Int J Clin Exp Pathol 2013;6(8):1603-1609
www.ijcep.com /ISSN:1936-2625/IJCEP1305026

Original Article

In vitro apoptosis effects of GnRHII on endometrial stromal cells from patients with endometriosis

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Received May 18, 2013; Accepted June 17, 2013; Epub July 15, 2013; Published August 1, 2013

Abstract: Purpose: To study the effect of gonadotropin-releasing hormone II (GnRHII) on the cell apoptosis of ectopic, eutopic and normal endometrial stromal cells cultured in vitro from endometriosis patients, and to provide theoretical basis for exploring new treatments for endometriosis (EMs). Methods: Ectopic, eutopic and normal endometrial stromal cells were isolated, cultured and identified in vitro, then treated with different concentrations of GnRHII (0, 10^{-10} M, 10^{-8} M and 10^{-6} M). Cell apoptosis was detected by Hoechst staining and flow cytometry. Results: GnRHII increased apoptosis in ectopic, eutopic and normal stromal cells in a dosage-dependent manner ($P<0.05$), and apoptosis of ectopic stroma cells was significantly higher than that of eutopic and normal cells ($P<0.05$); apoptosis in eutopic and normal cells had no different ($P>0.05$). Conclusion: GnRHII can significantly induce apoptosis in ectopic, eutopic and normal endometrial stromal cells from patients with endometriosis, especially to the ectopic.

Keywords: Endometriosis (EMs), gonadotropin-releasing hormone II (GnRHII), apoptosis, endometrial stromal cell (ESC), cell culture, in vitro

Introduction

Endometriosis (EMs) has become the research hotspot throughout the world due to its unclear pathogenesis, high incident and recurrent rates, and its difficulty of treatment [1-3]. Since GnRHa (gonadotropin-releasing hormone agonist) was used for EMs treatment in 1986 for the first time, in-depth studies on the mechanism of GnRHa in treating EMs have been carried out [4-6]. The proliferation rate of ectopic stromal cells from patients with EMs increased significantly while the apoptosis rate significantly decreased. GnRHa Leuprorelin could inhibit the proliferation of in vitro cultured eutopic stroma cells of EMs and promoted cell apoptosis, mediated by increased expression of the apoptosis-accelerating proteins Bax and FasL, and decreased expression of apoptosis-depressing protein Bcl-2 [7].

By virtue of its apoptosis-accelerating and proliferation-inhibiting effects, GnRHa is an effective drug for EMs treatment and is widely used in clinical at present. GnRHII is a recently discovered, another form of GnRH, which may also be the earliest form of GnRH in terms of its evolutionary history. The results of many studies on the application of GnRHII in tumor treatment have been published. GnRHII has a stronger anti-proliferative effect on oophoroma and endometrial carcinoma [8-10]. Endometriosis has similar characteristics of tumor implant, metastasis and recurrence. However, little is known about that GnRHII directly regulate apoptotic effect on endometriosis stromal cell in vitro?

Materials and methods

Materials

The source of ectopic, eutopic and normal endometrium: From June 2009 to September 2010, a total of 30 patients with endometriosis and 16 without endometriosis were included in this study and all patients had laparoscopic surgery. Formalin fixed tissue for histology pathology diagnosis and fresh tissue for cell culture were collected, the ectopic and eutopic endo-

metrium were from patients with endometriosis and normal endometrium was from benign ovarian cyst. The average age of endometriosis patients was 31.20±6.13 years (ranging from 23 to 47), the control group was 32.77±9.25 years (ranging from 21 to 49), there was no statistically significant difference between two groups' age ($p>0.5$). None of the patients received any hormonal therapy within 6 months before surgery. All samples were collected with patients informed consent and approval from the local ethics committee of the Second Xiangya Hospital, Central South University, Changsha, China.

Main reagents and instrumentation: GnRHII was obtained from Bachem (Switzerland), DMEM/F12 was from GIBCO (USA), IV-type collagenase and progesterone were from Sigma (USA), trypsin was from Amresco Inc. (USA), anti-human monoclonal antibodies against vimentin, keratin prolactin mouse were from Wuhan Boster (China), Hoechst 33258 staining kit was from Shanghai Biyuntian (China). The FACS Caliber flow cytometer was manufactured by Becton Dickinson (USA).

Methods

Isolation, culture, and identification of endometrial stromal cells: Surgical procedures were carried out in our hospital. Cell was cultured by the following steps described previously [11]: Endometriotic tissue was dissected after rinsing, then digested 2-3 h, until the tissue disappeared by adding 0.1% of type IV collagenase solution and 0.25% trypsin digestion (pH 7.4), at 37°C, and then 100 μm and 38 μm Strainer filtrated and isolated cells, then centrifuged (800 rpm) 5 min, removed the supernatant, added DMEM/F12 medium (containing 10% newborn bovine serum), finally cell morphology and growth conditions were observed under inverted microscope, 10^4/mL was vaccinated into 25 cm^2 cell culture plastic bottle, 37°C, 5% CO_2 incubator, semis replacement of medium after 2-3 d, till cell fusion, the culture of primary cells were completed. Identification of cultured cells using vimentin, keratin, and prolactin (PRL), due to PRL was produced only by ESC in non-pregnant but not glands and fibroblasts, so PRL was used to identify the ESC. We identified cells directly in 6-well cell culture plates, passages of cell adhesion, then replacement of medium, plus 10^{-8} mol/L progesterone stimulating 6 d. The ESCs were identified according to kit instructions by immunocytochemical ABC method.

Intervention group of endometrial stromal cells

The ectopic, eutopic and normal endometrial stromal cells were cultured to the third generations, and then harvested at 80% confluence by digestion in 0.1% trypsin containing 0.01% EDTA, and centrifugation at 800 rpm for 3 min. The supernatant was removed, and fresh DMEM/F12 medium containing 10% newborn calf serum was added, and the cells were resuspended. Then the cells were counted and seeded into 48-well culture plates at a density of 2×10^5 for each well. When the cells were close to 80% confluence, 0.5 ml of medium containing GnRHII at 10^{-10}, 10^{-8}, or 10^{-6} mol/L was added to each well. For a control group, only 0.5 mL/well of DMEM/F12 medium containing 2.5% newborn calf serum was added. After culturing for 24 h or 48 h, the culture medium was collected and stored at -20°C for subsequent assay of apoptosis.

Hoechst staining

After culturing for 24 h, 1 ml of Hoechst 33258 staining solution (at a concentration of 10 μg/ml) was added, and incubated at room temperature for 3-5 min. Staining solution was removed and then cells were washed with PBS. Anti-fluorescent mounting liquid was added and the cells were then observed under fluorescent microscope. The apoptotic rate was calculated as follows: 400 cells were randomly counted under optical microscope at 200× magnification and the apoptotic rate was expressed as apoptotic cell number/total cell numbers ×100%. A mean value was obtained from three parallel wells.

Flow cytometry

After washing cultured cells three times with PBS solution, the supernatants were collected. The remaining adherent cells were digested with 0.25% trypsin solution, and were added together to the previously collected supernatants, and were centrifugated at 800 rpm for 5 min. The supernatant fluid was discarded, then PBS was added for washing. The above steps were repeated once. Pre-cooled 70% ethanol

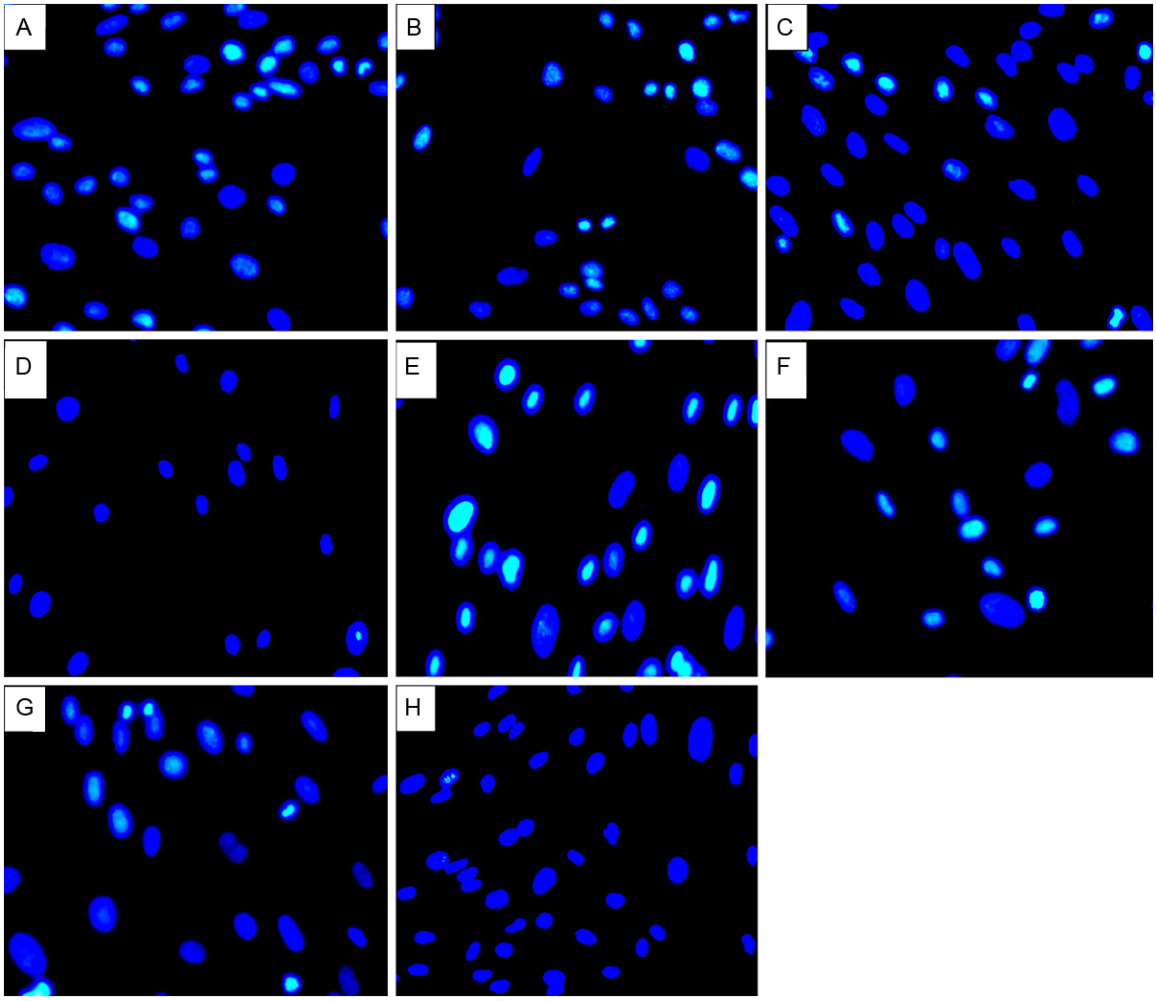

Figure 1. Pictures of the apoptosis of eutopic and ectopic endometrail stroma cells (ESC) induced by GnRHII at different concentrations in vitro by Hoechst staining (×200). A: Eutopic ESC treated with 10^{-6} M GnRHII; B: Eutopic ESC treated with 10^{-8} M GnRHII; C: Eutopic ESC treated with 10^{-10} M GnRHII; D: Negative control: eutopic ESC; E: Ectopic ESC treated with 10^{-6} M GnRHII; F: Ectopic ESC treated with 10^{-8} M GnRHII; G: Ectopic ESC treated with 10^{-10} M GnRHII; H: Negative control: ectopic ESC.

(4°C) solution was added and gently, evenly mixed, and allowed to fix at 4°C for 24-72 h. Cells were used for flow cytometry analysis. The apoptosis rate AI (%) = hypodiploid apoptotic cells/total cells ×100%, with the mean value of 3 wells.

Statistical analysis

SPSS™ 16.0 Software was used statistical analysis. Values are expressed as mean ±standard deviation ($\overline{\chi}$±S). The rate of apoptosis use two-factor variance analysis. The least significant difference method (LSD) was used for comparisons between groups. $P<0.05$ was considered statistically significant.

Results

The endometrial stromal cells were identified by immunocytochemical SABC staining according to criteria: i) vimentin positive; ii) keratin negative; and iii) and prolactin (PRL) positive.

Endometrial stroma cells were treated with GnRHII at different concentrations (0, 10^{-10}, 10^{-8} and 10^{-6} M), and dye with Hoechst 33258 was used for analysis of apoptotic morphology in the different groups. The results showed that the apoptosis of ectopic, eutopic and normal ESC occurred: karyorrhexis, karyolysis, karyopyknosis, or even with the formation of apoptotic bodies (**Figure 1A-H**). Which confirmed

Table 1. Comparisons of the apoptosis rates among different groups by Hoechst staining ($\overline{\chi}$±S)%

	Concentration of GnRHII			
	0 M	10^{-10} M	10^{-8} M	10^{-6} M
Eutopic cells	5.78±0.53$^{\Delta}$	18.62±2.59$^{*,\Delta}$	47.41±3.57$^{*,\Delta}$	66.16±6.46$^{*,\Delta}$
Ectopic cells	5.79±0.66	31.30±2.93$^{*,\blacktriangle}$	59.28±4.25$^{*,\blacktriangle}$	79.43±6.42$^{*,\blacktriangle}$
Normal cells	5.83±0.56	19.17±4.37*	46.77±3.48*	65.89±5.99*

Notes: With the increase of GnRHII concentration, the apoptosis rates of stroma cells were increased, in a dose-depenent manner in each group, $^{*}P<0.05$. GnRHII at the same concentration had a stronger apoptosis-inducing effect on ectopic stroma cells than that on eutopic and normal stroma cells, $^{\blacktriangle}P<0.05$. There is no difference between eutopic and normal stroma cells, $^{\Delta}P>0.05$.

Table 2. Comparisons of the apoptosis rates among different groups by flow cytometry ($\overline{\chi}$±S)%

	Concentrations of GnRHII			
	0 M	10^{-10} M	10^{-8} M	10^{-6} M
Eutopic cells	5.87±0.59$^{\Delta}$	19.30±3.28$^{*,\Delta}$	46.30±5.32$^{*,\Delta}$	69.70±8.77$^{*,\Delta}$
Ectopic cells	5.99±0.78	33.40±3.97$^{*,\blacktriangle}$	62.41±6.74$^{*,\blacktriangle}$	83.30±9.42$^{*,\blacktriangle}$
Normal cells	5.76±0.64	18.97±3.79*	45.66±4.62*	68.89±6.11*

Notes: With the increase of GnRHII concentration, the apoptosis rates of stroma cells were increased in a dose-depenent manner in each group, $^{*}P<0.05$. GnRHII at the same concentration had a stronger apoptosis-inducing effect on ectopic stroma cells than that on eutopic and normal stroma cells, $^{\blacktriangle}P<0.05$. There are no difference between eutopic and normal stroma cells, $^{\Delta}P>0.05$.

that GnRHII could induce the apoptosis of endometrial stroma cells in vitro. The apoptosis rates (%) of ectopic, eutopic and normal cells are in **Table 1**. The differences between ectopic and eutopic groups were statistically significance, the ectopic was highest ($p<0.05$). while eutopic and normal groups were not difference ($p>0.05$). Furthermore, with the increase of GnRHII concentration (0, 10^{-10} M, 10^{-8} M and 10^{-6} M), the apoptotic rate was increasing, and with a dose-dependence manner in each group ($p<0.05$) (**Table 1**).

Flow cytometric assay of apoptosis in different groups gave the similar results as the Hoechst 33258 staining. These results were in **Table 2** and **Figure 2A-D**. The apoptosis rate had a statistically significance difference among three groups with flow cytometry test, the ectopic was higher than the other two groups ($p<0.05$), while the eutopic and normal groups had no difference ($p>0.05$). What is more, with the increase of GnRHII concentration (0, 10^{-10} M, 10^{-8} M and 10^{-6} M), the apoptotic rate was increasing, and with a dose-dependence manner in each group ($p<0.05$) (**Table 2**).

Discussion

From the discovery of gonadotropin-releasing hormone GnRHI (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), scientists have isolated 22 variants of GnRH from vertebrates. They are divided into 3 types (according to binding to their receptors): GnRHI, II and III. GnRHIII has been found only in the forebrain terminal nerves of bony fishes.

GnRHII, pGlu-His-Trp-Ser-*His*-Gly-*Trp-Tyr*-Pro-Gly-NH_2 (the black italics differ from GnRHI), is also called chicken GnRH, and was isolated from chicken brains in 1984. The sequence of GnRHII is complete conserved from bony fish to human, through 500 million years of evolution, and is regarded as the earliest forms of GnRH [12].

GnRHa (that is GnRHI), because of its effective apoptosis-inducing and proliferation-inhibiting effects on endometrial stroma cells, has become the ideal drug for treatment of endometriosis at present. GnRHa has been reported to inhibit proliferation of eutopic and ectopic endometrial stroma cells in patients with endometriosis; promote their apoptosis; and decrease the expression of VEGF (vascular endothelial growth factor), all of which contribute to the effectiveness of endometriosis treatment [13, 14]. Results obtained from in vitro culture have proved that GnRHa Leuprorelin can inhibit eutopic endometrial stroma cells in patients with endometriosis and promote apoptosis of these cells. The mechanism might be associated with its up-regulation of Bax and

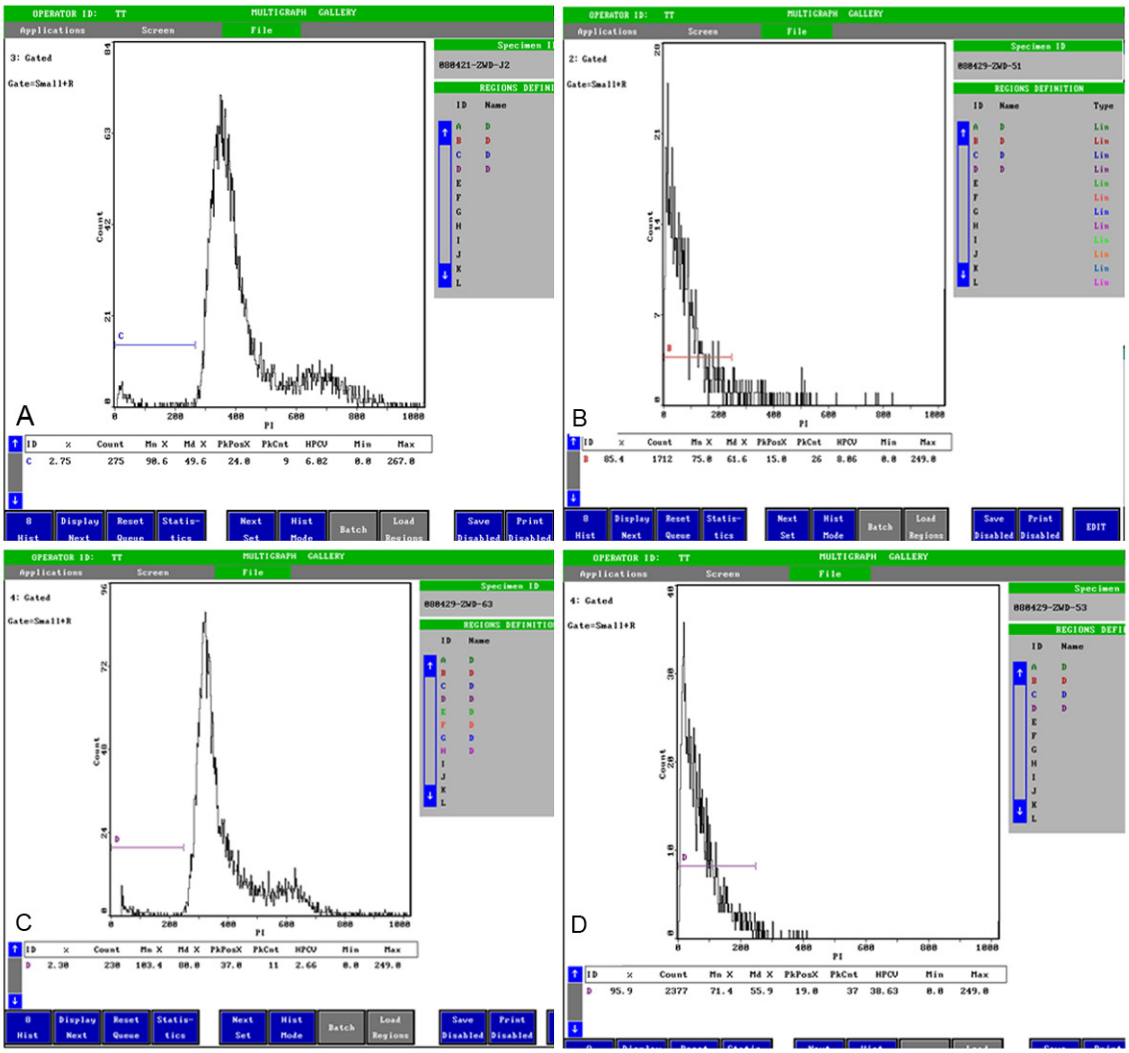

Figure 2. Diagrams of the apoptosis of eutopic and ectopic endometrial stroma cells of EMs after being treated with GnRHII in vitro by flow cytometry. A: Negative control: the apoptosis of eutopic endometrial stroma cells in vitro; B: An obvious hypodiploid apoptotic apex of eutopic endometrial stroma cells in vitro could be seen after application of 10^{-6} M GnRHII; C: Negative control: the apoptosis of ectopic endometrial stroma cells in vitro; D: An obvious hypodiploid apoptotic apex of ectopic endometrial stroma cells in vitro could be seen after application of 10^{-6} M GnRHII.

FasL and down-regulation of Bcl-2 [15]. GnRHI (100 ng/ml) has been shown to promote apoptosis of endometrial stroma cells from both endometriosis patients and normal controls, while antide (10^{-7} M) could block such an effect [16]. In a study of the different effects of GnRHa, LING21US (Levonorgestrel-releasing intrauterine system) and MPA (medroxyprogesterone acetate) on the apoptosis of endometrial stroma cells (using transmission electron microscopy, TEM), GnRHa had the most strongest effect among the three drugs [17].

GnRHII receptor mRNA was expressed in the tissues of ovarian cancer and endometrial carcinoma; GnRHII had a greater inhibitory effect on cell proliferation on these tumor cells than any other GnRHI agonist effect. GnRHII also displayed an inhibitory effect on the proliferation of SK-OV-3 cells (an endometrial carcinoma cell line, which is positive for GnRHII receptors but negative for GnRHI receptors) while GnRHI agonist (Triptorelin) had no such an effect [8]. Both GnRHI and GnRHII exert their effects via binding to GnRHI receptors and GnRHII receptors in marmosets. However, they have different degrees of affinities [18]: GnRHI binds to GnRHI receptor with 48 times higher affinity than GnRHII binds to GnRHI receptor, while GnRHII binds to GnRHII receptor with 421 times higher

affinity than GnRHI binds to GnRHII receptor. This advantage in binding affinity indicates that GnRHII might have much better treatment effect than GnRHI [19], and explains why, in recent years, GnRHII has become the hotspot of study.

The anti-tumor effect of GnRHII antagonist was confirmed in rats: GnRHII could significantly inhibit the growth of human endometrial carcinoma and oophoroma in rats, with no apparent side effects. These results indicate that, similar to GnRHI antagonist, GnRHII antagonist have anti-tumor effect both in vivo and in vitro [20].

What is more, the expression of GnRHII mRNA in patients with endometriosis (eutopic and ectopic) was lower than that in normal endometrial stroma cells, regardless of proliferative or secretary phase. And GnRHII could effectively inhibit the secretion of IL-8 protein and the expression of COX-2 mRNA and IL-8 mRNA in endometriosis cells, indicated that GnRHII has an anti-proliferative and anti-inflammatory effects on endometriosis cells, and that the decrease of endogenous GnRHII expression can lead to the initiation and development of endometriosis [21].

In this study, endometrial stroma cells were successfully cultured and identified by using an improved method. In vitro treatment of these cultured cells with GnRHII at different concentrations resulted in the apoptosis of normal, eutopic and ectopic endometrial stroma cells, occurring in the forms of karyorrhexis, karyolysis, karyopyknosis and apoptotic body formation. Hoechst staining confirmed morphologically that GnRHII could induce the apoptosis of in vitro cultured endometrial stroma cells, in a dose-dependent manner ($P<0.05$). The rates of apoptosis among three endometrial stroma cells exhibited significant differences, the ectopic was the steongest ($P<0.05$), indicating that GnRHII has a better apoptosis-inducing effect on ectopic cells than that on eutopic and normal cells. In addition, flow cytometry was used to detect cell apoptosis in our study, and showed similar results, which further validated our observation. Leuprorelin (GnRHIa) could promote the apoptosis of eutopic endometrial stroma cells in patients with endometriosis, and such an effect could be cancelled out by the GnRHI antagonist antide at 10^{-7} M. Like the above studies, our results suggest that GnRHII can directly promote the apoptosis of endometrial stroma cells in vitro, and in a dosage-dependent, especially to the ectopic. Which provide theoretical and experimental basis for exploring new treatments for endometriosis (EMs).

Acknowledgment

This work is granted by the health department of Hunan province (2010-016) and Hunan Province Natural Science Foundation Project (12jj3100).

Disclosure of conflict of interest

None.

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