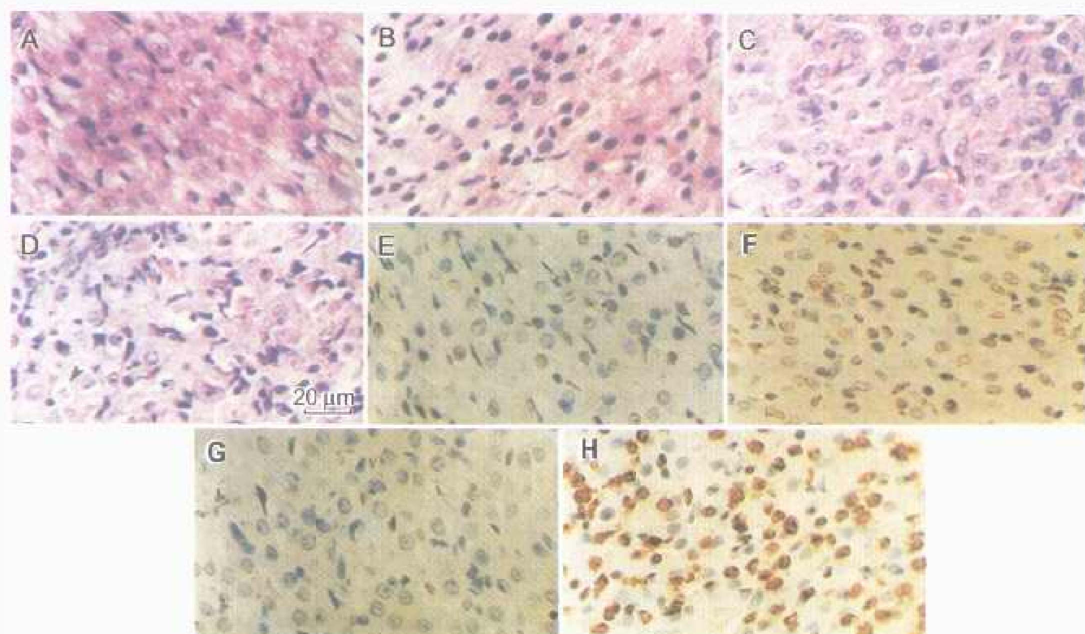

Fig 1. Apoptosis of luteal cells induced by droloxifene in pregnant rats. Pregnant rats were treated orally with droloxifene 20 mg·kg^{-1} (B, F, D, H) or 1 % CMC (A, E, C, G) on d 2. HE staining (A – D) and TUNEL detection (E – H) of ovarian sections were performed on d 4 (A, B, E, F) and d 8 (C, D, G, H). Condensed nuclei (indicated by arrows) appeared on d 4 of treatment, and apoptotic bodies (indicated by arrowheads) could be observed on d 8. TUNEL detection showed apoptotic luteal cells on d 4 and more apoptotic cells appeared on d 8 in the corpus luteum of droloxifene treated rats. ×500.

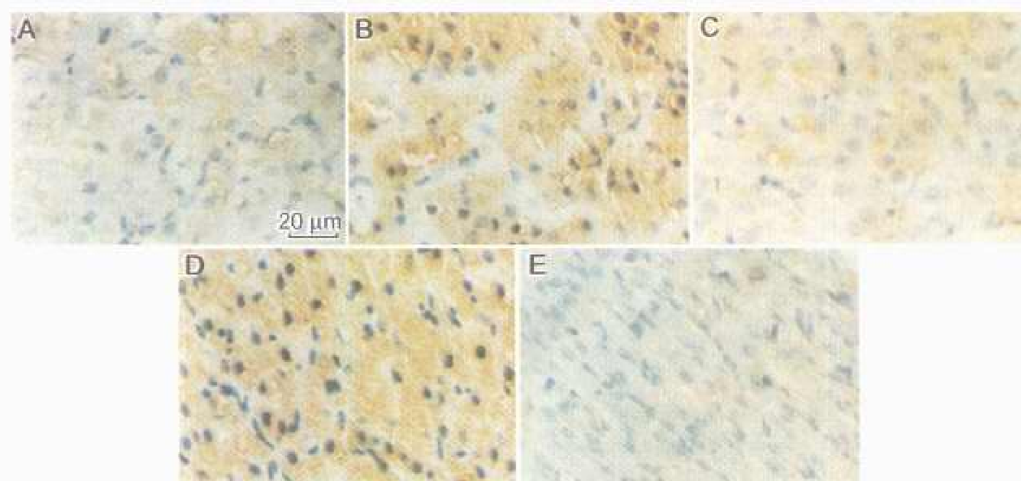

Fig 2. Effects of droloxifene on the expression of C-myc protein in corpus luteum of pregnant rats. Pregnant rats were treated orally with droloxifene 20 mg·kg^{-1} (B, D) or 1 % CMC (A, C) on d 2. C-myc protein immunohistochemistry was performed on d 4 (A, B) and d 8 (C, D). Negative control section (E) incubated with TBS in place of primary antibody was devoid of staining. ×500.

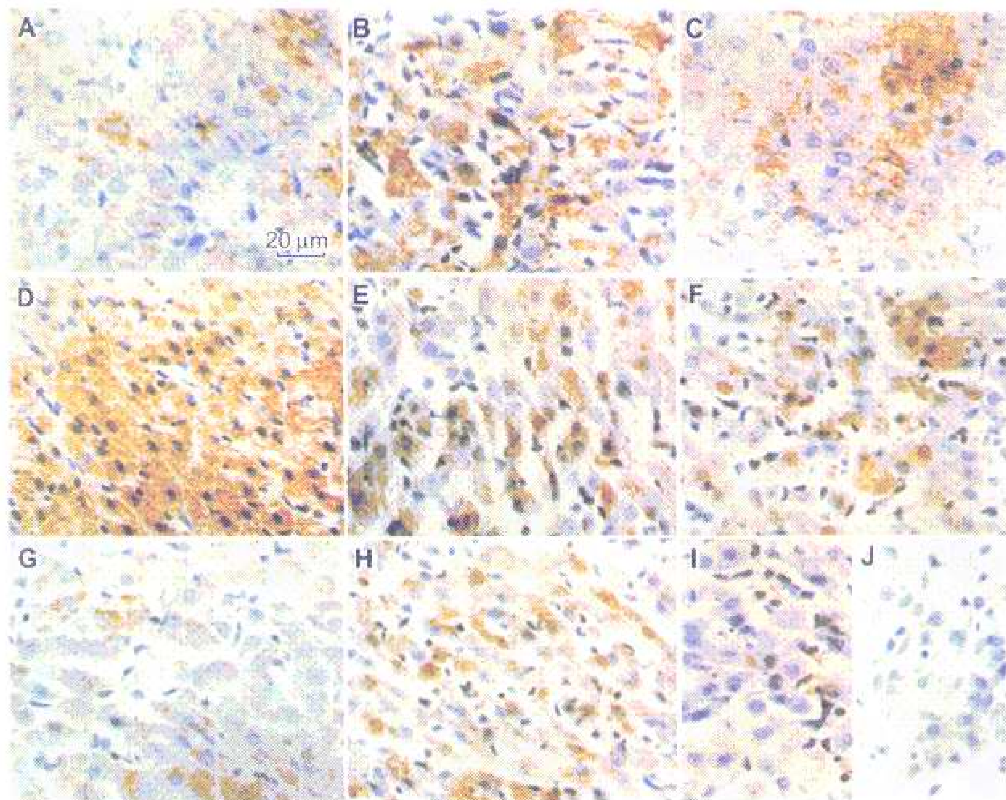

Fig 3. Effects of droloxifene on the expression of Bax (A – D) and Bcl-2 (E – H) protein in corpus luteum of pregnant rats. Pregnant rats were treated orally with droloxifene 20 mg·kg^{-1} (B, D, F, H) or 1 % CMC (A, C, E, G) on d 2. Bax and Bcl-2 immunohistochemistry was performed on d 4 (A, B, E, F) and d 8 (C, D, G, H). Negative control sections incubated with TBS in place of primary antibody of Bax (I) and Bcl-2 (J) were devoid of staining. ×500.

and localization of Bax protein was found on d 8 in corpus luteum of droloxifene treated rats.

The immunostaining of Bcl-2 protein which was brown could be observed predominantly in the cytoplasm of luteal cells, and some nuclei also exhibited positive staining. The expression of Bcl-2 protein could be observed in all of the sections examined. However, when pregnant rats were treated orally with droloxifene of 20 mg·kg^{-1} on d 2, no obvious differences in the intensity or localization of immunostaining for Bcl-2 could be found between the droloxifene treated rats and control group on d 4 or d 8.

DISCUSSION

Apoptosis, also known as programmed cell death, plays a complementary but opposing role to that of mitosis in regulating normal and neoplastic tissue growth and regression[4, 27]. The morphological features of apoptosis are cell shrinkage, condensation of nuclei, and formation of small, spherical, and membrane-bound organelles, referred to as apoptotic bodies[4]. Biochemically, apoptosis is characterized by the activation of Ca^{2+}/Mg^{2+}-dependent endonucleases, which cleave DNA into multiples of 185-bp fragments[28]. These fragments can be analyzed by separation of cellular DNA on agarose gels, where DNA laddering appears, or visualized *in situ* by DNA 3′-end labeling (TUNEL)[29].

In the present study, both morphological analysis and TUNEL detection demonstrated that apoptosis of luteal cells in pregnant rats could be induced by droloxifene when administered before implantation. HE stain showed that pyknotic luteal cells with condensed nuclei, which is the morphological character of apoptosis ap-

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peared on d 4, and the apoptotic bodies could be observed on d 8 in droloxifene treated rats. TUNEL detection also exhibited that DNA fragmentation in nuclei of luteal cells could be induced by the treatment with droloxifene. It is well known that in spontaneous or prostaglandin $F_{2\alpha}$ induced regression of corpus luteum apoptosis occurred after the decline of serum progesterone level and might be primarily associated with the structural luteolysis[9,10,30]. In this study, however, the serum progesterone concentration was found to be decreased significantly on d 8, while the apoptosis of luteal cells could be visualized on d 4 in droloxifene treated group. Accompanied with the occurrence of apoptosis of luteal cells, the ovarian fresh weight and protein contents declined in droloxifene treated rats. So, it may be suggested that droloxifene can induce regression of corpus luteum of preimplantation in pregnant rats and the apoptosis of luteal cells induced by droloxifene might be not only involved in the structural luteolysis, but also in the functional regression of corpus luteum.

The proto-oncogene c-*myc* has been shown to play a pivotal role in cell proliferation and DNA synthesis, but, paradoxically, in some tissues its expression under conditions of growth arrest is associated with the initiation of apoptosis[31]. The expression of c-*myc* gene in ovaries of human[32, 33], primate[18], and rat[20, 34] has been confirmed, and it may play a role in the molecular mechanisms of cell proliferation and apoptosis. In the hamster ovary cell line, c-*myc* is essential for the induction of apoptosis[35]. An immunohistochemical study by Fraser *et al*[18] also suggested that the increase in C-myc protein expression might be associated with the apoptosis of luteal cells induced by prostaglandin $F_{2\alpha}$ analogue or gonadotropin releasing hormone (GnRH) antagonist. Our previous studies showed that the expression of c-*myc* mRNA in cultured luteal cells of rats increased with the increasing concentrations or treatment duration of droloxifene. The increase in expression of c-*myc* mRNA might be one of the initiation factors in the apoptosis induced by droloxifene[25]. In the present study, the presence of C-myc protein in corpus luteum of pregnant rats was confirmed by immunohistochemical analysis. In corpus luteum of control rats, C-myc protein exhibited a weak expression, and no apoptotic cells could be found. When pregnant rats were treated with droloxifene 20 $mg \cdot kg^{-1}$ on d 2, apoptotic luteal cells appeared on d 4 and the intensity and localization of immunostaining for C-myc also arised significantly on this day. On d 8, accompanied with the appearance of more apoptotic luteal cells, the expression of C-myc protein in corpus luteum arised further. Therefore, it is suggested that similar to the *in vitro* results, the increase in expression of C-myc protein in corpus luteum of pregnant rats might be associated with the apoptosis of luteal cells induced by droloxifene.

Genes *bcl*-2 and *bax* are two members of *bcl*-2 gene family. Bcl-2 is known to protect against apoptosis triggered by a wide range of factors. However, the activity of Bcl-2 is known to be determined by the interaction with Bax, a M_r21 000 protein with a degree of homology to Bcl-2[36]. Bcl-2 forms heterodimers with Bax, resulting in a loss of its portative effect. Bax itself forms homodimers, which induce apoptosis. So it appears to be the relative levels of Bcl-2 and Bax which determine the fate of a cell, rather than the absolute concentrations of either. The c-*myc* proto-oncogene drives two coupled, however, opposing functions; proliferation and apoptosis. It is known that interaction of *bcl*-2 gene family interacted with c-*myc* gene and might be involved in the determination of the final outcome of cells. For example, activated *bcl*-2 gene could prevent apoptosis induced by c-*myc*[17], and *bax* was observed to be elevated when c-*myc* was overexpressed[37]. During the structural regression of corpus luteum in bovine, the level of *bax* mRNA was found to be increased, and might be associated with the apoptosis of cells[19]. Our previous studies also showed that an increased *bax*/*bcl*-2 mRNA ratio could be induced by droloxifene in cultured luteal cells of rats[25]. In the present study, the existence of Bax and Bcl-2 protein in corpus luteum of pregnant rats was confirmed by immunohistochemistry. Treatment with droloxifene 20 $mg \cdot kg^{-1}$ on d 2 of pregnant rats could cause a marked increase in the expression of Bax protein in corpus luteum on d 8, whereas the localization and intensity of immunostaining for Bcl-2 had no obvious changes. So, an increased Bax/Bcl-2 protein ratio induced by droloxifene might be involved in the apoptosis of luteal cells in pregnant rats, although its increase occurs later than the appearance of luteal cell apoptosis.

Droloxifene has been found to have an anti-implantation effect in rats. When pregnant rats were treated orally by droloxifene 20 $mg \cdot kg^{-1}$ on d 2, the inhibitory rate of implantation was observed to reach 100 % (unpublished). The present study showed that droloxifene at the same dose could induce apoptosis of luteal cells of preimplantation in pregnant rat and might be involved in the functional and structural regression of corpus luteum. It is suggested that the apoptosis of luteal cells induced by

droloxifene might be associated with the mechanisms of its anti-implantation effect, although more studies need to be done to elucidate the exact relationship between them.

In conclusion, droloxifene could induce apoptosis of preimplantation luteal cells in pregnant rats. An increased expression of C-myc and Bax protein in corpus luteum could be induced by the treatment with droloxifene when administered before implantation, while the Bcl-2 protein level did not change. It is suggested that the elevated expression of C-myc and Bax/Bcl-2 ratio might be associated with the apoptosis of luteal cells induced by droloxifene.

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屈洛昔芬对妊娠大鼠黄体细胞凋亡和 C-myc、Bax、Bcl-2 蛋白表达的影响[1]

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关键词 屈洛昔芬；黄体；细胞凋亡；妊娠；大鼠；原癌基因蛋白质 c-*myc* 类；原癌基因蛋白质 c-*bcl*-2 类；原癌基因蛋白质类

目的：观察屈洛昔芬对妊娠大鼠黄体细胞凋亡的影响，并分析黄体中 C-myc，Bax 和 Bcl-2 蛋白表达与屈洛昔芬所诱导的黄体细胞凋亡间的可能联系．**方法**：大鼠于妊娠第 2 天口服给予屈洛昔芬 20 mg·kg^{-1}后，分别于第 4 天和第 8 天取卵巢，HE 染色和 TUNEL 法检测黄体中凋亡细胞的存在，免疫组织化学方法观察黄体中 C-myc，Bax 和 Bcl-2 蛋白的表达，同时测定卵巢重量、蛋白质含量及血中孕酮水平．**结果**：大鼠经屈洛昔芬处理后，第 4 天卵巢黄体中出现明显的凋亡细胞，第 8 天时更加明显．卵巢重量、蛋白质含量及血清孕酮水平在第 8 天时显著下降．屈洛昔芬处理组大鼠卵巢黄体中 C-myc 蛋白表达在第 4 天即显著增加，Bax 蛋白表达的显著增加在第 8 天可观察到，而 Bcl-2 蛋白在卵巢黄体中的表达无明显改变．**结论**：屈洛昔芬可诱导妊娠大鼠着床前黄体细胞凋亡，C-myc 蛋白及 Bax/Bcl-2 蛋白表达的增加可能与该过程有关．

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