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Effect of Ovarian Stimulation on the Endometrial Apoptosis at Implantation Period

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ABSTRACT

Background: Apoptosis is a process that plays an important role during early stage of implantation. The aim of this study was to investigate the incidence of apoptosis in mice endometrium after ovarian stimulation at implantation period. **Methods:** NMRI female mice were divided into two groups: 1) control group, which were rendered pseudopregnant by vaginal stimulation and 2) experimental group, which were stimulated using an intrapritoneal injection of 10 IU hMG followed by another injection of 10 IU hCG after 48 h. In the evening of the second injection, the mice were rendered pseudopregnant the same as control group. Samples were obtained from 1/3 middle part of uterine horns during implantation period. Apoptosis was assessed in two groups at implantation period using light and electron microscopic studies, TUNEL staining and semiquantitative RT-PCR. **Results:** Our morphological and ultrastructural results showed apoptosis in both groups, while TUNEL analysis showed that the percentage of TUNEL-positive cells was higher in stimulated group than in the control group ($P \leq 0.05$). The expression of P53, Fas and FasL mRNA was similar in two groups but Bax and Bcl2 were much higher in control group than in the stimulated group ($P \leq 0.05$). The ratio of Bax/Bcl2 expression was much higher in stimulated group than in the control group ($P \leq 0.05$). **Conclusion:** The ovarian stimulation could change the expression of some apoptosis-related genes and enhance the incidence of endometrial apoptosis at implantation period; thus, it could affect on the implantation rate and endometrial receptivity. *Iran. Biomed. J. 14 (4): 171-177, 2010*

Keywords: Apoptosis, Endometrium, Gene expression

INTRODUCTION

Within a short time which is considered as "implantation window," the endometrium undergoes specific changes for embryo attachment and implantation [1].

Several studies showed that after ovarian stimulation, the implantation rate decreases compared to normal groups [2-4]. One suggestion in this regard is due to absence of synchronization between morphological, ultrastructural, and molecular alterations in endometrium with implantation of embryo [5-9]. During ovarian stimulation, a large number of follicles develop and result in supraphysiological levels of estrogen [5-9] and high estradiol concentration leads to a decrease in number of estrogen and progesterone receptors compared to a natural cycle [2, 3, 5-7]. These changes cause interference to implantation process and results in diminish the implantation and pregnancy rate [5, 8, 9].

Apoptosis is a kind of programed cell death with morphological changes in the nucleus, cytoplasm and cell membrane including irregular nuclei, condensing and margination of chromatin of nuclei, Ruffle cell membrane and formation of apoptotic bodies [10]. It plays an important role during the early stage of implantation in some species of animals such as monkey, mouse and canine [11-16]. However, the uterus homeostasis that is regulated by various hormone and cytokines is closely related to apoptosis [17].

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Ovarian hormones are the important regulatory factors for uterine apoptosis and proliferation [13, 18-20], and the balance between pro-apoptotic (p53, FasL, Fas) and anti-apoptotic (Bcl-2) genes is regulated by these hormones [14].

It was shown that the abnormal cellular apoptosis of the endometrium at the early stages of gestation may result in failure of pregnancy and spontaneous abortion [21].

Although there have been several studies in apoptosis during estrus cycle and implantation period, none of them have focused on the incidence of apoptosis in the endometrium during implantation period in ovarain stimulated model [11-16]. Therefore, this study was designed to investigate, the incidence of apoptosis in ovarian stimulated mice endometrium during implantation period using light microscopy, electron microscopy and TUNEL assay in parallel with apoptosis-related gene expression by smiquantitative RT-PCR.

MATERIALS AND METHODS

Animals. NMRI female mice, aged between 6 to 8 weeks, were kept under 12 h light/12 h dark conditions. The mice were randomly divided into two groups: 1) control group, which were rendered pseudopregnant by vaginal stimulation and 2) experimental group, which were stimulated using an intrapritoneal injection of 10 IU hMG followed by another injection of 10 IU hCG after 48 h. In the evening of the second injection, the mice were rendered pseudopregnant in the same as control group.

Tissue preparation. The mice from each group were sacrificed by cervical dislocation at implantation period, 4.5 days after hCG injection or pseudopregnancy. The samples were obtained from 1/3 middle part of the uterine horns and prepared separately for light microscopy, electron microscopy, TUNEL staining and RT-PCR analysis.

Light microscopy. The tissues were fixed using formaldehyde and embedded in paraffin wax. The paraffin sections were prepared and routine hematoxylin and (H & E) staining was carried out for morphological study.

Electron microscopy. Samples (n= 5) were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 2 h. Then, they were fixed with 1% osmium tetroxide in the same buffer for 2 h. After dehydration in a graded ethanol series, specimens were placed in propylene oxide and embedded in Epon 812 (TAAB, UK). Semi-thin sections (0.5 µm) were stained with toluidine blue and ultrathin sections were contrasted with uranyl acetate and lead citrate, then they were examined by a transmission electron microscope (Zeiss EM 900, Germany).

TUNEL staining. Samples (n = 5 in each group) were fixed in 10% (w/v) PBS-buffered formaldehyde and embedded in paraffin. Sections (5 µm) were serially cut from each block and from each 10 sections, one of them was counted serialy. TUNEL staining was performed using a TUNEL detection kit according to the manufacturer's instructions (Roche Diagnostic, Germany). Briefly, sections rehydrated, incubated in 3% H_2O_2 for 10 minutes, and incubated with proteinase k (Roche Diagnostic, Germany) at 37°C for 15 minutes. TUNEL reaction mixture was added to the samples, incubated at 37°C for 60 minutes. Afterwards, converter-POD was added and samples were incubated at 37°C for 30 minutes and then treated with diaminobenzidine tetrahydrochloride for 5 minutes. A negative control was similarly performed except for omitting TUNEL reaction mixture. For quantitative analysis, percentage of TUNEL-positive endometrial cells was taken in each group.

Semiquantitative RT-PCR. The expression of apoptosis-related genes including p53, FasL, Fas, Bcl-2 and Bax was analyzed in stimulated and control groups (n = 5 in each group) at the same time (4.5 days after hCG injection or pseudopregnant). β2M was also considered as a housekeeping gene and evaluated in both groups. Total RNA was extracted (Kit Qiagen, USA) and treated with DNase. RT-PCR was carried out with the following condition: 95°C for 5 minutes, followed by amplification rounds consisting of 95°C for 30 s, 58°C for 30 s (annealing) and 72°C for 50 s (extension) for 35 cycles. The specific primers used for RT-PCR are listed in Table 1 [22]. The PCR products were analyzed on a 1.5% agarose gel (Isolab, Germany) and visualized by ethidium bromide staining.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 Software. Data are shown as mean ± SD or as indicated. The tests were repeated five times and the results were compared by Mann-Whitney test ($P \leq 0.05$).

Table 1. Sequence of the primers.

Gene	Sense	Anti-sense	Size (bp)
β2M	5'TGACCGGCTTGTATGCTATC-3'	5'CACATGTCTCGATCCCAGTAG-3'	316
p53	5'AGAGACCGCCGTACAGAAGA-3'	5'GCATGGGCATCCTTTAACTC-3'	227
FasL	5'TCCCAGGGTGGGTCTACTTACTAC-3'	5'CCCTCTTACTTCTCCGTTAGGA-3'	200
Fas	5'GCTGCAGACATGCTGTGGATC-3'	5'TCACAGCCAGGAGAATCGCAG-3'	419
Bcl-2	5'ACCGTCCTGACTTCSCACAC-3'	5'CGTGTGCAGATGCCGGTTCCA-3'	240
Bax	5'TGCGCAGACATGCTGTGGATC-3'	5'TCACAGCCAGGAGAATCGCAC-3'	160

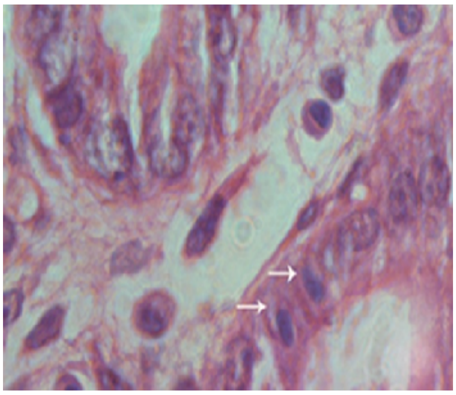

Fig. 1. Light microscope photograph of mouse endomerium by hematoxylin and eosin (H & E) staining. Arrows show the apoptotic cell (magnification × 1,000).

RESULTS

Light and electron microscopy. Morphologic examination by H & E staining showed characteristic of apoptotic cells (Fig. 1). Apoptotic cells in two groups were morphologically identifiable in tissue sections stained with H&E, shrunk with eosinophilic-condensed cytoplasm and condensed chromatin [23]. Results of semi-thin sections stained with toloidine blue also confirmed these results (Fig. 2). In transmission electron microscopy, a sign of apoptosis was observed. Nuclei of normal cells were regular and euchromatin and cell's membrane was monotonous (Fig. 3A), but the nuclei of apoptotic cells were irregular in shape and showed a condensed and marginated chromatin. Also, formation of apoptotic bodies was observed (Fig. 3B).

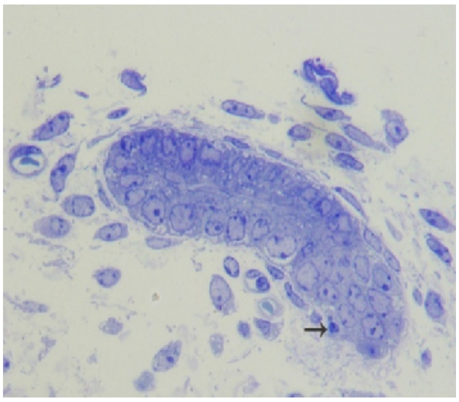

Fig. 2. Semi-thin section of mouse endometrium. Apoptotic cell is visible with shrinkage of cytoplasm and condensed nuclei (arrow); magnification ×400.

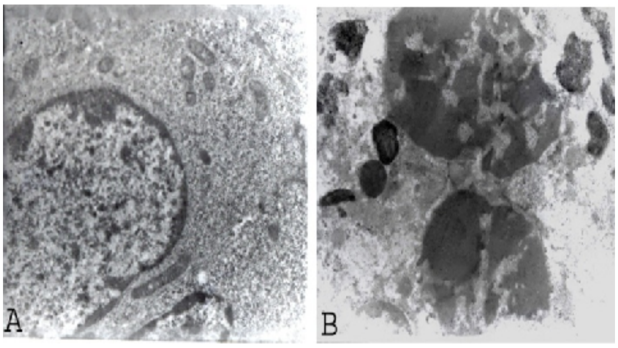

Fig. 3. Electron micrograph of an apoptotic cell in mouse endometrium. **(A)** Normal cell with monotonous membrane and regular and euchromatin nuclei can be observed (magnification ×12000). **(B)** The nuclei of apoptotic cell shows condensed and marginated chromatin and apoptotic bodies formation is visible (magnification ×7000).

TUNEL. TUNEL-positive cells were obtained from stimulated and control groups (Fig. 4). The percentage of TUNEL-positive cells in the control and stimulated groups was 1.24 ± 0.305 and 3.429 ± 0.8597, respectively (Table 2). The statistical differences between stimulated and control groups were significant ($P \leq 0.05$).

Semiquantitative RT-PCR. Results of semi-quantitative RT-PCR showed that all genes were expressed in two groups (Fig. 5). Ratio of gene expressions (target gene/housekeeping gene) was calculated and compared between two groups [24]. These ratios for expression of Bax and Bcl2 genes were much higher in the control than the stimulated groups ($P \leq 0.05$) and the other gene expression did

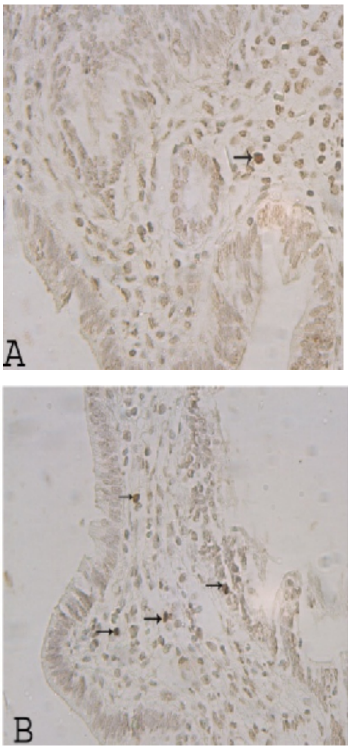

Fig. 4. Micrograph of mouse endomerium with TUNEL staining. **(A)** control group and **(B)** stimulated group (more apoptotic cells can be observed). The arrows show the apoptotic cell with brown nuclei (magnification × 200).

not have any significant difference in both groups ($P \leq 0.05$). However, the ratio of Bax/Bcl2 was much higher in the stimulated group than the control group ($P \leq 0.05$, Table 3).

DISCUSSION

In the current study, we aimed to evaluate the incidence of endometrial apoptosis after ovarian stimulation at implantation period. Therefore, we have used different methods such as semiquantitative RT-PCR, light and electron microscopy, and TUNEL staining to detect the cells undergoing apoptosis. Apoptotic cells with shrinkage, eosinophilic condensed cytoplasm and nuclei with condensed chromatin were observed at the light microscopic level. Results of electron microscopic studies also confirmed these results. The irregular nuclei associated with peripheral, condensed chromatin and formation of apoptotic bodies were signs of apoptosis in these cells. Also with TUNEL staining, the nuclei of apoptosis cells were stained in brown. Sign of apoptosis was observed in the structural and ultrastructural of endometrial cell in both groups of study. It seems that in stimulated group, numbers of apoptosis cells are prominent and quantitative analysis of TUNEL-positive cells also confirmed these observations ($P \leq 0.05$).

Table 2. Percent of TUNEL-positive cell.

Groups	TUNEL-positive cell (%)
Control group	1.240 ± 0.3050
Stimulated group	3.429 ± 0.8597[a]

Data are given as mean ± SD; [a]significant difference with control group ($P \leq 0.05$).

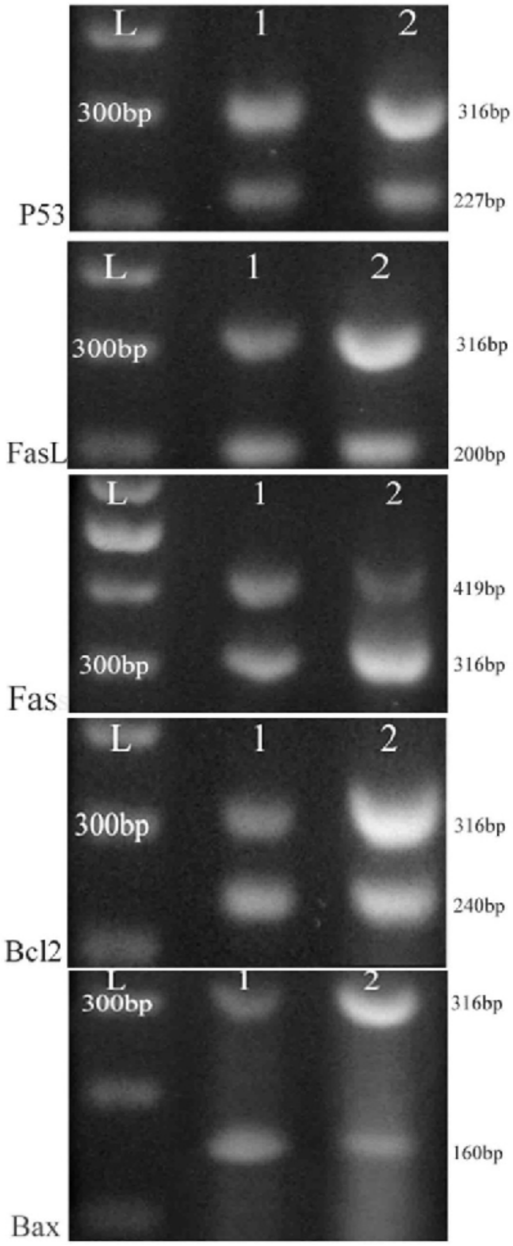

Fig. 5. RT-PCR analysis of apoptosis-specific gene expression in control group (line 1) and stimulated group (line 2). β2m gene (316 bp), p53 gene (227 bp), FasL gene (200 bp), Fas gene (419 bp), Bcl2 gene (240 bp) and Bax gene (160 bp). Line L, molecular weight marker (100 bp).

Table 3. Ratio of gene expression using semiquantitative RT-PCR.

Ratio of gene expression	P53/β2m	Bax/β2m	Bcl2/β2m	Fas/β2m	FasL/β2m	Bax/Bcl2
Control group	0.1210 ± 0.04831	0.5052 ± 0.04863	0.5830 ± 0.06846	0.1852 ± 0.01893	0.1893 ± 0.02380	0.8610 ± 0.1158
Stimulated group	0.1872 ± 0.03700	0.3093 ± 0.02792[a]	0.2884 ± 0.02136[a]	0.1947 ± 0.02836	0.1917 ± 0.03126	1.208 ± 0.09166[a]

Note: Data are given as mean ± SD; [a]Significant difference with control group ($P\leq0.05$).

These differences may be due to the increased level of steroid hormones in ovarian stimulated group. During ovarian hyperstimulation, a large number of follicles develop and result in high levels of estrogen. It has been shown that small doses of stradiol potentate the progesterone effect, whereas higher concentration of this steroid almost completely blocks expression of progestational response [6]. During the early stage of implantation, apoptosis plays an important role and it is closely related to several types of hormones, as it has been reported in some species [12, 16, 17, 25-31]. Despite of several studies, the signaling mechanisms that are involved in regulation of apoptosis by sex steroids are still largely unknown [32]. Therefore, an increase in the rate of apoptosis may have a role in the implantation failure.

Moreover, the expression ratio of Bax and Bcl2 in control group was more than the stimulated group and the difference between these two groups was significant ($P\leq0.05$). Conversely, there was no significant difference in ratio of Fas and FasL between stimulated and control groups ($P\leq0.05$). Since the cell's fate is dependent on the ratio of Bax/Bcl2 expression [29], it can be concluded that the ovulation stimulation may activate the apoptosis pathway via Bax/Bcl2 genes not via Fas/FasL genes. The expression of apoptosis-related genes such as P53 affects on the number of endometrial apoptotic cells [31, 32]. Results of this research showed that there was not any siginificant difference between the expression ratio of P53 gene in stimulated and control groups; thus, it seems that ovarian stimulation did not have any effects on apoptosis rate of endometrium via P53 gene.

Several studies showed that endometrial apoptosis is regulated by the coordination of Bax, Bcl2, Fas, FasL, and P53 genes expression during estrus cycle and pregnancy [14, 16, 25, 27, 31, 32] and the expression of apoptosis-related molecules (such as Fas/FasL, Bax/Bcl2 and P53) is correlated with a number of endometrial apoptotic cells [31, 32]. The role of estrogen and progesterone in regulating endometrial expression of Bax and Bcl2 is not well understood. It has been suggested that estrogen might up-regulate Bcl2 expression, while progesterone down-regulate it [33]. Balance between expression of Bax and Bcl2 genes is regulated by progesterone, since the application of antagonist results in a higher Bax to Bcl2 ratio and enhances decidual apoptosis [14].

In the current study, we showed that the balance between pro-apoptotic and apoptotic genes in endometrium is changed by ovarian stimulation. In related studies, it was shown that ovarian induction could affect on granulosa cell apoptosis [34, 35].

In conclusion, the ovarian stimulation could change the expression of some apoptosis-related genes and enhance the incidence of endometrial apoptosis at implantation period; thus, it could affect on the implantation rate and endometrial receptivity. It is suggested to investigate the effect of ovarian stimulation protocol on apoptosis occurrence rate in other parts of reproduction system.

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