

Induction of mitochondrial apoptotic pathway by raloxifene and estrogen in human endometrial stromal ThESC cell line

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Abstract

Introduction: Endometrial hyperplasia is a condition that occurs as a result of hormonal imbalance between estrogen and progesterone. Morphological disturbance of endometrial cells occurs consequently leading towards endometrial cancer. In therapy of endometrial hyperplasia SERMs are used to suppress effects of locally high estrogen level in uterus. There is strong evidence suggesting that estrogen could be involved in cell death – apoptosis. There are no experimental data demonstrating the direct apoptotic effect of both raloxifene and estrogen on the ThESC cell line. The aim of our study was to investigate both cytotoxic and apoptotic mechanism of raloxifene and estrogen – induced death in the ThESC cell line.

Material and methods: In order to determine their cytotoxic and apoptotic effects, various doses of raloxifene and estrogen were applied to the ThESC cell line for 24 h. After the treatment MTT assay, FACS analysis and immunofluorescence method were conducted.

Results: The results of this study for the first time demonstrated the cytotoxic and apoptotic effects of raloxifene and estrogen on human endometrial stromal cell line suggesting the involvement of the inner, mitochondrial apoptotic pathway.

Conclusions: Our results demonstrated apoptotic effects of investigated drugs in the ThESC cell line through increasing the Bax/Bcl-2 ratio and activation of caspase 3.

Key words: apoptosis, raloxifene, estrogen, endometrial hyperplasia, inner apoptotic pathway.

Introduction

It is fascinating that small molecules such as hormones can orchestrate the crucial events in someone's life [1]. In order to express its abundant effect on different cells and organs, an obligatory step for estrogen action is binding of estrogen to estrogen receptors (ER), both α and β . Different expression of ER in various tissues determines multiple diverse effects of estrogen and results in activation of the ligand-dependent estrogen signaling. As a result of estrogen-receptor interaction, the cell activates transcription of the co-regulatory proteins and promotion of the estrogen-responsive genes [1]. Due to its ability to modulate transcription of the genes crucial for either cell survival or cell death, estrogen is

a powerful hormone whose levels determine the fine border between health and disease development. The primary physiological effects of estrogen are observed in cell proliferation, differentiation, activation of vasculogenesis and induction of a higher mitotic cell index, consequently leading to a higher level of incidence for transcriptional mistakes resulting in tissue tumor genesis [2]. Our aim was to determine the effect of different doses of estrogen treatment on the viability rate of the ThESC cell line. Treatment of a breast cancer cell line with high estrogen causes a higher proliferation rate compared to the proliferation rate in case of lower dose estrogen treatment. Deprivation of estrogen induces apoptotic changes in MCF-7:5C cells [3]. Although the molecular mechanisms of estrogen-induced apoptosis are not fully understood, evidence indicates that mitochondria-related caspase pathways are involved [4]. In our experiment we evaluated both cytotoxic and apoptotic effects of different doses of estrogen in ThESC cells that have not yet been investigated.

In order to partially exclude the unwanted proliferative estrogen effects the intensive development of new anti-estrogenic drugs such as the selective estrogen receptor modulators (SERM) was conducted during the past decade. The SERM express either agonistic or antagonistic estrogen effects in different tissues depending on their structural differences and tissue expression of ER [5]. The SERM were initially developed for chemoprevention and chemotherapy of breast cancer tissue [6]. Although tamoxifen-treated patients expressed higher risk for uterine cancer development due to its agonistic effect on uterus cells, this drug has been used for the treatment of hormone-responsive breast cancer due to its antagonistic effect on the breast tissue [7]. These negative effects of tamoxifen led to the development of raloxifene. According to numerous studies, raloxifene showed an advantage over tamoxifen [8] due to its dual effects on ER (acting through both binding and modulating ER), consequently exhibiting both agonistic (bone and adipose tissues) and antagonistic effects on estrogen-dependent tissues (uterus and breast tissues) [9].

Raloxifene binds to both ER α and ER β with high affinity. However, the binding affinity to ER α is four times higher compared to β [10]. In non-reproductive organs, bone and liver, raloxifene shows a purely agonistic effect [11]. In addition, raloxifene inhibits proliferation and induces apoptotic cell death in different cancer cell lines [12, 13]. Moreover, the SERM are used in the treatment of endometrial hyperplasia [5]. However, there are no experimental data showing the cytotoxic and apoptotic effects of raloxifene on a human endo-

metrial stromal ThESC cell line isolated from the patient's myoma uteri.

Apoptosis is crucial for tissue homeostasis and embryogenesis, and its deregulation leads to autoimmune disorders, immunodeficiency, or cancer. It can be induced by various stimuli activating either the extrinsic or intrinsic apoptotic pathway [14]. The anti-apoptotic family of Bcl-2 proteins serves as a strength marker of the survival mechanism of the cell. Granville *et al.* [15] found that the high expression of anti-apoptotic Bcl-2 proteins preserves the integrity of the mitochondrial outer membrane, inhibits translocation of the pro-apoptotic protein Bax from the cytosol to the outer membrane of mitochondria, and blocks the release of cytochrome c from the intermembrane space of mitochondria into the cytosol, utterly inhibiting apoptosis [15, 16]. Activation and translocation of Bax to mitochondria could be considered as a marker for the mitochondrial apoptotic pathway [15]. Following the initiation of the inner apoptotic pathway, cytochrome c with APAF-1 β forms a functional apoptosome that activates pro-caspase 9. Active caspase 9 directly activates executor pro-caspases 3 and 7, which finalize the apoptotic process [15]. Although numerous studies have demonstrated an apoptotic effect of raloxifene on certain cell lines [12, 13] and patients [17], the exact apoptotic mechanism of raloxifene is not yet fully understood. Liu *et al.* [13] suggested that low doses of raloxifene inhibit the growth of cultured leiomyoma cells due to downregulation of the expression of anti-apoptotic Bcl-2 [13]. However, its exact apoptotic mechanism has not been determined. In the same study, along with the increasing concentrations of raloxifene, the effect of estrogen on cell proliferation was also determined, suggesting a positive effect of estrogen (10^{-7} M) on cell proliferation but a paradoxical downregulatory effect on the expression of Bcl-2.

The aim of this study was to investigate both cytotoxic and apoptotic effects of both raloxifene and estrogen on the human endometrial stromal ThESC cell line. Both raloxifene and estrogen treatment significantly inhibited proliferation of ThESC cells and induced apoptosis in ThESC cells through downregulating Bcl-2 expression and triggering the activation of Bax and caspase 3 compared to untreated cells.

Material and methods

Cell culture

The human endometrial stromal cell ThESC cell line (ATCC, 4003TM) derived from a patient with myoma, immortalized by reversible human telomerase transcriptase (hTERT), was used. The cells were cultured and maintained in DMEM complete

growing medium. Before the treatment, cells were divided into a control group, grown on regular medium, and an experimental group treated with different doses of raloxifene (from 10^{-5} M to 10^{-10} M) or estrogen (10^{-9} , 10^{-8} , 10^{-7} and 10^{-4} M) for a 24 h period.

MTT assay

The cytotoxic effect of different doses of raloxifene and estrogen was investigated by (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) MTT assay. Cells were resuspended in a medium, seeded in a 96-well microtiter plate and incubated with MTT solution for 4 h. After centrifugation, cells were resuspended with 200 μ l DMSO (Sigma Chemical, ST. Lois, Mo.) per well and incubated for 30 min. The absorbance was measured at a wavelength of 595 nm (multimode micro plate detector, Zenith 3100). The percentage of cytotoxic cells was calculated using the formula: Cytotoxicity (%) = $(1 - (\text{experimental group (absorbance)} / \text{control group (absorbance)})) \times 100$.

FACS analysis

To analyze the apoptotic effect of different doses of both raloxifene and estrogen on the ThESC cell line, the annexin V FITC PI assay was performed according to the method described by Nikolic *et al.* [16].

Immunofluorescence microscopy

To analyze the expression of anti-apoptotic Bcl-2, active pro-apoptotic Bax and active caspase 3, fluorescence microscopy was conducted according to Nikolic *et al.* [16]. Cells were incubated for one hour with different anti-rabbit primary antibodies: Bax (N20, sc-493, Santa Cruz Biotech. Inc), Bcl-2 (DC21, sc-783, Santa Cruz Biotech. Inc), caspase-3 (9661, Cell Signaling Technology, USA)

and β -actin (A5316, Sigma Aldrich, Germany). Following incubation, cells were washed in 1xPBS, incubated in the dark for one hour and stained with secondary fluorescent antibodies conjugated with Alexa 488 (11001, Invitrogen, USA) and Alexa 594 (gift from Dr Ljubica Ivanisevic, Ottawa, Canada). Cells were visualized by fluorescence microscopy at 100x and 400x magnifications on an Olympus microscope (model BX51).

Results

To determine the effect of investigated substances on the viability of the ThESC cell line, the percentage of cytotoxic cells was determined by MTT assay following 24 h treatment with different doses of raloxifene (from 10^{-10} to 10^{-5} M) and estrogen (10^{-9} , 10^{-8} , 10^{-7} and 10^{-4} M) (Figures 1 A and B). The results demonstrated that raloxifene inhibited the proliferation of the ThESC cell line in a dose-dependent manner. Raloxifene at the dose of 10^{-10} M exhibited 2.82% cell cytotoxicity, while at the highest dose of 10^{-5} M it induced significant cell cytotoxicity of 29.94% compared to the control group (1.07%). According to the MTT assay, a statistically significant difference in the percentage of cytotoxic cells (29.94% and 2.82%) was present between the highest (10^{-5} M) and the lowest (10^{-10} M) doses of raloxifene applied for the 24-hour period (Figure 1 A). Next, we investigated cytotoxic effects of different doses of estrogen on cells. The results showed that estrogen exerted the highest cytotoxic effect (29.13%) at the dose of 10^{-9} M, while the lowest (8.29%) cytotoxicity was induced at the highest investigated dose of estrogen of 10^{-4} M.

Following the findings demonstrating the cytotoxic effects of raloxifene and estrogen on ThESC cells, we next examined the type of cell death using the annexin V FITC PI staining method (Figures 2 and 3). Our results demonstrated that the increas-

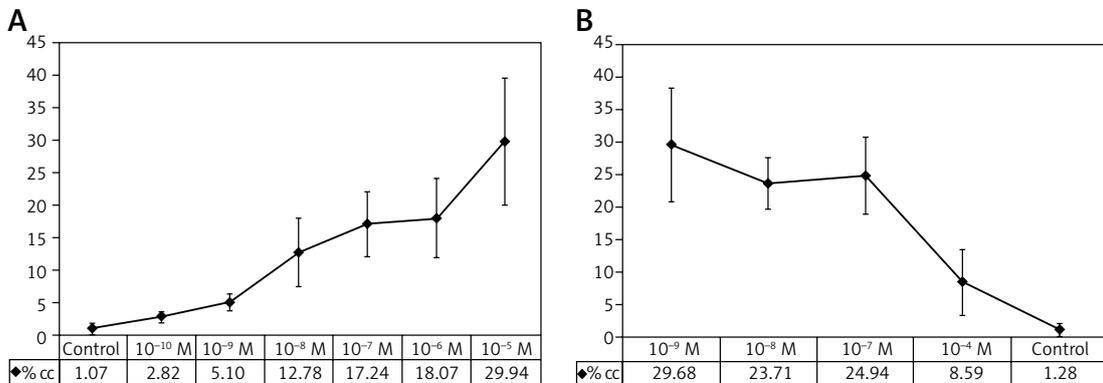


Figure 1. MTT assay was performed after 24 h treatment with increasing doses of raloxifene (A) and estrogen (B) on the ThESC cell line. A dose of 10^{-9} M raloxifene caused 5.10% cytotoxic cells, while at the highest dose (10^{-5} M), raloxifene induced significant 30% cytotoxicity, compared to the control group. Estrogen at the highest investigated dose (10^{-4} M) caused 8.29% cytotoxic cells compared to the 29.13% cytotoxic cells in the lowest investigated dose of 10^{-9} M

ing doses of raloxifene and the decreasing doses of estrogen induced apoptosis in ThESC cells. Induction of apoptosis in ThESC cells treated with

raloxifene was statistically significant in all doses tested, with the highest percentage of apoptotic cells of 19.21% at a dose of 10^{-6} M compared to

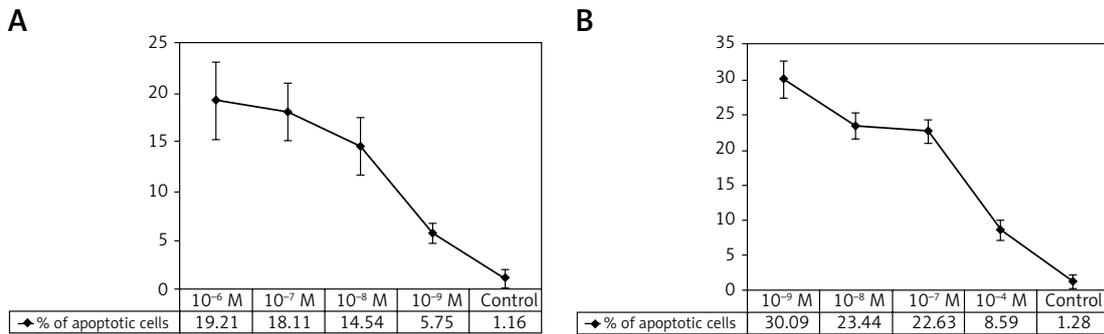


Figure 2. Apoptotic effects of raloxifene and estrogen on ThESC cell line. Cells were treated with different concentrations of raloxifene (A) and estrogen (B) for 24 h period at 37C°. The percentage of apoptotic cells in the group treated with 10^{-9} M of raloxifene for a 24 h period were six times higher compared to the control group. For the investigated dose of estrogen 10^{-4} M the percentage of apoptotic cells was 8.61%

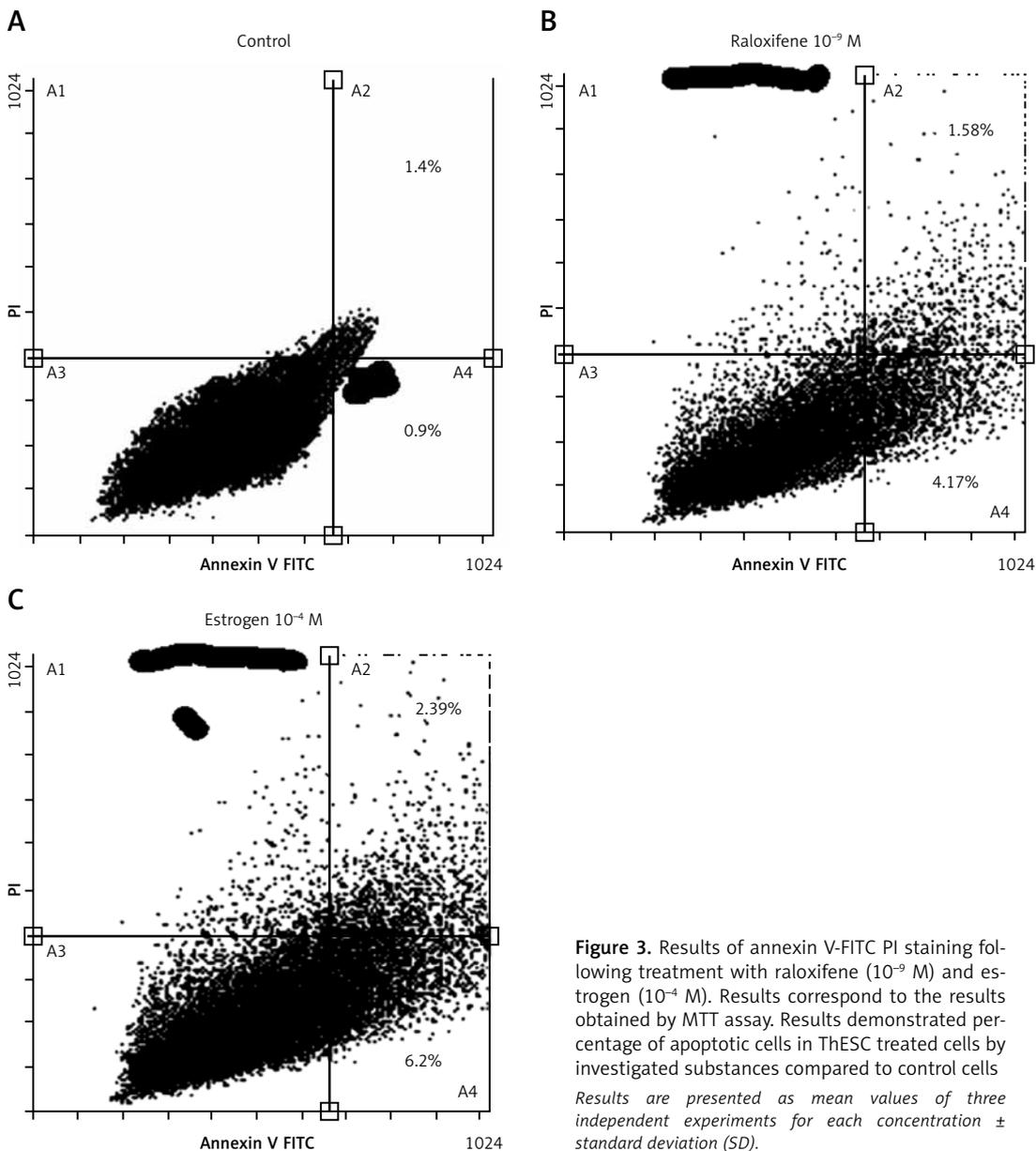


Figure 3. Results of annexin V-FITC PI staining following treatment with raloxifene (10^{-9} M) and estrogen (10^{-4} M). Results correspond to the results obtained by MTT assay. Results demonstrated percentage of apoptotic cells in ThESC treated cells by investigated substances compared to control cells. Results are presented as mean values of three independent experiments for each concentration \pm standard deviation (SD).

control cells. Cells treated with different doses of estrogen induced significant apoptotic changes of the ThESC cell line ranging from 8.6% (10^{-4} M) to 30.1% (10^{-9} M) of apoptotic cells compared to the control group of cells. Results presented in Figure 3 show the percentage of apoptotic cells in ThESC cells treated with investigated substances compared to control cells.

Based on the results obtained by both MTT assay and FACS analysis, next we applied the immunofluorescence method to determine the mechanism of apoptosis induced by raloxifene and estrogen by investigating the level of expression of the crucial anti-apoptotic Bcl-2 protein. Anti-apoptotic Bcl-2 is associated with prolonged cell survival through inhibition of a common pathway of cell death. One of the first indicators of ongoing apoptotic changes in treated cells is downregulation of anti-apoptotic Bcl-2 protein expression. As a result of reduced Bcl-2 expression, survival mechanisms of treated cells are diminished, resulting in their death. The applied doses of raloxifene (10^{-9} M) and estrogen (10^{-4} M) used in this experiment did not exceed 10% of their both cytotoxic and apoptotic effects. Immunofluorescence assay revealed the highest level of Bcl-2 expression of 77% in untreated cells. Both raloxifene and estrogen decreased the Bcl-2 protein expression to 61% and 48% respectively compared

to untreated cells (Figure 4 A). Reduction of Bcl-2 expression in treated cells suggests diminished survival that ultimately leads to apoptosis.

Pro-apoptotic Bax is mainly localized in the cytosol of the healthy mammalian cells, and upon induction of apoptotic signaling it translocates from the cytosol to the mitochondria where it forms transition pores and allows release of cytochrome c that further commits cells to death. When Bax is activated, it undergoes conformational changes that expose its N terminus, and this alteration can be detected by the conformation-specific antibodies. To investigate the level of expression of activated Bax, we performed immunofluorescence assay using an antibody specific to the N terminus of Bax in treated and control cells (Figure 4 B). Bax was found to be more activated in cells treated with raloxifene and estrogen compared to the untreated cells. Precisely, 20% and 27% of ThESC cells treated with investigated substances showed staining of active Bax localized at the mitochondria, respectively, compared to untreated cells (Figure 4 B). Activation of Bax in control cells was probably due to the spontaneous apoptosis in these cells.

After we had determined involvement of Bax and Bcl-2 in treated cells, in the next experiment we determined the expression of the main apoptosis executioner, caspase 3. Immunofluorescence

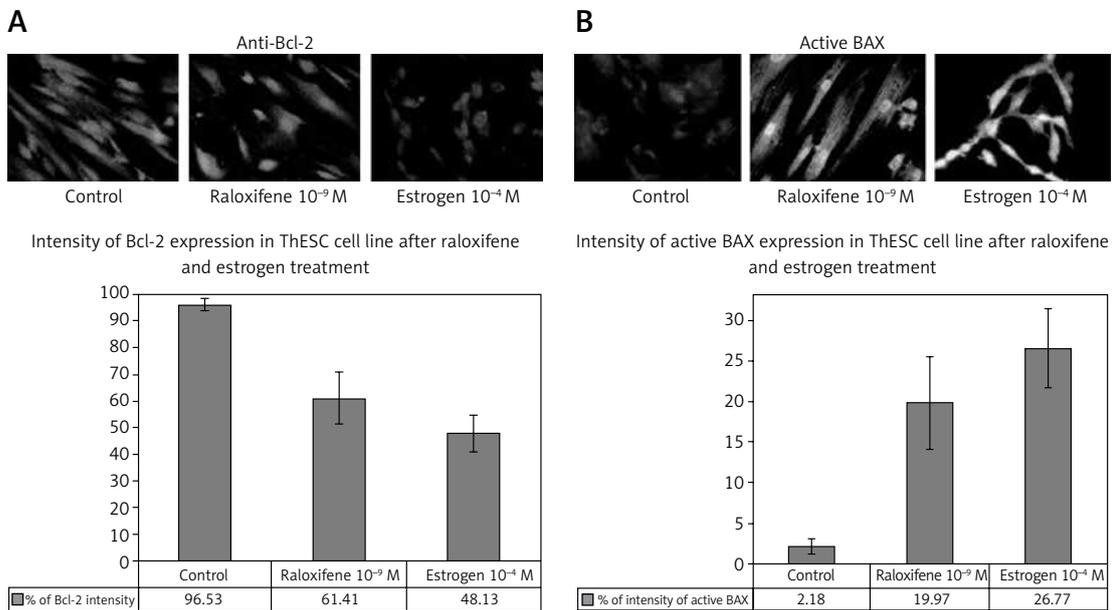


Figure 4. A – Expression of Bcl-2 protein in ThESC cells after treatment with both raloxifene and estrogen for 24 h period. Based on our results expression of Bcl-2 protein in control cells is statistically higher compared to the cells treated with investigated substances, indicating downregulation of Bcl-2 protein in treated cells. In the cells treated with both raloxifene 10^{-9} M and estrogen 10^{-4} M level of the expression of Bcl-2 protein was 1.5 and 3 times lower compared to the control cells. **B** – Expression of active mitochondrial Bax in ThESC cell line after 24 h treatment with different doses of raloxifene and estrogen. In control cells, active Bax is mainly localized in cytosol compare to both raloxifene and estrogen treated cells, which show redistribution of active Bax to the mitochondria. Redistribution of Bax from cytosol to the mitochondria resulted in a change in the permeability of the mitochondrial membrane of treated cells compared to the control cells. Intensity of active Bax was calculated using ImageJ software (active Bax = 100 – mean of measured fluorescence)

was performed with an antibody specific to the cleaved active form of caspase-3 following the 24 h treatment of 10^{-9} and 10^{-6} M of raloxifene in ThESC cells. Our results demonstrated significantly higher expression (activation) of caspase 3 in ThESC cells treated with raloxifene (10^{-9} M) and estrogen (10^{-4} M) compared to the untreated cells (Figure 5).

Discussion

Endometrial hyperplasia is a condition most often related to the hormonal imbalance of estrogen and progesterone with the combination of

the morphological disturbance of endometrial cell integrity (both glandular and stromal). The main goal of the endometrial hyperplasia treatments is the reduction of patients' irregular bleeding and volume of hyperplastic change, thus preventing the malignant transformation of endometrial hyperplasia to endometrial cancer. This aim of medical treatment is usually achieved through the activation of apoptosis. Endometrial hyperplasia develops as a result of prolonged endometrial stimulation by estrogen in the absence of a progesterone effect locally in the uterus. During the postmenopausal period, the systemic level of estrogen is decreased, while locally in the uterus,

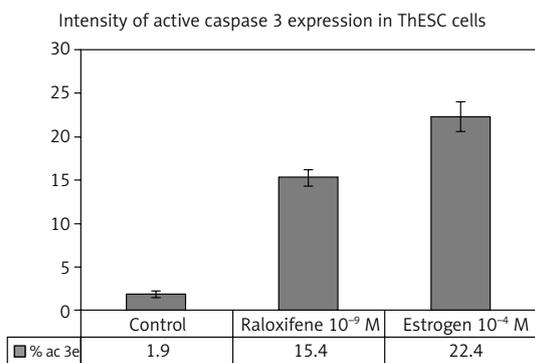
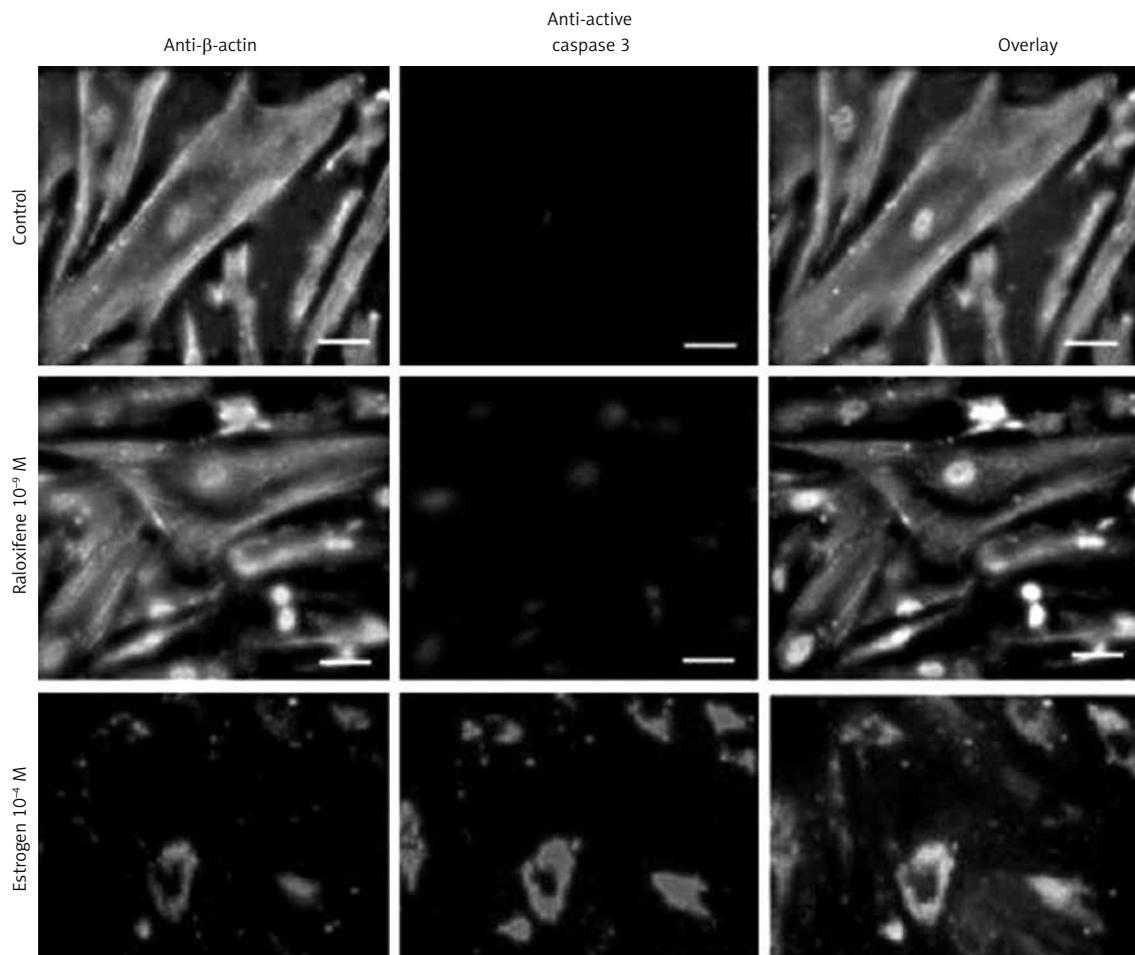


Figure 5. Expression and localization of active caspase 3 both in control and treated cells with raloxifene and estrogen. Expression of active caspase 3 in control cells is statistically lower compared to the expression of active caspase 3 in both raloxifene- and estrogen-treated cells. Expression of active caspase 3 in the cells treated with both raloxifene 10⁻⁹ M and estrogen 10⁻⁴ M is significantly higher compared to the expression of active caspase 3 in control cells

it is otherwise. Due to this hormonal imbalance, the morphological changes of the endometrial cell integrity begin to take their course towards uncontrolled proliferation, named hyperplasia. According to the literature data, there is a direct correlation indicating that higher levels of estrogen induce synthesis of new ER, causing an increase in their expression [18]. In our study, we used a human endometrial stromal ThESC cell line, isolated from the patient with myoma, as a cell model for endometrial hyperplasia. Until now, no studies have investigated and determined the effects of raloxifene and estrogen on cells originating from the endometrium that were locally exposed to increased levels of estrogen, such as ThESC cells. Here, for the first time, we investigated both cytotoxic effects and apoptotic mechanisms of raloxifene and estrogen on ThESC cells.

During the past decade, investigations concerning the medical treatment of endometrial hyperplasia were mainly directed towards either the elimination or the reduction of the primary stimulating effect of estrogen. Even though raloxifene, a selective estrogen receptor modulator, was registered for the treatment of osteoporosis, ongoing investigations have not yet revealed the exact mechanism of apoptotic action of raloxifene on endometrial hyperplasia.

As a potent female sex hormone, estrogen stimulates growth and inhibits apoptosis in estrogen-dependant tissues through ER-mediated mechanisms. Interestingly, there is strong evidence suggesting estrogen's induction of apoptosis in breast cancer and other cancer cell types [2–4]. However, similar to raloxifene, the precise mechanism of estrogen's induced apoptosis has not yet been precisely explained. Recent investigations suggested the involvement of both the extrinsic (Fas/FasL) and the intrinsic (mitochondria) apoptotic pathways in this process. Moreover, a recent study showed that long-term estrogen deprivation (LTED) of estrogen-dependent MCF-7 breast cancer cells caused them to undergo adaptive changes in which estrogen switched from being a proliferative agent to paradoxically a growth-inhibiting agent and inducer of apoptosis [4]. In the study conducted by Liu *et al.* [13] the authors demonstrated that treatment of cultured leiomyoma cells with different doses of raloxifene and estrogen at a dose of 10^{-7} M lowers the expression of anti-apoptotic Bcl-2 protein, thus inducing cell death. The results of our study correlate with the findings of Liu *et al.* [13].

In this study we investigated the cytotoxic effects of different doses of raloxifene and estrogen on the ThESC cell line. Our results suggested that estrogen showed different effects on the viability of the ThESC cell line in a dose-dependent manner.

Administration of estrogen at the highest applied dose of 10^{-4} M induced the cytotoxicity of ThESC cells below 10%, whereas the lowest estrogen dose of 10^{-9} M caused a statistically significant increase in the percentage of cytotoxic cells compared to the control. These results suggested an anti-proliferative effect of estrogen similar to the inhibiting growth changes observed in the estrogen-deprived cells as described earlier [4].

Furthermore, the literature data concerning the effect of raloxifene on viability of cell lines indicated that raloxifene showed a significant inhibitory effect on the viability of the human prostate cancer cell line [12] and of the primary isolated cell line derived from myoma uteri [13]. It is well known that the modulation of ER by raloxifene caused the transcriptional activity of the genes crucial for the synthesis of proteins that play a key role in either cell proliferation or death [8]. Specifically, the presence of aspartate at position 351 in the ligand-binding domain (LBD) of ER α is crucial for formation of the hydrogen bond of raloxifene with LBD of ER, and therefore it is responsible for the antiestrogen activity of raloxifene/ER complex [19]. In addition, Liu *et al.* conducted one of the first studies that demonstrated a direct effect of raloxifene on both proliferation and apoptosis in human leiomyoma cells cultured *in vitro* [13]. According to our results, raloxifene showed a cytotoxic effect on the ThESC cell line in a dose-dependant manner. The highest cytotoxic effect of raloxifene was 18.04% at a dose of 10^{-6} M, while 5.09% of cytotoxic cells were present at the lowest applied dose of raloxifene of 10^{-9} M. Thus, our results correlated with the findings of Liu *et al.* [13] and indicated that raloxifene treatment caused a significant decrease in the percentage of viable ThESC cells compared to the control cells. So far, the mechanism of the apoptotic effect of raloxifene and estrogen on the ThESC cell line has not been studied. Here, in our experiments we explored the apoptotic mechanism of raloxifene and estrogen in ThESC cells by determining both expression and the cell's localization of the key regulatory apoptotic proteins including Bax, Bcl-2 and active caspase 3. In order to determine apoptotic effects of investigated substances on the ThESC cell line, annexin V FITC/PI staining was performed after 24-hour treatment. Some of the well-described changes in the cell morphology caused as a result of ongoing apoptosis include cell shrinkage, blebbing, aggregation of chromatin, nucleus fragmentation and formation of apoptotic bodies [14]. In addition, one of these crucial changes in the apoptotic cell include the translocation of phosphatidylserine from the inner to the outer side of the plasma membrane where it can be detected by annexin V FITC/PI staining applying the flow cytometry method.

According to our results obtained with annexin V FITC/PI staining, the cytotoxic effect of raloxifene and estrogen on the ThESC cell line was mediated by induction of apoptosis. The range of the percentage of apoptotic cells for increasing doses of raloxifene was from 5.75% to 19.21%, and for decreasing doses of estrogen was from 8.6% to 30.1%.

In order to determine the apoptotic pathway involved in raloxifene- and estrogen-induced apoptosis in the ThESC cell line, both the expression or the activation and the cell's localization of crucial pro- and anti-apoptotic proteins were determined using the immunofluorescence method. Thus, the expression or the activation and the cell's localization of Bcl-2, Bax and caspase 3 were determined following 24-hour treatment with the investigated substances. The available literature data showed that the expressions of both Bcl-2 protein and PCNA (proliferating cell nuclear antigen) in cultured leiomyoma cells were decreased following raloxifene and estrogen administration, indicating the potent role of the investigated substances in the induction of apoptosis [13]. In line with a previous study [13], our results demonstrated a statistically significant decrease in the expression of Bcl-2 in ThESC cells treated with raloxifene and estrogen compared to the untreated cells. Moreover, the study conducted by Robertson *et al.* showed that raloxifene induced apoptosis through interaction and modulation of the ER receptor [20].

In addition, one of the crucial events in the mitochondrial apoptotic pathway is activation and translocation of pro-apoptotic Bax from the cytosol to the outer membrane of mitochondria and the subsequent release of cytochrome c from the inner membrane space of mitochondria into the cytosol, formation of the apoptosome and the activation of the executor caspase 3. Our study showed that raloxifene and estrogen caused activation of the mitochondrial apoptotic pathway by inducing activation of BAX, causing it to translocate into the mitochondria, which activated downstream apoptotic events leading to the subsequent activation of caspase 3 and execution of apoptosis.

The results of our study, for the first time, demonstrated the cytotoxic and apoptotic effects of raloxifene and estrogen on human endometrial stromal cell ThESC cells and suggested the involvement of the inner mitochondrial apoptotic pathway in both raloxifene- and estrogen-treated ThESC cells.

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Conflict of interest

The authors declare no conflict of interest.

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