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Effects of droloxifene on apoptosis and Bax, Bcl-2 protein expression of luteal cells in pseudopregnant rats[1]

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KEY WORDS droloxifene; corpus luteum; rats; pseudopregnancy; apoptosis; proteins

ABSTRACT

AIM: To study the effects of droloxifene on apoptosis and the expression of Bax and Bcl-2 protein in corpus luteum of pseudopregnant rats. **METHODS**: HE staining was used to examine the histological changes of ovaries. Apoptosis detection *in situ* was performed with TUNEL method. Expression of Bax and Bcl-2 protein was observed by immunohistochemistry analysis. **RESULTS**: Apoptosis of luteal cells during the spontaneous regression of corpus luteum of pseudopregnant rats appeared on d 13 of pseudopregnancy, and the marked increase of apoptotic luteal cells could be observed on d 15. When pseudopregnant rats were treated with droloxifene 20 $mg \cdot Kg^{-1}$ on d 2, apoptosis of luteal cells could be observed on d 8, and the duration of pseudopregnancy could be shortened from (15.5 ± 1.1) d to (12.8 ± 1.6) d. In pseudopregnant rats, the expression of Bax and Bcl-2 protein was found in the cytoplasm of luteal cells. However, no obvious differences in the intensity or localization could be found during various days of the pseudopregnancy, while an increase in Bax and a decrease in Bcl-2 protein expression could be induced by droloxifene treatment. **CONCLUSION**: Droloxifene could facilitate apoptosis of luteal cells in pseudopregnant rats and shorten the period of pseudopregnancy. An increased Bax/Bcl-2 ratio might be involved in the facilitation of apoptosis induced by droloxifene in corpus luteum of pseudopregnant rats.

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INTRODUCTION

Corpus luteum (CL) is one of the few adult tissues which exhibit periodic growth and regression in mammals. It primarily synthesizes and secretes progesterone which is essential for the establishment and maintenance of pregnancy. If the ovulated oocyte is not fertilized and pregnancy does not occur, the CL regresses to maintain the normal estrous cycles.

Apoptosis is a kind of physiological death which plays a complementary but opposite role to mitosis in the regulation of animal cell populations[1,2]. Recently, it was found to be associated with the spontaneous or $PGF_{2\alpha}$-induced luteolysis, including rat[3], cattle[4,5], rabbit[6], ewe[7], non-human primate[8] and human[9,10]. As a gene-directed process, apoptosis is modulated by the expression of a number of regulatory genes, such as p53, c-*myc* and *bcl*-2 family[11]. In the ovary, increased expression of *bax*, a pro-apoptotic *bcl*-2 gene family member, had been correlated with the occurrence of apoptosis in luteal cells during CL regression[12]. In human, the expression of Bax and Bcl-2 protein had also been demonstrated in CL, although its content remained unchanged throughout the luteal phase[13,14].

Droloxifene is an anti-estrogenic compound whose effect on the therapy of human breast cancer had been studied widely[15]. Our previous study firstly demonstrated that it could induce apoptosis of rat luteal cells *in vitro*[16]. Further studies showed that the apoptosis of luteal cells of preimplantation in pregnant rats could be induced by droloxifene, and its anti-implantation effect on rats also had been confirmed (unpublished). However, the effects of droloxifene on apoptosis of luteal cells in pseudopregnant rats were unknown. Therefore, in the present study, apoptosis of luteal cells during the spontaneous regression of pseudopregnant CL and the effects of droloxifene were investigated, and the expression of Bax and Bcl-2 protein were observed in CL to analyze the

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possible roles of them in apoptosis of luteal cells.

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. Programmed Cell Death Assay kits and 3′, 3-diaminobenzidine (DAB) were purchased from Sino-American Biouechnology. Rabbit anti-mouse Bax polyclonal antibody (sc-526) and rabbit anti-mouse Bcl-2 polyclonal antibody (sc-492) were purchased from Santa Cruz biotechnology. Biotinylated goat antirabbit IgG and ABC kit were obtained from VECTOR Laboratories.

Animals and treatments Sprague Dawley-rats (Grade II, ♀, $n = 80$, 220 ~ 250 g, ♂, $n = 10$, 300 ~ 350 g, SIPPR/BK Ltd, Certificate № 02-49-2) were housed in an air-conditioned room with controlled lighting conditions (12 h light/dark) and allowed free access to water and rat chow. Pseudopregnant rats were obtained by using a method modified from Lu *et al*[17]. Briefly, the male rats were treated by gavage of α-chlorohydrin 10 $mg \cdot Kg^{-1}$ for 5 d. On the sixth day, female rats cohabited with these males in a 1:2 (male: female) ratio. The appearance of sperm in the vaginal smear on the next morning was considered as d 1 of pesudopregnancy. Male rats were administered with α-chlorohydrin once daily until the end of mating experiment.

Experimental design Exp 1: Twenty-five rats were sacrificed respectively on d 9, d 11, d 13, d 15, and d 17 of the pseudopregnancy ($n = 5$/group). Ovaries of each rat were dissected and fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and mounted onto glass sides. These sections were used for histological examination, apoptosis detection *in situ* and immunohistochemistry analysis of Bax and Bcl-2 proteins.

Exp 2: Pseudopregnant rats were administered orally droloxifene 20 $mg \cdot Kg^{-1}$ (treated group) or 1 % CMC (control group) on d 2. Six rats in each group were dissected on d 8. Histological examination and apoptosis detection *in situ* were carried out, and the immunohistochemistry analysis of Bax and Bcl-2 protein was also performed on the ovaries. In the remaining rats (10 rats in control group and 11 rats in treated group), effects of droloxifene on the duration of pseudopregnancy were estimated. Vaginal smear was performed every morning from the first day of pseudopregnancy until the day when regular estrous cycles resumed. The duration of pseudopregnancy was expressed as the number of days that the continued diestrus remained. Results were expressed as $\bar{x} \pm s$ and compared by t-test. Differences were considered to be statistically significant if $P < 0.05$.

Histological examination and apoptosis detection *in situ* Tissue sections were deparaffinized and hydrated. For histological examination, sections were stained with hematoxylin and eosin according to standard procedures. Other sections were utilized to examine apoptosis of luteal cells in ovaries by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) kits. The detection was carried out according to the instructions of the supplier. Briefly, tissue sections were treated with proteinase K 20 $mg \cdot L^{-1}$ for 15 min at 37 ℃ and washed with distilled water three times. 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 (dUTP) for 1 h 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 four washes with PBS. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3′, 3-diaminobenzidine (DAB) 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, which contained DNA fragments were stained brown.

Immunohistochemistry 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 three washes with TBS (Tris-HCl 0.05 $mol \cdot L^{-1}$, 0.9 % NaCl, 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, rabbit anti-mouse Bax polyclonal antibody or rabbit anti-mouse Bcl-2 polyclonal antibody was applied at 1:50 dilution. For control sections, TBS was used in place of primary antibody. After incubation for 20 h at 4 ℃, the sections were incubated with biotinlyated goat anti-rabbit IgG (1:50, 37 ℃, 30 min), followed by avidin-biotin-peroxidase complex (1:100, 37 ℃, 30 min) from a Vector ABC Kit. The sections were subsequently incubated with DAB 0.5 $g \cdot L^{-1}$ (RT, 10 min), counterstained by hematoxylin and observed under light microscopy.

RESULTS

Histological examination and apoptosis detection during spontaneous regression of CL in pseudopregnant rats Healthy luteal cells with abundant cytoplasm and regular, circular nuclei were shown by HE staining on d 9, d 11, and d 13 of pseudopregnancy, while degenerating luteal cells with condensed nuclei and a low ratio of cytoplasm to nucleus appeared on d 15. On d 17 of pseudopregnancy, apoptotic bodies and enlarged intercellular spaces could be observed. By TUNEL detection, it was found that the sections of ovaries exhibited no apoptotic cells in CL on d 9 and d 11 of pseudopregnancy, and a few apoptotic cells could be observed on d 13. A marked increase in apoptotic cells appeared in CL of pseudopregnant rats on d 15 and d 17 (Fig 1).

Expression of Bax and Bcl-2 protein in CL of pseudopregnant rats Photomicrographs of immunohistochemical localization of Bax, Bcl-2 protein in CL and the control sections are shown in Fig 2. The nega-

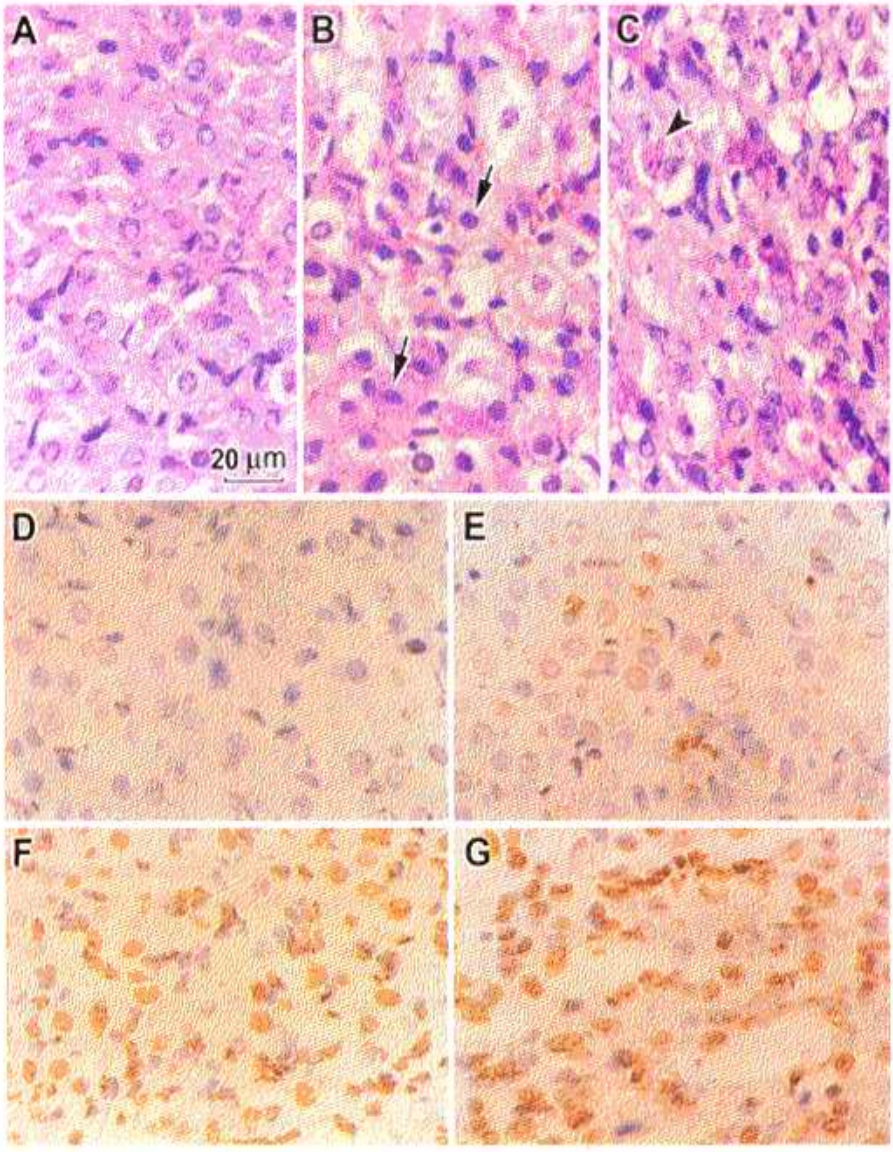

Fig 1. Histological examination and apoptosis detection of CL in pseudopregnant rats. HE staining (A – C) and TUNEL detection (D – G) of ovarian sections were performed on d 9, d 11, d 13, d 15, and d 17 of pseudopregnancy. No morphological changes appeared on d 9 (A). Pyknotic nuclei (indicated by arrows) appearedon d 15(B), and some apoptotic bodies (indicated by arrowheads) appearedon d 17 (C). TUNEL detection showed that no apoptotic cells appeared on d 9 (D), and a few apoptotic cells appeared on d 13 (E), while more apoptotic cells could be observed on D15 (F) and D17 (G). × 500.

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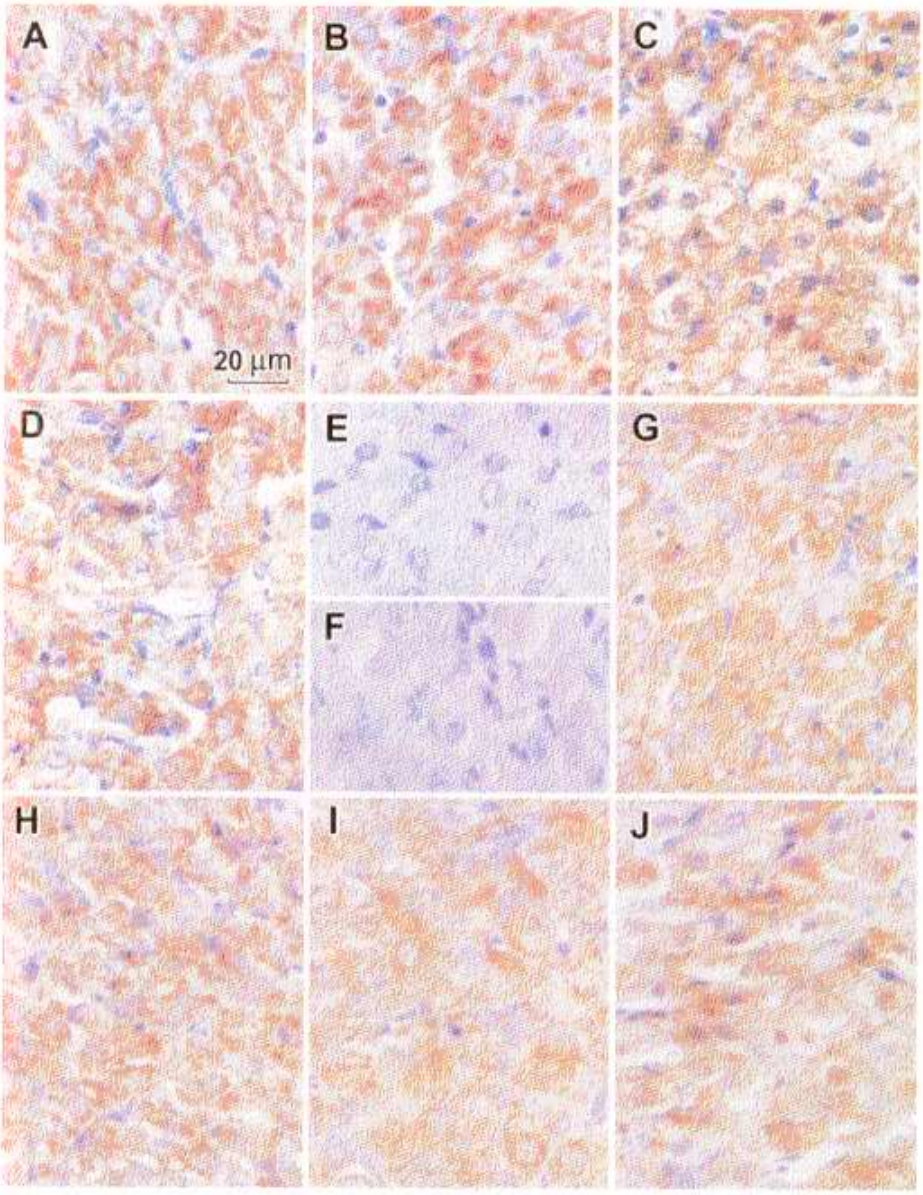

Fig 2. Immunostaining of Bax (A – E) and Bcl-2 (F – J) proteins in CL of pseudopregnant rats on day9 (A, G), d 13 (B, H), d 15(C, I) and d 17 (D, J). Negative control sections incubated with TBS in place of primary antibody of Bax (E) and Bcl-2 (F) were free from staining. × 500.

tive control sections were consistently free of staining, and positive staining for Bax and Bcl-2 was found primarily in the cytoplasm of luteal cells in all of the sections examined. However, no obvious differences in the intensity or localization of immunostaining for Bax or Bcl-2 could be observed during the various days of the pseudopregnancy.

Effects of droloxifene on apoptosis of luteal cells in pseudopregnant rats Pseudopregnant rats were treated with droloxifene 20 mg · Kg^{-1} on d 2, the morphological characters and apoptosis were detected on d 8. HE staining showed that marked degenerating luteal cells with condensed nuclei appeared in droloxifene treated group, while healthy luteal cells with circular and regular nuclei were apparent in control rats. By TUNEL detection, apoptotic luteal cells whose nuclei were stained positively could be found in droloxifene treated group on d 8 of pseudopregnancy, whereas ovarian slides of control group exhibited no apoptotic cells in CL (Fig 3).

Effects of droloxifene on expression of Bax

and Bcl-2 protein in CL of pseudopregnant rats Immunostaining for Bax and Bcl-2 was found primarily in the cytoplasm of luteal cells. Negative control sections incubated with TBS in place of primary antibody were consistently free from immunostaining. In CL of pseudopregnant rats which were treated with droloxifene 20 $mg \cdot Kg^{-1}$ on d 2, an increased intensity and localization of immunostaining for Bax could be observed on d 8. Conversely, the expression of Bcl-2 protein was found to be decreased (Fig 4).

Effects of droloxifene on pseudopregnancy period of rats The duration of pseudopregnancy was (15.5 ± 1.1) d in control group, whereas a significantly shortened duration of (12.8 ± 1.6) d was found in droloxifene treated rats ($P < 0.01$).

DISCUSSION

Corpus luteum plays a key role in the establishment and maintenance of pregnancy in mammals. In human, production of progesterone by this gland is essential for the first 8 gestation weeks[18]. The abnormal regression of CL will disturb or even terminate the implantation process and early pregnancy. So, it is of great significance to study the drugs that can induce CL regression. Droloxifene had been found to induce apoptosis of luteal cells *in vitro* and in pregnant rats. In the present study, the effects of droloxifene on apoptosis of luteal cells in pseudopregnant rats were investigated, and the possible relationships between the expression of Bax and Bcl-2 protein and apoptosis of luteal cells were analyzed.

The pseudopregnant rats could be obtained by copulating with male rats whose fertility had been deprived by the treatment with α-chlorohydrin[17]. Because of the absence of embryos, the pseudopregnant CL could only be maintained for certain days, then, spontaneous regression occurred. In this study, the existence of apoptotic cells was examined during the spontaneous regression of CL in pseudopregnant rats. The results showed that no apparent morphological changes of luteal cells could be observed till d 11 of pseudopregnancy, and a few apoptotic cells could be observed by TUNEL detection on d 13. On d 15 and d 17 of pseudopregnancy, abundant apoptotic cells deeply stained appeared in CL. So, it is suggested that the apoptosis of luteal cells during the spontaneous regression of pseudopregnant CL of rats appeared on d 13 and the marked increase of apoptotic luteal cells could be found on d 15.

Then, the effects of droloxifene on apoptosis of luteal cells in pseudopregnant rats were studied. After being treated with droloxifene 20 $mg \cdot Kg^{-1}$ on d 2 of pseudopregnancy, the occurrence of apoptosis of luteal cells was examined on d 8. Many apoptotic cells which exhibited condensed nuclei and stained positively by TUNEL method could be found in droloxifene treated group, while no apparent apoptotic cells existed in the control group. Further study showed that the duration of pseudopregnancy of rats was shortened from (15.5 ± 1.1) d in control group to (12.8 ± 1.6) d after being treated with droloxifene 20 $mg \cdot Kg^{-1}$ on d 2. The facilitation of apoptosis of luteal cells in pseudopregnant rats might be attributed to the shortened duration of pseudopregnancy induced by droloxifene.

Droloxifene has been found to have an anti-implantation effect in rats. When pregnant rats were treated orally droloxifene 20 $mg \cdot Kg^{-1}$ on d 2, the inhibitory rate of implantation could reach 100 % (unpublished). The present study showed that droloxifene of the same dose could facilitate the apoptosis of luteal cells in pseudopregnant rats. This facilitated regression of CL caused by droloxifene may be associated with the mechanisms of its anti-implantation effects.

Bcl-2 and Bax are two members of the *bcl*-2 gene family[19]. Bcl-2 is known to protect against apoptosis triggered by a wide range of factors. However, the inhibitory effect of Bcl-2 on apoptosis is determined by the interaction with Bax, a M_r 21 000 protein with a degree of homology to Bcl-2. Bcl-2 can form heterodimers with Bax and lose its protective effect. When Bcl-2 is in excess, cells are protected from apoptosis, and when Bax is in excess and homodimers of Bax dominate, cell are susceptible to programmed cell death. So, it appears that the relative ratio of Bcl-2 and Bax determines the fate of cell, rather than the absolute concentrations of either. In the present study, the expressions of Bax and Bcl-2 protein in CL of pseudopregnant rats were analyzed by immunohistochemistry. Both Bax and Bcl-2 protein were found in CL, however, the intensity and localization of immunostaining were consistent during the spontaneous regression of the pseudopregnant CL of rats. This finding was similar to the consistent expression of Bax and Bcl-2 protein during CL maintenance or regression in human[13,14], but contrary to the increased *bax* mRNA during CL regression of bovine[12], which suggested that mechanisms leading to apoptosis of luteal cells might be different in various species. The consistent expression of Bax and Bcl-2 suggested that the relative ratio of Bax and Bcl-2 had no changes during the spontaneous regression

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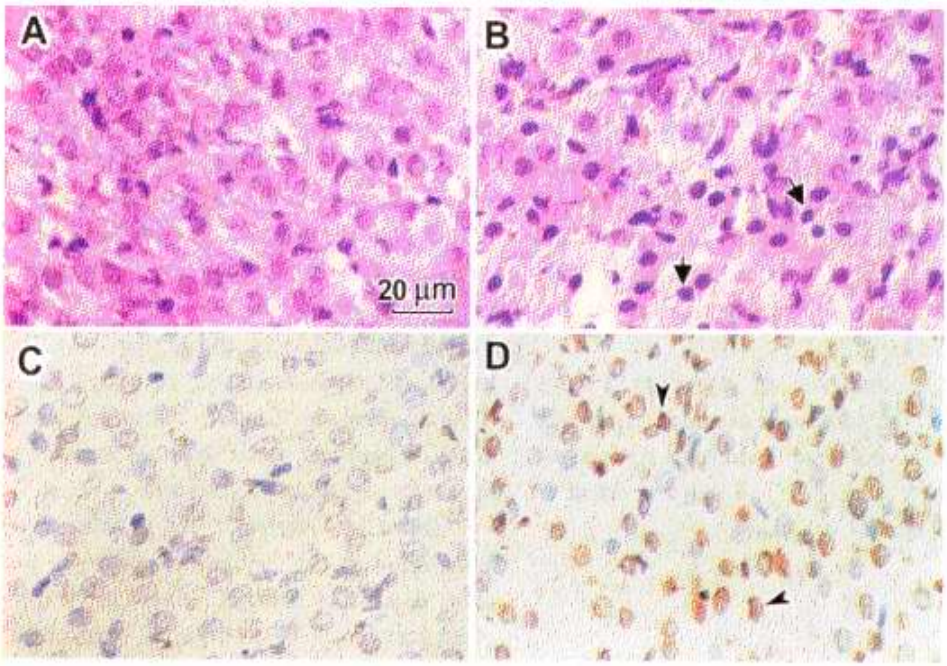

Fig 3. Histological examination and apoptosis detection *in situ* of CL in pseudopregnant rats treated by droloxifene. Rats were treated by gavage of 1 % CMC (A,C) or droloxifene 20 mg·kg^{-1} (B,D) on d 2 of pseudopregnancy. HE staining (A,B) and TUNEL detection (C,D) of ovarian sections were performed on d 8. Luteal cells exhibited pyknotic nuclei (indicated by arrows) and apoptotic luteal cells (indicated by arroeheads) were observed in droloxifene treated group. × 500.

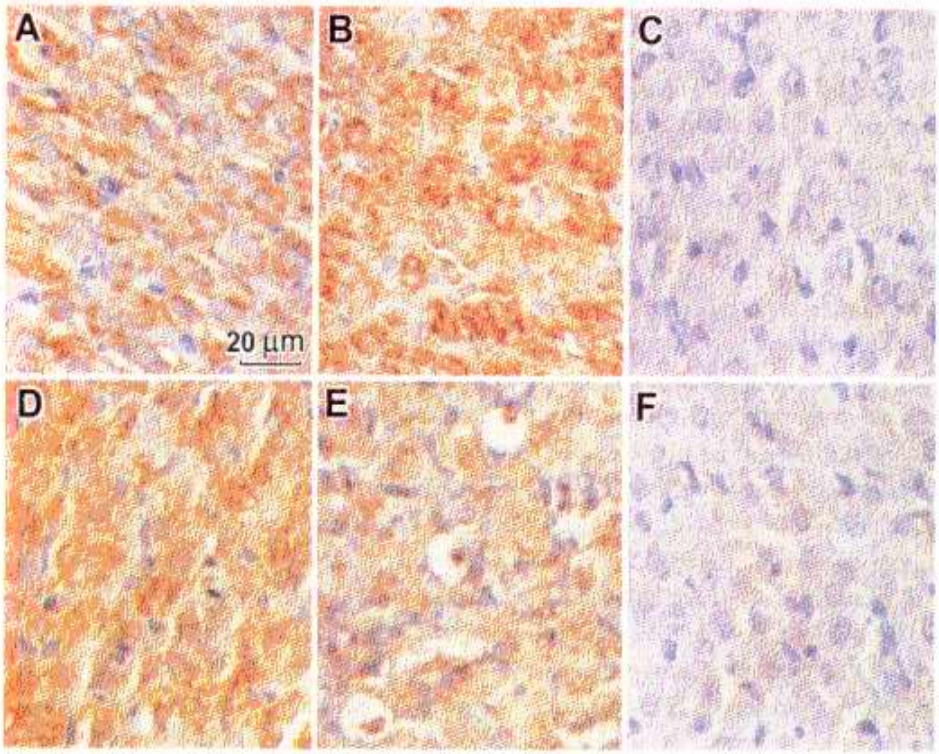

Fig 4. Immunostaining of Bax (A – C) and Bcl-2 (D – F) proteins in CL of pseudopregnant rats treated by gavage of 1 % CMC (A,D) or droloxifene 20 mg·kg^{-1} (B,E) on d 2 of pseudopregnancy. Negative control sections incubated with TBS in place of primary antibody of Bax (C) and Bcl-2 (F) were free from staining. × 500.

of the pseudopregnant CL of rats. However, other members of the *bcl*-2 family such as *mcl*-1[20], *bak*[21] and *bcl*-x[22] can form heterodimers with Bax and Bcl-2 to promote cell death or survival. So, clarification of the role of Bcl-2 and Bax in the regression of CL of rats may involve further investigations on other members of the *bcl*-2 family.

Treatment with droloxifene 20 mg·Kg^{-1} on d 2 of pseudopregnancy could increase the expression of Bax protein in CL, while the expression of Bcl-2 protein was found to be decreased when the examination was performed on d 8. So, an increased Bax/Bcl-2 ratio could be induced by droloxifene, which might be one of the mechanisms of luteal cells apoptosis facilitated by droloxifene in pseudopregnant rats.

In conclusion, apoptosis of luteal cells occurred during the spontaneous regression of CL in pseudopregnant rats. Droloxifene could facilitate the apoptosis of luteal cells in pseudopregnant rats and shorten the period of pseudopregnancy. The expression of Bax and Bcl-2 protein had no obvious changes during the various days of the pseudopregnancy, while an increased Bax/Bcl-2 ratio may be involved in the facilitation of apoptosis induced by droloxifene in CL of pseudopregnant rats.

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屈洛昔芬对假孕大鼠黄体细胞凋亡和 Bax、Bcl-2 蛋白表达的影响[1]

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关键词 屈洛昔芬; 黄体; 大鼠; 假孕; 凋亡; 蛋白质类

目的: 研究屈洛昔芬对假孕大鼠黄体细胞凋亡和 Bax、Bcl-2 蛋白表达的影响. **方法**: HE 染色观察大鼠卵巢黄体组织学变化,TUNEL 法检测黄体中凋亡细胞的存在,免疫组织化学方法观察假孕大鼠卵巢黄体中 Bax 和 Bcl-2 蛋白的表达. **结果**: 成年假孕大鼠卵巢黄体自然退化中黄体细胞凋亡出现在假孕第 13 天,15 天时可观察到凋亡细胞的显著增加。Bax 和 Bcl-2 蛋白在黄体细胞中的表达在整个黄体自然退化中无显著改变. 20 mg·kg^{-1}假孕大鼠第 2 天口服给予 20 mg·kg^{-1}屈洛昔芬后,第 8 天观察时黄体中出现凋亡细胞,且假孕期从(15.5 ± 1.1)天缩短至(12.8 ± 1.6)天. 经屈洛昔芬作用后,假孕大鼠黄体中 Bax 蛋白的表达增加而 Bcl-2 蛋白表达下降. **结论**: 成年假孕大鼠卵巢黄体自然退化中有细胞凋亡的发生,屈洛昔芬可加速假孕大鼠黄体细胞凋亡的出现并可缩短假孕期. Bax 和 Bcl-2 蛋白的表达在假孕不同时间时无明显改变,而屈洛昔芬所诱导的 Bax/Bcl-2 的增加可能与该药物对黄体细胞凋亡的促进作用有关.

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