ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 May; 20 (5): 400 - 404
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Effects of anordrin, droloxifene, nomegestrol, and mifepristone on cultured rat luteal cell apoptosis

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KEY WORDS corpus luteum; cultured cells; apoptosis; necrosis; flow cytometry; DNA; anordrin; droloxifene; nomegestrol; mifepristone

ABSTRACT

AIM: To study the effect of four kinds of antifertility agents anordrin (Ano), droloxifene (Dro), nomegestrol (Nom), and mifepristone (Mif) on luteal cell apoptosis. **METHODS**: Cultured rat luteal cells were incubated with different agents. HE stain was used to observe morphological changes. Extracted DNA was electrophoresed on agarose gel. Apoptotic cells were quantitated by flow cytometry. **RESULTS**: All 4 drugs reduced cell viability. Dro induced apoptosis while the other 3 drugs induced necrosis. Typical DNA ladders were observed after cells were incubated with Dro and there were 15.4 %, 75.4 %, or 90.5 % apoptotic cells after treatment with Dro 1.25, 2.5, or 3.75 mg·L^{-1}, respectively. **CONCLUSION**: Dro induced apoptosis while Ano, Nom, and Mif induced necrosis in cultured rat luteal cells.

INTRODUCTION

Apoptosis occurs during $PGF_{2\alpha}$-induced or spontaneous regression of corpus luteum (CL)[1-4]. To screen the compounds which induce apoptosis of luteal cells may contribute to the development of new agents of terminating implantation and early pregnancy.

Anordrin (Ano), droloxifene (Dro), nomegestrol (Nom), and mifepristone (Mif) represent 4 types of hormone characteristic compounds. Ano has an estrogenic activity[5] while Dro is an anti-estrogenic agent[6] which has been found to have an anti-implantation effect in our lab recently (unpublished). Nom is a potent progestogen[7] and Mif is an anti-progestogen[8]. All 4 agents have antifertility activities[5,7,8]. In the present study, we investigated whether apoptosis could be induced by these 4 drugs on luteal cells.

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Received 1998-07-05 Accepted 1998-11-24

MATERIALS AND METHODS

Drugs and reagents Mif was manufactured by Zhejiang Xianju Pharmaceutical Co. Ano was manufactured by Shanghai № 19 Pharmaceutical Factory. Dro and Nom were synthesized by Prof XIA Peng (Department of Organic Chemistry, Shanghai Medical University). Collagenase (type Ⅱ) and McCoy's 5A medium were purchased from Sigma Chemical Co.

Rats Sprague Dawley-rats (Grade Ⅱ, ♀, n = 360, 22 - 25 d, 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. Each rat was injected sc pregnant mare serum gonadotropin (PMSG) 65 IU, and 65 h later sc human chorionic gonadotropin (hCG) 35 IU. On d 5 after sc hCG, rats were killed by cervical dislocation for collection of corpora lutea.

Luteal cell culture[9] Both ovaries were excised from pseudopregnant rats. Corpora lutea were separated from ovaries under a dissection microscope and incubated with collagenase (100 kU·L^{-1}) at 37 ℃ for 20 min. Dispersed luteal cells were seeded ($0.5-1$) × 10^5 cells/well into 24-well plates in 0.5 mL of McCoy's 5A medium supplemented with 10 % FBS and were cultured in 5 % CO_2 at 37 ℃ for 24 h. Culture medium was changed into McCoy's 5A medium

containing Ano, Dro, Nom, or Mif. The control group was medium or vehicle (0.2 % Me_2SO, vol/vol). After a 24-h incubation, cell viability was assessed with trypan blue dye exclusion.

HE stain Luteal cells were cultured on 35-mm^2 dishes. After treatment with 4 drugs for 24 h, cells were fixed in 10 % buffered formalin and stained by hematoxylin-eosin. Apoptotic cells were examined under light microscope.

DNA isolation and analysis Luteal cells were incubated with Dro 1.25, 2.5, or 3.75 $mg \cdot L^{-1}$ for 24 h and collected after treatment with trypsin-edetic acid solution. DNA was extracted from cells using a technique modified from others[4]. Briefly, cells were lysed in lysis buffer at 37 ℃ for 1 h and centrifuged at $8500 \times g$ for 10 min. The supernatants were extracted three times with saturated phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and EtOH precipitated. The precipitate was rinsed with 70 % EtOH and resuspended in Tris-edetic acid buffer. Samples were electrophoresed in 2 % agarose gel at 40 V for 3 h. The gels were photographed under UV light.

Assessment of apoptotic cells Luteal cells were collected by treatment with trypsin-edetic acid solution after incubation with Dro 1.25, 2.5, or 3.75 $mg \cdot L^{-1}$. The vehicle was 0.2 % Me_2SO (vol/vol). Having been fixed with citric acid buffer for at least 1 h and incubated with propidium iodide for 15 min, cells were analyzed with an FACS Calibur Flow Cytometer (Becton-Dickinson, San Jose CA). The apoptotic cell number was analyzed using Cellquest and Modfit LT for mac V1.01 (Becton-Dickinson).

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by t test. ED_{50} of cultured cells was calculated by Bliss method.

RESULTS

Viability of rat luteal cells Having been incubated with vehicle (0.2 % Me_2SO vol/vol) for 24 h, the viability of rat luteal cells was unchanged *vs* medium. With the increasing concentration of Ano, Dro, Nom, or Mif, the viability of cells decreased. ED_{50} of the four agents was showed in Tab 1. The inhibitory effect of Dro was the strongest while Nom was the weakest.

Morphological changes Luteal cells incubated with vehicle had no morphological change *vs* medium. After incubation with Dro 0.3625, 0.625, 1.25, or 2.5 $mg \cdot L^{-1}$ for 24 h, cells showed typical apoptosis. The cell size was obviously reduced, cytoplasm shrinked but the plasma membrane was integral. The nuclei were pyknotic and marginated to the periphery of cell membrane which indicated condensation of chromatin. Some nuclei were divided into several pieces and the apoptotic bodies appeared. After treatment with Nom 40 $mg \cdot L^{-1}$, Ano or Mif 2.5 or 10 $mg \cdot L^{-1}$, few cells showed apoptosis, but necrosis was seen in some cells with the morphological changes of swollen cell and collapsed membrane. Intracelluler contents were released. (Fig 1)

Tab 1. Inhibitory effects of anordrin, droloxifene, nomegestrol, and mifepristone on the viability of rat luteal cells *in vitro*.

	ED_{50}/$mg \cdot L^{-1}$	95 % confidence limits/$mg \cdot L^{-1}$
Anordrin	10.9	7.3 – 16.3
Droloxifene	2.9	2.3 – 3.8
Nomegestrol	28.5	17.0 – 47.7
Mifepristone	7.1	4.8 – 10.7

DNA electrophoresis Typical "DNA ladder" representing oligonucleosomes was obviously viewed after incubating luteal cells with Dro 1.25, 2.5, or 3.75 $mg \cdot L^{-1}$. No "DNA ladder" was found in control groups of medium and vehicle. DNA extracted from HL-60 cells after incubation with camptothecine 1 $\mu mol \cdot L^{-1}$ for 4 h was used as positive control. (Fig 2)

Apoptotic cell number The number of apoptotic cells after treatment with Dro 1.25, 2.5, or 3.75 $mg \cdot L^{-1}$ for 24 h was assessed by flow cytometry. There were only 2.3 % and 3.3 % apoptotic cells in medium and vehicle control groups, respectively. But the apoptotic cell number elevated with the increasing concentration of Dro. There were 15.4 %, 75.4 %, or 90.5 % apoptotic cells after treatment with Dro 1.25, 2.5, or 3.75 $mg \cdot L^{-1}$, respectively. The difference between the three Dro groups and the vehicle control group was significant ($P < 0.01$). (Tab 2)

DISCUSSION

Apoptosis is a kind of physiological cell death

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 May; 20 (5)
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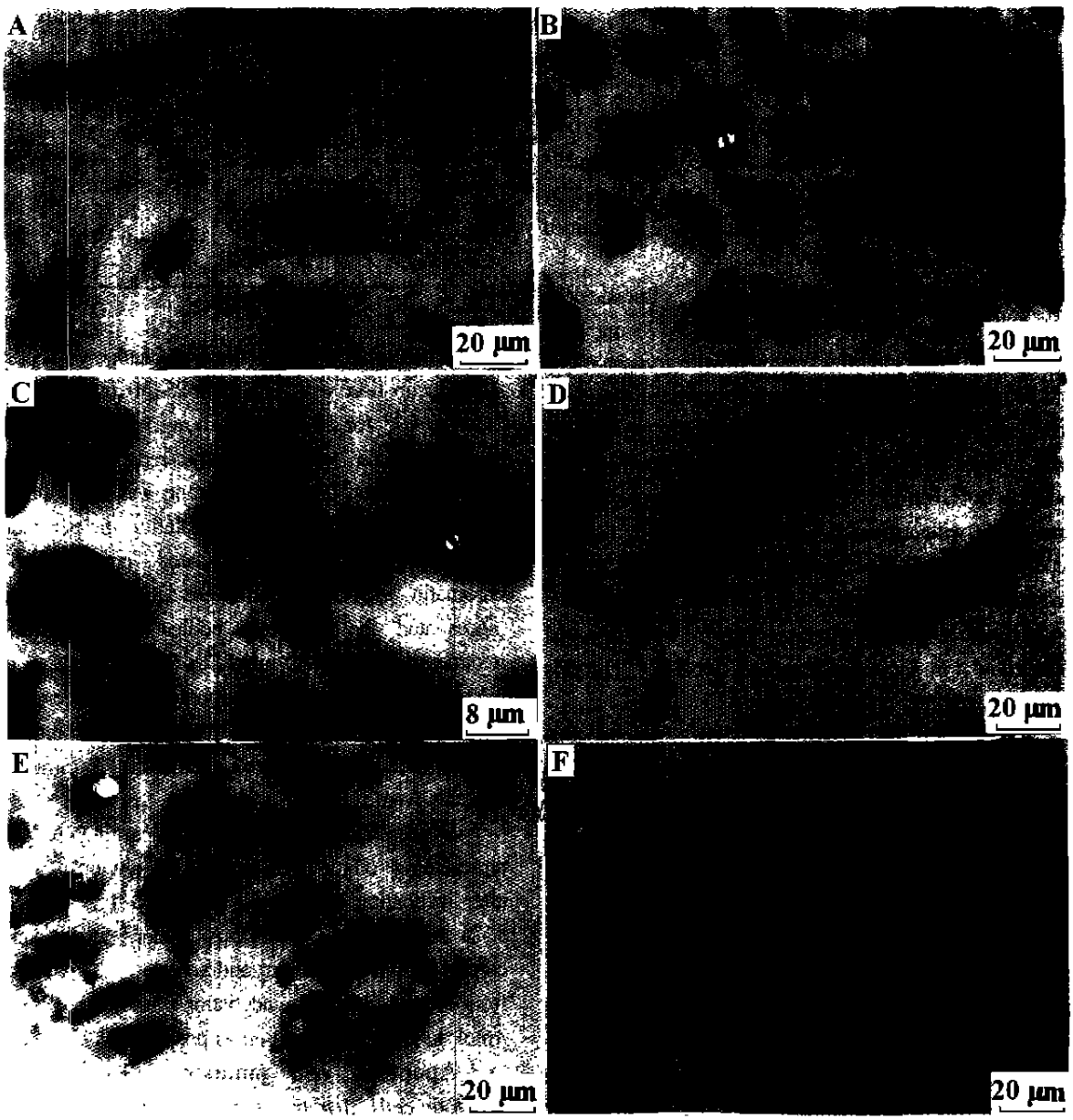

Fig 1. Rat luteal cells after 24-h incubation with vehicle (A), Dro 0.625 mg·L^{-1}(B,C), Nom 40 mg·L^{-1}(D), Ano 2.5 mg·L^{-1}(E), and Mif 10 mg·L^{-1}(F). Many apoptotic cells showed cytoplasm shrinkage and nuclear pyknosis in B and C where the arrows showed apoptotic bodies, and there were some necrotic cells which had swollen cytoplasm and collapsed membrane in D, E, and F. HE stain, A, B, D, E, F×500; C×1250.

which played a complementary but opposite role to mitosis in the regulation of animal cell populations[10]. It is an active cellular process of gene-directed self-destruction and there are obvious morphologic and biochemical differences between apoptosis and necrosis[11]. The important role of apoptosis in luteal regression has been demonstrated in recent years, especially in spontaneous and $PGF_{2\alpha}$-induced luteal regression[1-4].

In the present research, we studied the effect of four kinds of antifertility agents on cultured rat luteal cells. The results showed that each of the four agents

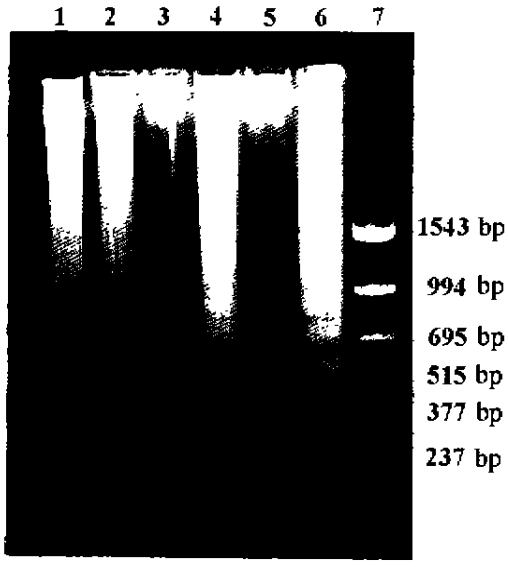

Fig 2. Agarose gel eletrophoresis of DNA isolated from cultured rat luteal cells. Lane 1) droloxifene 2.5 mg·L^{-1}; 2) 1.25 mg·L^{-1}; 3) medium; 4) droloxifene 3.75 mg·L^{-1}; 5) Me_2SO; 6) camptothecine 1 μmol·L^{-1}; 7) DNA mark.

Tab 2. Apoptotic cells after incubation with droloxifene for 24 h. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* vehicle.

		Apoptotic cell percentage/ %
Medium control		2.3 ± 1.8[a]
Vehicle control		3.3 ± 2.9
Droloxifene	1.25 mg·L^{-1}	15.4 ± 4.9[c]
	2.50 mg·L^{-1}	75.4 ± 2.0[c]
	3.75 mg·L^{-1}	90.5 ± 1.1[c]

reduced cell viability and the viable cell number declined with the increasing concentration. But not all of them caused cell death through inducing apoptosis. HE stain showed that only cells treated with Dro exhibited apoptotic morphologic characters while the cells exposed to other three agents showed necrosis. Besides the morphologic changes, a most striking feature of apoptosis is the activation of Ca^{2+}/Mg^{2+}-dependent endonuclease, which specifically cleaves cellular DNA between regularly spaced nucleosomal units and produces DNA fragment in size multiples of 185 - 200 bp[12]. Separated by agarose gel electrophoresis, a distinct ladder of DNA bands were visualized. In the present research, the presence of "DNA ladder" in agarose gel could be detected in cells treated with various concentration of Dro, and there was no "DNA ladder" in control groups. The quantitative analysis of apoptotic cell number by flow cytometry showed that the apoptotic cell number increased with the increasing concentration of Dro. So, all of those results demonstrated that Dro could induce apoptosis in rat luteal cells *in vitro*. But the relationship between the apoptosis induced by Dro and its anti-estrogenic effect is not clear, and more study should be done to illustrate the physiologic and pharmacologic significance of the apoptosis induced by Dro. In conclusion, Dro, Ano, Nom, and Mif have different effects on rat luteal cells *in vitro*. Dro induced apoptosis of cells while the other three agents induced necrosis.

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400 – 404

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 May; 20 (5)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

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双炔失碳酯、屈洛昔芬、诺美孕酮及米非司酮对离体培养大鼠黄体细胞凋亡的影响

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关键词 黄体; 培养的细胞; 凋亡; 坏死; 流动血细胞记数; 脱氧核糖核酸; 双炔失碳酯; 屈洛昔芬; 诺美孕酮; 米非司酮

目的: 研究双炔失碳酯、屈洛昔芬、诺美孕酮和米非司酮是否可诱导离体培养的大鼠黄体细胞凋亡. **方法**: 离体培养的大鼠黄体细胞经各种药物作用后 HE 染色, 观察其形态学变化. 抽提细胞 DNA 进行琼脂糖凝胶电泳. 流式细胞仪定量分析凋亡细胞数. **结果**: 四种药物均降低细胞存活率, 屈洛昔芬诱导细胞凋亡, 其它三种药物引起细胞坏死. 细胞经屈洛昔芬作用后发生 DNA 断裂, 电泳后显示典型的 "DNA ladder", 屈洛昔芬 1.25, 2.5 或 3.75 mg·L^{-1}分别引起15.4 %, 75.4 %或 90.5 %的细胞凋亡. **结论**: 屈洛昔芬诱导离体培养的大鼠黄体细胞凋亡, 而双炔失碳酯、诺美孕酮和米非司酮诱导细胞坏死.

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