RRPC89020080 (12P)

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※
※細胞凋亡和 Bcl-2 family 在 GnRHa 治療子宮內膜異位※
※之不孕症婦女的顆粒細胞與異位子宮內膜表現之研究※
※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：■個別型計畫　□整合型計畫

計畫編號：NSC89－2314－B－038－031－

執行期間：88 年 08 月 01 日至 89 年 07 月 31 日

計畫主持人：曾 啟 瑞

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：台北醫學院 婦產科

中　華　民　國　89 年　09 月　　　日

行政院國家科學委員會專題研究計畫成果報告

國科會專題研究計畫成果報告撰寫格式說明

Preparation of NSC Project Reports

計畫編號：NSC 89-2314-B-038-031-

執行期限：88 年 08 月 01 日至 89 年 07 月 31 日

主持人：曾啟瑞　　台北醫學院 附設醫院 婦產科

共同主持人：XXXXXX　執行機構及單位名稱

計畫參與人員：陳惠文　台北醫學院 附設醫院 婦產科

一、中文摘要

細胞凋亡在胚胎發育、組織更新、荷爾蒙造成的組織萎縮、和許多病理狀態下都扮演相當重要的角色，之前的研究報告指出 GnRHa 會造成大鼠卵巢內顆粒細胞的凋亡，但對於其在人類 IVF-ET 過程中所造成細胞凋亡的影響卻鮮少被研究。因此、在本研究計劃中我們經由測定子宮內膜異位病患，在 IVF 過程中接受長效型 GnRHa 治療後，其顆粒細胞凋亡的比例，以及測定其中與凋亡相關之因子(BCl-2, Bax, MCl-1)的表現，並研究與子宮內膜異位之相關性，期望借此可評估其卵子及胚胎的數量與品質。

關鍵詞：細胞凋亡、顆粒細胞、子宮內膜異位、GnRHa、IVF-ET、BCl-2、Bax、Mcl-1

Abstract

Apoptosis is important during embryonic development, tissue renewal, hormone-induced issue atrophy, and many pathological conditions. GnRHa had been reported to induce apoptosis in the granulosa cells of the rat ovary. There have been few reports concerning apoptosis in human IVF-ET programs until recently. In the present study, through measuring apoptosis and apoptotic-related proteins (Bcl-2, Bax, and Mcl-1) in cumulus granulosa cells following long acting GnRHa treatment in the patients with endometriosis in IVF program, the incidence of apoptosis of granulosa cells might be a marker to predict the outcome of oocyte and embryo quality.

Keywords: Apoptosis; Bax; Bcl-2; Endometriosis; GnRH analogue; Granulosa cells; IVF-ET; Mcl-1.

二、Introduction

Apoptosis (or programmed cell death) is important during embryo development, metamorphosis, tissue renewal, hormone-induced tissue atrophy, and many pathological conditions. It was reported that apoptosis was found in the ovary not only during the natural cycles, but also during the gonadotropin-stimulated cycles (1). Gonadotropins such as hMG and/or FSH have been used to obtain multiple oocytes for in vitro fertilization (IVF). Moreover, gonadotropin-releasing hormone analogues (GnRHa) which induced pituitary desensitization, recently the long acting GnRHa had been included in the treatment of endometriosis and increased clinical pregnancy rates. GnRHa had been reported to induce apoptosis in the granulosa cells of the rat ovary whereas hMG and FSH block apoptosis (2). The direct ovarian effects of GnRHa are profound in rodents as compared with humans. There have been few reports concerning apoptosis in human IVF-ET programs until recently (3). Prior studies had shown that the incidence of apoptotic bodies in membrana granulosa cells of patients with endometriosis was significantly higher than that of the control group which without endometriosis and the apoptotic cells increased as the stage of the revised AFS classification advanced (3-4). Bcl-2-related anti- and proapoptotic proteins are important in the decision step of the intracellular death program upstream from the

caspases. Targeted overexpression of Bcl-2 in ovarian somatic cells of transgenic mice leads to decreased apoptosis of granulosa cells and is associated with higher ovulation rates (5). In the present study, through measuring apoptosis and apoptotic-related proteins (Bcl-2, Bax, and Mcl-1) in cGCs following GnRHa treatment in the patients with endometriosis, the incidence of apoptosis of cGCs might be a marker to predict the outcome of ovulation process, oocyte quality, and further cleavage rate.

三, MATERIALS & METHODS

Patients and Granulosa Cells Collection A case-control study enrolled 8 women with GnRHa-treated endometriosis and 4 control (male factor infertility patients) undergoing IVF. The cGCs were collected after follicular aspiration, oocyte isolation, and cumulus separating process.

Detection of Apoptosis Detection of apoptotic nuclei was accomplished by in situ nuclear labelling with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) reaction (Boehringer Mannheim, Laval, PQ, Canada). cGCs were fixed in 3.7 % paraformaldehyde solution in PBS for 30 min, rinsed twice in phosphate buffered saline (PBS), and permeated with 0.1 % Triton-X 100 in 0.1 % sodium citrate for 2 min on ice. cGCs were incubated in 50 μl TUNEL reaction mixture or in 50 μl TUNEL label alone as a negative control for 60 min at 37 °C in a humidified atmosphere. Positive controls were incubated with 50 U/ml RNase-free DNase (Promega, Madison, WI) for 20 min at 37 °C before exposure to the TUNEL reaction mixture. cGCs were rinsed twice in PBS and examined with the fluorescent microscope.

Immunohistochemistry The expression of Bcl-2, Bcl-$x_{S/L}$, Bax, and Mcl-1 protein products in cGCs were examined by immunohistochemistry with specific antibodies. cGCs were fixed in 3.7 % paraformaldehyde solution in PBS for 30 min, rinsed twice in PBS, and permeated with 0.1 % Triton X-100/PBS for 10 min. cGCs were blocked with PBS containing 3 % goat serum for 30 min and then incubated for 4 h at room temperature with 1:200 dilution of either the primary antibodies of Bcl-2, Bax, and Mcl-1 (Santa Cruz, Biotechnol Inc., CA) in a humidified chamber. After washes with PBS, the cGCs were incubated for 1 h with the secondary antibody, biotinylated anti-rabbit-IgG or anti-mouse-IgG (Santa Cruz, Biotechnol Inc., CA) and then incubated with ABC mixture (Victor,) for 1.5 h. The cells were washed again with PBS and the antigen-antibody complexs were visulized by immersion in a DAB solution. The cell nuclei were then counterstained with haematoxyllin, dehydrated in graded ethanol baths and xylene, and mounted with a coverslip for light microscope. Negative controls were processed in the same manner but were not incubated with the primary antibody. All antibodies have been previously characterized through immunocytochemistry and western blot analysis as specific for Bcl-2, Bax or Mcl-1 (Santa Cruz, Technical notes).

Statistical Analysis Data are presented as mean ± SEM. A Student's t test was used to evaluate statistical significance of differences between two paired observations. A value of $P < 0.05$ was considered statistically significant.

RESULTS

A typical apoptotic bodies of cGCs, which included fragmented and shrunken nuclei stained with the TUNEL technique, was observed under a fluorescence microscope (Fig.1a). The GnRHa-treated endometriosis showed more TUNEL positive stained cells (Fig. 1b), than the control group (Fig. 1c). Table 1 summerized the incidence of apoptotic cells and apoptotic bodies in the patients with GnRHa treated endometriosis and compared with the control cases. The results indicated that GnRHa-treated cases had higher incidence of apoptotic cells and apoptotic bodies in cGCs than the control group ($P<0.05$). We also found that the patients with higher incidence of apoptotic cGCs had smaller number of harvested oocyte and matured oocytes ($P<0.05$), but no significantly influence in the embryo development ($P>0.05$).

The expression of Bax and Mcl-1 were higher than the Bcl-2 in the cytoplasm and nuclei of cGCs indicated that the balance of

these proteins might be involved in the mechanism of apoptosis in cGCs (Fig.2).

CONCLUSIONS

The TUNEL technique provide more specific, sensitive, and objective way to examine the apoptotic cells and apoptotic bodies formation in GCs than the Hoechst 33258 staining (3). Results indicated that GnRHa-treated endometriosis might increase the rates of apoptosis in GCs and showed no significant change in embryo development and pregnancy rate compared with the control group. The results suggested that the treatment of endometriosis with GnRHa before the induction of ovulation would modify the apoptosis in GCs, decrease oocyte number, but still retain the same cleavage rates.

In this report, using immunocytochemical staining, we have shown for the first time the distribution of Bcl-2, Bax, and Mcl-1 in cGCs from patients with endometriosis undergoing IVF-ET program. The results indicated that Bax and Mcl-1 were highly expressed in the cGCs, suggested that the reguation of these proapoptotic or anti-apoptotic protein might be involved in the apoptotsis of cGCs.

REFERENCES

(1). PALUMBO A., YEH J. In situ localization of apoptosis in the rat ovary during follicle atresia. Biology Reproduction 51; 888-895, 1994. (2). BILLIG H., FURUTA I., HSUEH AJ. Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of deoxyribonucleic acid fragmentation in granulosa cells. Endocrinology 134; 245-252, 1994. (3). NAKHARA K., SAITO H., SAITO T., ITO M., OHTA N., TAKAHASHI T., HIROI M. The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. Fertility & Sterility 68(2); 312-317, 1997. (4). NAKHARA K., SAITO H., SAITO T., ITO M., OHTA N., TAKAHASHI T., HIROI M. Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. Fertility & Sterility 69(5); 931-935, 1998. (5). KAIPIA A., HSU SY., HSUEH AJ. Expression and function of a proapoptotic Bcl-2 family member Bcl-XL/Bcl-2-associated death promoter (BAD) in rat ovary. Endocrinology 138(12); 5497-5504, 1997.

ILLUSTRATIONS

Table 1.

	N	Age	Apoptotic cells (%)	Apoptotic bodies (%)	Oocyte number	Cleavage rates (%)
Control	4	33 ± 2	4.1 ± 0.9	0.7 ± 0.1	12.0 ± 2.0	60.9 ± 2.2
GnRHa-treated Endometriosis	8	33± 4	13.6 ± 2.4*	1.6 ± 0.3*	5.4 ± 1.1*	78.3 ± 7.4
Significance by student t-test		*P*=0.61	**P*=0.01	**P*=0.02	**P*=0.004	*P*=0.12

Each data represents the mean ± s.e.mean. *: A value of $P < 0.05$ was considered statistically significant.

Figure 1. Fluorescence micrograph of fragmented nuclei of apoptotic cells and apoptotic bodies stained with TUNEL. (A) cGCs from the control group also stained with TUNEL and (B) Fluorescence micrograph of cGCs collected from the patients with GnRHa treated endometriosis and following IVF-ET .

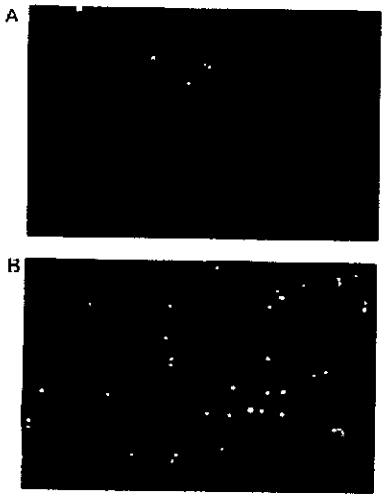
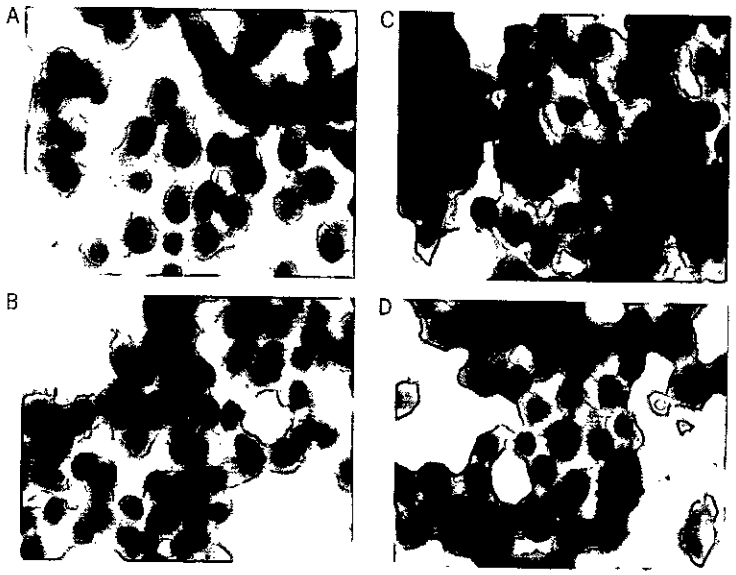


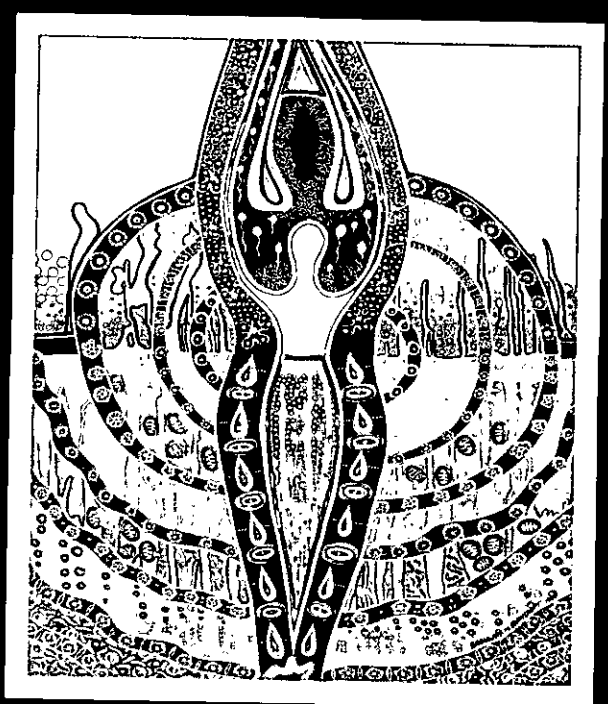
Figure 2. cGCs were stained with specific antibodies. (a) negative control; (b) Bcl-2; (c) Bax; and (d) Mcl-1.



REPRINTED FROM:

11th World Congress on IN VITRO FERTILIZATION HUMAN REPRODUCTIVE GENETICS

Sydney (Australia), May 9-14, 1999



MONDUZZI EDITORE

International Proceedings Division

11th World Congress on
In Vitro Fertilization & Human Reproductive Genetics

Increased Apoptosis in Granulosa Cells Following GnRHa-Treatment in Patients with Endometriosis

Tzeng Chii-Ruey, Chen Huei-Wen, Jiang Wen-Sheng, Wu Ko-Yi, Chang Shu-Ju, Wen J.Y. and Au Heng-Kien

Center for Reproductive Medicine, Department of Obs and Gyn
Taipei Medical College, Taipei, Taiwan

Summary

Using TdT-mediated dUTP nick end labelling (TUNEL) technique, we can evaluate the apoptosis of cGCs on cases of endometriosis with GnRH analog (GnRHa) treatment, and compared with the control cases. Results showed that the GnRHa-treated endometriosis had higher incidence of apoptotic cells and apoptotic bodies in cGCs than the control group ($P<0.05$). The patients with higher incidence of apoptosis in cGCs had less number of harvested oocytes and matured oocytes ($P<0.05$), but no influence in the embryo development ($P>0.05$). Additionally, the apoptotic-related proteins, Bax and Mcl-1, were highly expressed in the cGCs treated with GnRHa, but Bcl-2 had less expression levels in the cGCs. The results indicated that Bax and Mcl-1 might play the role in the mechanism of apoptosis in cGCs, and the treatment of endometriosis with GnRHa before the induction of ovulation would modify the apoptosis in cGCs, influence the oocyte number, but still retain the same cleavage rates of embryos.

Introduction

Apoptosis (or programmed cell death) is important during embryo development, metamorphosis, tissue renewal, hormone-induced tissue atrophy, and many pathological conditions. It was reported that apoptosis was found in the ovary not only during the natural cycles, but also during the gonadotropin-stimulated cycles (1). Gonadotropins such as hMG and/or FSH

have been used to obtain multiple oocytes for in vitro fertilization (IVF). Moreover, gonadotropin-releasing hormone analogues (GnRHa) which induced pituitary desensitization, recently the long acting GnRHa had been included in the treatment of endometriosis and increased clinical pregnancy rates. GnRHa had been reported to induce apoptosis in the granulosa cells of the rat ovary whereas hMG and FSH block apoptosis (2). The direct ovarian effects of GnRHa are profound in rodents as compared with humans. There have been few reports concerning apoptosis in human IVF-ET programs until recently (3). Prior studies had shown that the incidence of apoptotic bodies in membrana granulosa cells of patients with endometriosis was significantly higher than that of the control group which without endometriosis and the apoptotic cells increased as the stage of the revised AFS classification advanced (3-4). Bcl-2-related anti- and proapoptotic proteins are important in the decision step of the intracellular death program upstream from the caspases. Targeted overexpression of Bcl-2 in ovarian somatic cells of transgenic mice leads to decreased apoptosis of granulosa cells and is associated with higher ovulation rates (5). In the present study, through measuring apoptosis and apoptotic-related proteins (Bcl-2, Bax, and Mcl-1) in cGCs following GnRHa treatment in the patients with endometriosis, the incidence of apoptosis of cGCs might be a marker to predict the outcome of ovulation process, oocyte quality, and further cleavage rate.

Materials and Methods

Patients and Granulosa Cells Collection. A case-control study enrolled 8 women with GnRHa-treated endometriosis and 4 control (male factor infertility patients) undergoing IVF. The cGCs were collected after follicular aspiration, oocyte isolation, and cumulus separating process.

Detection of Apoptosis. Detection of apoptotic nuclei was accomplished by in situ nuclear labelling with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) reaction (Boehringer Mannheim, Laval, PQ, Canada). cGCs were fixed in 3.7 % paraformaldehyde solution in PBS for 30 min, rinsed twice in phosphate buffered saline (PBS), and permeated with 0.1 % Triton-X 100 in 0.1 % sodium citrate for 2 min on ice. cGCs were incubated in 50 ml TUNEL reaction mixture or in 50 ml TUNEL label alone as a negative control for 60 min at 37°C in a humidified atmosphere. Positive controls were incubated with 50 U/ml RNase-free DNase (Promega, Madison, WI) for 20 min at 37°C before exposure to the TUNEL reaction mixture. cGCs were rinsed twice in PBS and examined with the fluorescent microscope.

Immunohistochemistry The expression of Bcl-2, Bax, and Mcl-1 protein products in cGCs were examined by immunohistochemistry with specific antibodies. cGCs were fixed in 3.7 % paraformaldehyde solution in PBS for 30 min, rinsed twice in PBS, and permeated with 0.1 % Triton X-100/PBS

for 10 min. cGCs were blocked with PBS containing 3 % goat serum for 30 min and then incubated for 4 h at room temperature with 1:200 dilution of either the primary antibodies of Bcl-2, Bax, and Mcl-1 (Santa Cruz, CA) in a humidified chamber. After washes with PBS, the cGCs were incubated for 1 h with the secondary antibody, biotinylated anti-rabbit-IgG or anti-mouse-IgG (Santa Cruz, CA) and then incubated with ABC mixture (Victor, Burlingame, CA) for 1.5 h. The cells were washed again with PBS and the antigen-antibody complexs were visulized by immersion in a DAB solution (Victor, Burlingame, CA). The cell nuclei were then counterstained with haematoxyllin, dehydrated in graded ethanol baths and xylene, and mounted with a coverslip for light microscope. Negative controls were processed in the same manner but were not incubated with the primary antibody. All antibodies have been previously characterized through immunocytochemistry and western blot analysis as specific for Bcl-2, Bax or Mcl-1 (Santa Cruz, Technical notes).

Statistical Analysis Data are presented as mean ± SEM. A Student's t test was used to evaluate statistical significance of differences between two paired observations. A value of $P < 0.05$ was considered statistically significant.

Results

A typical apoptotic bodies of cGCs, which included fragmented and shrunken nuclei stained with the TUNEL technique, was observed under a fluorescence microscope (Fig.1a). The GnRHa-treated endometriosis showed more TUNEL positive stained cells (Fig. 1b), than the control group (Fig. 1c). Table 1 summerized the incidence of apoptotic cells and apoptotic bodies in the patients with GnRHa treated endometriosis and compared

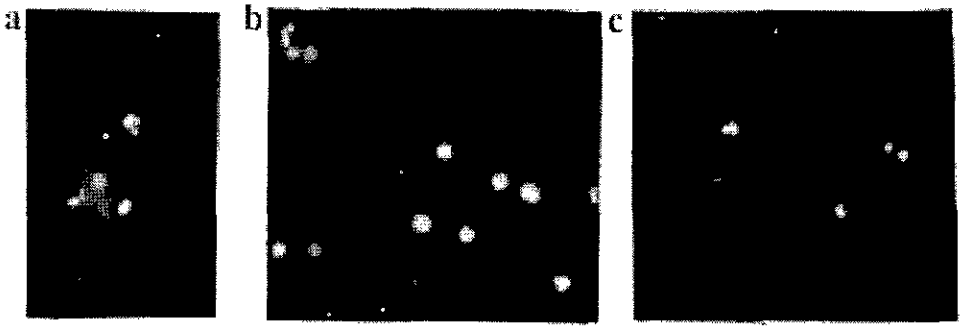


Figure 1. (a) Fluorescence micrograph of fragmented nuclei of apoptotic bodies stained with TUNEL. (b) Fluorescence micrograph of cGCs collected from the patients with GnRHa treated endometriosis and following IVF-ET and (c) cGCs from the control group also stained with TUNEL.

Table 1

	N	Age	Apoptotic cells (%)	Apoptotic bodies (%)	Oocyte number	Cleavage rates (%)
Control	4	33 ± 2	4.1 ± 0.9	0.7 ± 0.1	12.0 ± 2.0	60.9 ± 2.2
GnRHa-treated Endometriosis	8	33± 4	13.6 ± 2.4*	1.6 ± 0.3*	5.4 ± 1.1*	78.3 ± 7.4
Significance by student t-test		P=0.61	*P=0.01	*P=0.02	*P=0.004	P=0.12

Each data represents the mean ± s.e.mean. *: A value of $P < 0.05$ was considered statistically significant.

with the control cases. The results indicated that GnRHa-treated cases had higher incidence of apoptotic cells and apoptotic bodies in cGCs than the control group (P<0.05). We also found that the patients with higher incidence of apoptotic cGCs had smaller number of harvested oocyte and matured oocytes (P<0.05), but no significantly influence in the embryo development (P>0.05).

The expression of Bax and Mcl-1 were higher than the Bcl-2 in the cytoplasm and nuclei of cGCs indicated that the balance of these proteins might be involved in the mechanism of apoptosis in cGCs (Fig.2).

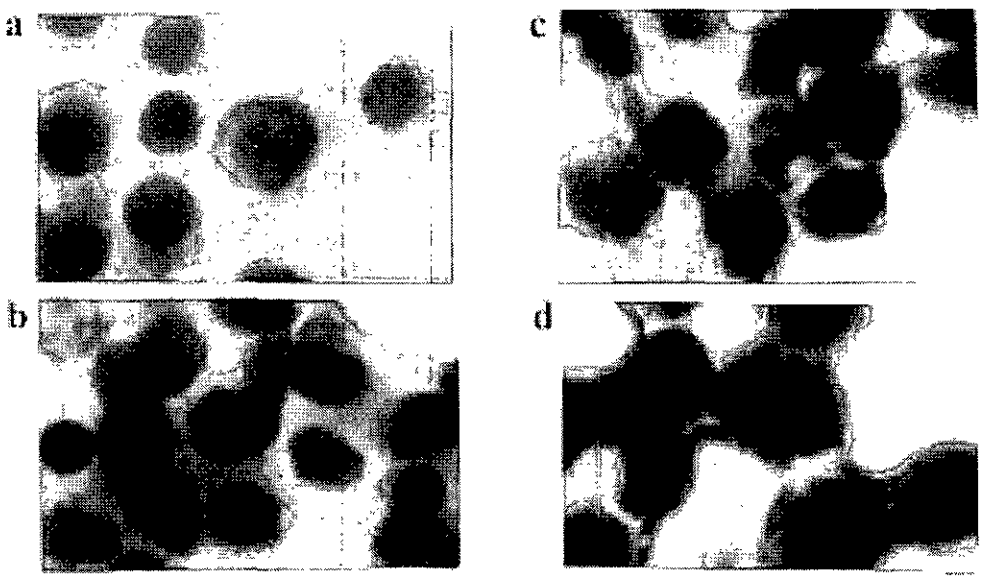


Figure 2. cGCs were stained with specific antibodies. (a) negative control; (b) Bcl-2; (c) Bax; and (d) Mcl-1.

Conclusions

The TUNEL technique provide more specific, sensitive, and objective way to examine the apoptotic cells and apoptotic bodies formation in GCs than the Hoechst 33258 staining (3). Results indicated that GnRHa-treated endometriosis might increase the rates of apoptosis in cGCs and showed no significant change in embryo development and pregnancy rate compared with the control group. The results suggested that the treatment of endometriosis with GnRHa before the induction of ovulation would modify the apoptosis in cGCs, decrease oocyte number, but still retain the same cleavage rates.

In this report, using immunocytochemical staining, we have shown for the first time the distribution of Bcl-2, Bax, and Mcl-1 in cGCs from patients with endometriosis undergoing IVF-ET program. The results indicated that Bax and Mcl-1 were highly expressed in the cGCs, suggested that the reguation of these apoptotic-related protein might be involved in the apoptotsis of cGCs.

References

(1). PALUMBO A., YEH J. In situ localization of apoptosis in the rat ovary during follicle atresia. Biology Reproduction 51; 888-895, 1994.

(2). BILLIG H., FURUTA I., HSUEH AJ. Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of deoxyribonucleic acid fragmentation in granulosa cells. Endocrinology 134; 245-252, 1994.

(3). NAKHARA K., SAITO H., SAITO T., ITO M., OHTA N., TAKAHASHI T., HIROI M. The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. Fertility & Sterility 68(2); 312-317, 1997.

(4). NAKHARA K., SAITO H., SAITO T., ITO M., OHTA N., TAKAHASHI T., HIROI M. Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. Fertility & Sterility 69(5); 931-935, 1998.

(5). KAIPIA A., HSU SY., HSUEH AJ. Expression and function of a proapoptotic Bcl-2 family member Bcl-X_L/Bcl-2-associated death promoter (BAD) in rat ovary. Endocrinology 138(12); 5497-5504, 1997.

MONDUZZI EDITORE

Via Ferrarese, 119/2 – 40128 Bologna, Italy

Tel ++ 39-051 4151123 – Fax ++ 39-051 4151125