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Original paper

The Effects of Estradiol Valerate and Progesterone on the Activity of Caspase -8 and -9 and Evaluating the Expression of *BAX*, *BCL2*, and *P53* Genes in the Apoptosis Pathway of HT29 Cancer Cells in the Cell Culture

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Abstract

Background and aim: Studies have shown that sex steroid hormones affect the proliferation of cancer cells. This study aimed to evaluate the effect of estradiol valerate and progesterone on caspase-8 and -9 activity and expression of *BAX*, *BCL2*, and *P53* genes in HT29 cells compared to control groups.

Materials and methods: HT29 cells were treated with cytotoxic concentration of estradiol valerate (1mg/ml) and progesterone (0.1 mg/ml). *BAX*, *BCL2*, and *P53* gene expression was evaluated using Real-Time PCR and caspase -8 and -9 activity level was measured using ELISA kits. Data were compared between groups using student's t-test.

Results: The results showed that 0.1 mg/ml progesterone and 1 mg/ml of estradiol valerate significantly increased the expression level of apoptotic BAX gene and decreased the expression level of *BCL2* gene in HT29. Treatment with progesterone led to significant decrease in expression level of P53 gene and treatment with estradiol valerate resulted in significant increase in expression level of the P53 gene in HT29 cells. Treatment with progesterone and estradiol valerate resulted in increased caspase-8 and caspase-9 activity level in HT29 cells, respectively.

Conclusion: Estradiol valerate and progesterone have apoptotic effects on colon cancer cells *in vitro* which is mediated by intrinsic and extrinsic pathway, respectively.

Keywords: *Estradiol valerate, Progesterone, BAX, BCL2, P53, HT29 Cell line, Caspase-8, Caspase-9*

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Introduction

Colorectal cancer (CRC) is the third most common cause of cancer death in men and women in the United States and the second most common when men and women combine [1], [2], [3]. HT29 colon cancer cells grow under standard conditions and form undifferentiated cell layers, which provide a perfect model for investigating the effects of apoptosis on these cells [4]. Although apoptosis is programmed cell death, not all programmed deaths are apoptotic [5], [6]. Therefore, the primary purpose of apoptosis studies is to focus on understanding the molecular components and regulatory mechanisms of apoptosis. Caspase enzymes are also involved in apoptosis. Caspases are synthesized as inactive zymogenes and undergo proteolytic failures during apoptosis. Efficient removal of apoptotic cells plays an essential role in forming three-dimensional structures, homeostasis, and the removal of abnormal, harmful, and dysfunctional cells [7]. Caspase-9 activates executive caspases (caspases-6, -3, and -7), and as a result, executive caspases act on their substrates, and apoptosis takes place [8]. *BAX*, *P53*, and *BCL2* genes are among the most important genes involved in the mitochondrial pathway of apoptosis. There is a direct link between the expression of these genes and the cancer process. Therefore, the study of changes in the expression of these genes can be considered a therapeutic or diagnostic target in cancer studies [9], [10], [11], [12], [13], [14], [15], [16].

Steroid-sex hormones, especially estradiol valerate, progesterone, and testosterone, are the most popular hormones in the reproductive system. The association between sex steroids and cancer has been demonstrated in a number of past and recent studies. Steroid hormones can stimulate cancer cells or inhibit their growth and proliferation. Estradiol valerate and progesterone can prevent metastasis or stimulate metastasis in cancer cells [17], [18]. Studies show that steroid hormones can play inhibitory or stimulatory roles in developing gastrointestinal tumors. However, the mechanism of these roles is not very clear in terms of cellular and molecular basis [19], [20]. Some studies show an association between sex hormones and the expression of *BAX*, *BCL2*, and *P53* genes [21], [22].

Although many studies have been carried out to investigate the link between sex steroid hormones and cancer development, few *in vitro* studies have been performed to study the effects of sex steroid hormones on colon cancer cells. The aim of this study was to investigate the effects of estradiol valerate and progesterone on caspase-8, -9 activity and expression of *BAX*, *BCL2*, and *P53* genes in HT29 cancer cells *in vitro*.

Material and Methods

Hormones

Progesterone and estradiol were purchased from Abu Reihan Pharmaceutical Company, Tehran, Iran and kept at room temperature. 1 mg of pure powder of each hormone was dissolved in 1 ml of DMSO and 1 ml of TWEEN 80 % and 7 ml of phosphate-buffered saline (PBS) (Sigma-Aldrich).

Cell Culture and Treatment

Colorectal adenocarcinoma cells (HT29) were obtained from Pasteur Institute cell bank, Tehran, Iran. The samples were transferred to the laboratory in a nitrogen tank and stored under standard conditions. The cells were cultured in DMEM supplemented with 10 % Foetal Bovine Serum (FBS) and 1 % antibiotics (penicillin/streptomycin). Cells were then preserved in an incubator (37 °C, 5 % CO2 atmosphere). HT29 cells were seeded in a 6-well plate at a density of about 105.000 cells per well, and after 24 hours of incubation, the culture medium (DMEM supplemented with 10% FBS) was discarded. The cells were treated with effective cytotoxic concentrations of

progesterone (0.1 mg/ml), and estradiol valerate (1 mg/ml) (The effective concentrations of the hormones have been evaluated in our past experimental studies).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To study the gene expression levels by RT-PCR, the primers were designed using Analysis Primer Analysis 7 software and the NCBI database (Table 1). The cells were cultured in 6-cell plates (105,000 cells per cell) and incubated for 24 h, and total RNA was extracted and reverse transcribed to cDNA. Finally, the AB Applied Biosystem PCR machine was used to evaluate the expression levels of the genes. The 2ΔΔCt method was used to calculate the relative gene expression [23], [24].

Table1: Primers sequences for *P53*, *BCL-2*, *BAX*, and *GAPDH*.

Gene	Primer		GC (%)	Tm (°C)	PCR Product
P53	F	5′-GCCATGGCCATCTACAAGAA-3′	50	51	198 bp
	R	5′-CTCGGGTGGCTCATAAGGTA-3′	55	54	
BCL2	F	5'-TGTGGATGACTGAGTACCTGAACC-3'	50	61	122 bp
	R	5'-CAGCCAGGAGAAATCAAACAGAG-3'	47	59	
BAX	F	5'-TTGCTTCAGGGTTTCATCCAG-3'	47	58	101 bp
	R	5'-AGCTTCTTGGTGGACGCATC-3'	55	60	
GAPDH	F	5'-ACCCACTCCTCCACCTTTGA-3'	55	60	101 bp
	R	5'-CTGTTGCTGTAGCCAAATTCGT-3'	45	59	

F: forward primer; R: reverse primer; Tm: annealing temperature.

Assessment of Caspase -8 and -9 Activity

The caspase- 8 and -9 activity was assessed using ELISA reader at 405 OD wavelength with a caspase colorimetric protease test kit (Abnova, Taiwan). HT29 cells were treated with progesterone (0.1 mg/ml) and estradiol valerate (1 mg/ml). After 24 hours, the cells were taken from the plate's bottom and lysed using Lysine buffer. The precipitate was discarded and added to the protein soup, DTT complex, and reaction buffer. The suspension was collected and transferred to a 96-well microtiter plate and incubated at 37 °C for 2 h before being measured at 405 nm using an ELISA microplate reader.

Statistical Analysis

Data were analyzed using SPSS (version 21.0; SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to examine the normal distribution of data. Differences between the groups were tested using student’s t-test. Value differences were considered significant if $p< 0.05$.

Results

Evaluation of the Effects of Progesterone and Estradiol Valerate on the Expression of BAX, BCL2, and P53 Genes in HT29 Cells

The results showed that 0.1 mg/ml of progesterone and 1 mg/ml of estradiol valerate significantly increased the expression level of apoptotic gene *BAX* in HT29 cells compared with the control group ($p<0.001$).

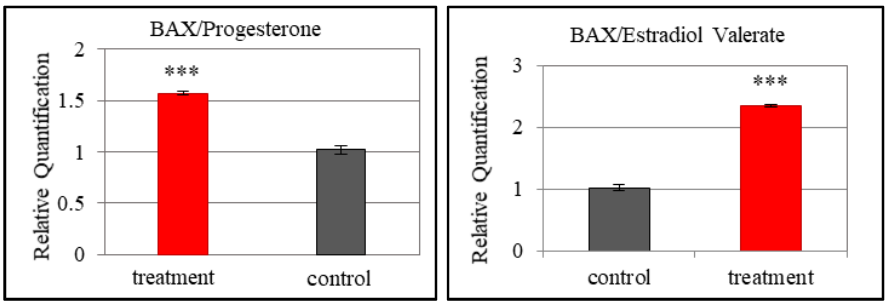

Figure 1. The effect of progesterone and estradiol valerate on *BAX* gene expression in HT29 cells. * indicates a significant difference compared to control group (***:$p<0.001$)

According to Figure 2, 0.1 mg/ml of progesterone and 1 mg/ml of estradiol valerate significantly decreased the expression level of *BCL2* in HT29 cells compared with control group ($p<0.05$).

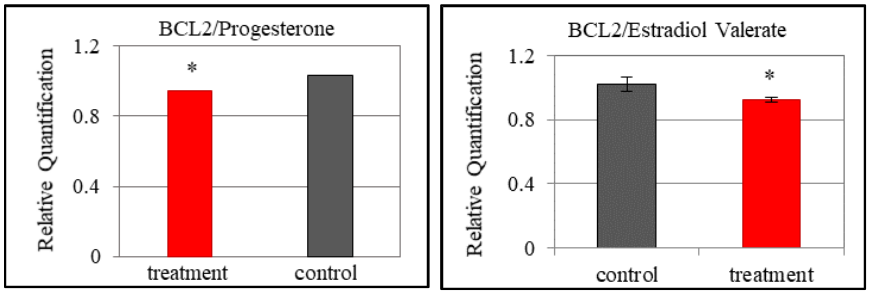

Figure 2. The effect of progesterone and estradiol valerate on *BCL2* gene expression in HT29 cells. * indicates significant difference compared to the control group (*:$p<0.05$)

Treatment of HT29 cells with 0.1 mg/ml of progesterone was followed by a significant decrease in expression level of the *P53* gene compared with control group ($p<0.001$), however, treatment of HT29 cells with 1 mg/ml of estradiol valerate significantly increased the expression level of the *P53* gene compared to control group ($p<0.01$) (Figure 3).

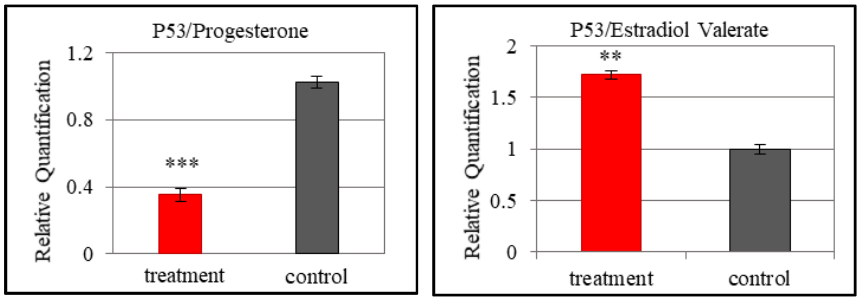

Figure 3. The effect of progesterone and estradiol valerate on *P53* gene expression level in HT29 cells. * indicates significant difference compared to control group (***: $p<0.001$, **:$p<0.01$)

Evaluation of the Effects of Progesterone and Estradiol Valerate on Caspase-8 and -9 Activity Levels in HT29 Cells

Treatment of HT29 cells with 0.1 mg/ml of progesterone and 1 mg/ml of estradiol valerate resulted in significant increase ($p<0.001$) and dcrease ($p<0.05$) of caspase-8 activity level, respectively (Figure 4).

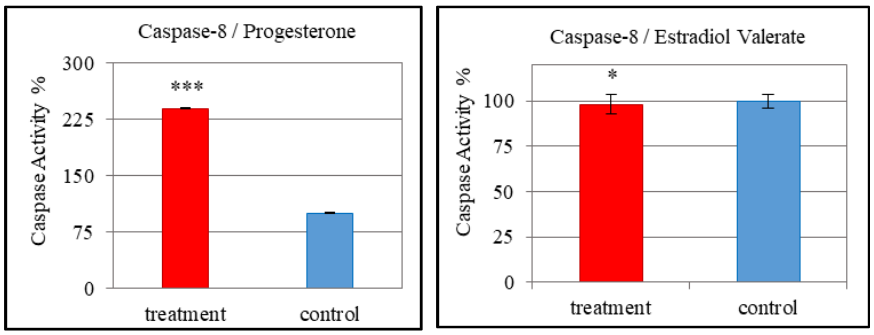

Figure 4. The effect of progesterone and estradiol valerate on caspase-8 activity level in HT29 cells. * indicates a significant difference compared to control group (***:$p<0.001$), *:$p<0.05$).

The level of caspase-9 activity significantly decreased in HT29 cells treated with 0.1 mg/ml of progesterone ($p<0.05$), however, significantly increased when 1mg/ml of estradiol valerate ($p<0.001$) compared to the control group (Figure 5).

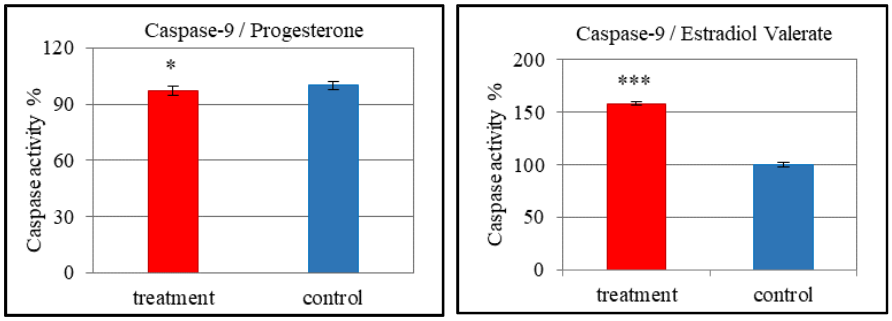

Figure 5. The effect of progesterone and estradiol valerate on caspase-8 activity level in HT29 cells. * indicates a significant difference compared to control group (***: $p<0.001$), *: $p<0.05$).

Discussion

Our finding indicated that the cytotoxic concentration of progesterone and estradiol valerate increases apototic *BAX* gene expression and decreases antiapoptotic *BCL2* gene expression level in HT29 cells. Accordingly, it can be said that estradiol valerate and progesterone can induce *BAX*-dependent apoptosis. In consistent with our findings, it has been shown that progesterone has an antiproliferative impact colon cancer cells [25]. A study on the impact of steroid hormones on the proliferation of human colon cancer cells in cell culture media showed that increasing the amount of estradiol in a cell culture medium containing estrogen or estradiol completely inhibited the growth of cancer cells [26]. Also, research on the effect of sex hormones on the risk of cervical cancer due to human papillomavirus has shown that estrogen receptors' expression level increases the *BAX* gene's expression level and decreases the expression level of the *BCL2* gene [27]. It has been reported that the estrogen receptor signaling pathway has a physiological role in reproduction and the development of breast and uterine cancer via acting on expression of the *BCL2* gene [28].

Estradiol stimulates the proliferation of colon cancer cells using different genomic and non-genomic pathways [29].

Our findings showed that the effect of progesterone on HT29 cells was not mediated by *P53*-dependent apoptosis, however, estradiol valerate can induce *P53*-dependent apoptosis in colon cancer cells. In line with this finding a study examining the relationship between sex hormones and breast cancer and *P53* gene expression level showed that *P53* gene expression increased causing the cells to move toward apoptosis [30]. It has also been found that increased sex steroids in HT29 cell line can increase the expression of *P53* gene leading to cancer cell death [31]. In a research, progesterone was reported to inhibit the normal breast epithelial cell proliferation *in vivo*, just like in breast cancer cells *in vitro* [32].

We have shown that the cytotoxic concentration of progesterone increased caspase-8 activity and decreases caspase-9 activity level, however, estradiol valerate cytotoxic concentration resulted in decreased caspase-8 and increased caspase-9 activity level, therefore, it can be concluded that the apoptotic effects of the estradiol valerate on HT29 cells is mediated by intrinsic pathway, however of progesterone is mediated by extrinsic pathway. In this regard, in a study that investigated the role of steroid hormones in inducing apoptosis in human liver cells, the researchers found that steroid hormone does not affect the receptor pathway and caspase-8; on the other hand, this study showed that steroid hormone only affects the mitochondrial pathway and induces apoptosis in human liver cells after the release of the caspase-9 and triggering the intrinsic apoptotic pathway [33]. Another study on breast cancer cells found that the steroid anti-inflammatory drug-induced apoptosis was mediated by intrinsic pathway and increased caspase-9 activity level [34].

Conclusion

This study showed that the cytotoxic dose of progesterone and estradiol valerate increased the expression of the *BAX* gene and decreased the expression of the *BCL2* gene level in HT29 cells, indicating that these sex steroid hormones can induce *BAX*-dependent apoptosis in colon cancer cells. Increased caspase-8 activity level in HT29 cells treated with progesterone and increased caspase-9 activity level in HT29 cells treated with estradiol valerate revealed that apoptotic effects of progesterone and estradiol valerate on colon cancer cells is mediated by extrinsic and intrinsic pathways, respectively. Tumor suppressing P53 expression level in colon cancer cells was also increased by progesterone, however, decreased by estradiol valertae. The apoptotic effects of the estradiol valerate on HT29 cells is mediated by increased caspase-9 activity level and intrinsic pathway, however of progesterone is mediated by increased caspase-8 activity level and extrinsic pathway.

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Conflict of interests

The authors declare that there are no competing interests.

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