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ORIGINAL ARTICLE

UV irradiation induces apoptosis in the human endometrial stromal cell line (ThESC)

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Summary

Purpose: *The treatment options of endometrial hyperplasia consist of surgical, interventional and medical therapies including apoptosis-inducing agents. The purpose of the study was to evaluate the effects of ultraviolet (UV) radiation on the viability and the type of cell death on the human endometrial stromal cells (ThESC) line.*

Methods: *We investigated the effect of UV exposure on human endometrial stromal cell line (ThESC) on cell viability using MTT assay as well as changes in cell morphology using phase microscopy and acridine orange (AO)/ethidium bromide (EB) cell staining.*

Results: *UV treatment significantly decreased the percentage of the viable ThESC cells compared to the viability of untreated control cells using MTT assay ($p<0.05$). In addition, UV treatment of ThESC cells for 60 and 90 min induced high level of cell morphology disruption, followed with loss of both the cell shape and the presence of defragmented debris and stained with intense red color.*

Conclusions: *The obtained results suggest the potential role of UV light application as additional treatment option of benign endometrium hyperplasia alone or in combination with other treatment modalities.*

Key words: *cytotoxic effect, endometrial stromal cell line, MTT assay, acridine orange/ethidium bromide staining*

Introduction

Apoptosis, an important and highly controlled form of programmed cell death, may be induced by various biological and chemical agents under both physiological and pathological conditions [1]. UV radiation represents a powerful cytotoxic and apoptotic agent due to its ability to induce DNA damage. UV light exerts distinct mutagenic properties in the cell due to the different UV wavelength types that include UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm) [2]. The key molecular mechanisms of UV-induced apoptosis in cells are presented through the activation of the mitochondrial intrinsic pathway, resulting in the formation and accumulation of reactive oxidative species (ROS), direct DNA damage, and the clustering of death receptors on the cell surface (extrinsic pathway) that consequently lead to the irreversible cell damage and death [2,3]. Phototherapy induces apoptotic changes of certain cells or organs through their local exposure to UV rays. The adverse effect of UV exposure on both healthy and damaged cells is used for phototherapy in the treatment of different types of diseases including cutaneous graft versus host disease [4], sclerotic skin diseases and localized infections [5,6]. Activation of apoptosis in the cell with UV treatment implements various

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morphological and metabolic changes including disturbance of the mitochondrial metabolism, cell cycle arrest, DNA damage, cytoplasmic changes, hydrolysis of the plasma membrane that consequently result in the ceramide accumulation at the cell surface [7,8]. Furthermore, in the UV-treated cells, actin filaments undergo specific apoptotic morphological changes that include their impaired adherence and disruption, resulting in cell instability [9]. UV-C ray disrupts the genetic integrity of the cell by directly causing damages to DNA, due to its shortest wave length and the highest energy. In order to determine the effect of UV light exposure on different type of cells, numerous studies have been evaluating the efficiency of UV radiation on induction of apoptosis. In the experiments conducted by Kimura et al, the effect of UV radiation on induction of apoptosis and the cell viability was evaluated in human osteosarcoma cells, HT-1080 human fibrosarcoma cells, Lewis lung carcinoma (LLC) and XPA-1 human pancreatic cancer cells *in vitro* [10]. Based on their results, UV-C irradiation of cancer cells decreased the percentage of the viable cells due to apoptotic induction. Kulms et al [11] evaluated the effect of UV-B radiation combined with low temperature on apoptosis induction in the HeLa human epithelial carcinoma cell line. Based on their results, low temperature combined with UV radiation promoted cell death exclusively by affecting and disrupting the cell membrane. Dai et al [12] investigated the effect of UV radiation on the contents of sphingomyelin (SM) in subcellular compartments of HeLa cell line. Their results demonstrated that UV irradiation of HeLa cells dramatically increased the levels of SM in all cellular compartments, especially in mitochondria. In experiments conducted by Nijhawan et al [13] exposure of HeLa cells to UV radiation in the range of 254 nm (corresponding to wavelength UV-C) induced apoptosis in HeLa cells via mitochondrial pathway. In their study, the activation of the inner apoptotic pathway was carried out by the inhibition in the antiapoptotic Mcl-1 protein's synthesis that is crucial for the antiapoptotic Bcl-xl's cytosolic localization. As a result of UV radiation and subsequent inhibition of Mcl-1 synthesis, cytosolic Bcl-xL translocates to the outer membrane of the mitochondria, consequently leading to changes of mitochondrial membrane permeabilization and eventually to events responsible for the activation and execution of apoptosis including activation of apoptotic Bax, cytochrome C release from the mitochondria and caspase 3 activation [13-15].

The aim of this study was to investigate the effect of different exposure times to direct UV-C radiation on the viability and on the induction of apoptosis in the endometrial stromal cell line ThESC.

Methods

In our experiments we used human endometrial stromal cell line (fibroblast like) immortalized by reversible human telomerase transcriptase (hTERT), ThESC cell line (ATCC ®: CRL-4003tm). The cells were cultured and maintained in DMEM complete growing medium containing 4.5 g of glucose/L, 2% L-glutamine (2mM), 1% penicillin/streptomycin, 1% of non-essential amino acids, 1% of insulin transferin supplement and 10% fetal bovine serum (FBS) in control environment at 37C° and 5% CO_2. Before the exposure to UV-C, cells were washed three times in 1xPBS.

UV-C exposure

A lamp emitting direct UV-C radiation at a wavelength of 253.7 nm and an intensity of 36 W (180 J/m^2) was used in the experimental cell group. The cells were divided into two major groups, first, the experimental group (UV-C treated cells) and control group of cells (UV-C untreated cells). Cell exposure to UV-C was conducted in three different time periods of 30, 60 and 90 min after which both the viability and the cell morphology changes were assessed using MTT assay and acridine orange (AO)/ethidium bromide (EB) staining.

MTT assay

MTT assay was used in order to determine the effect of UV-C radiation on the viability of experimental cells in comparison to control cells. The percentage of viable cells in the experimental group was calculated and compared to the percentage of viable cells in the control group. Specifically, both control and experimental cells were resuspended in DMEM medium (1.8×10^4 cells/200µl medium), seeded in 4x12 well micro titer plates (1 plate for the control cells, while the remaining plates were exposed to UV-C radiation depending on the incubation time) and exposed to UV-C light for 30, 60 and 90 min (the lid of the plate in the experimental group was removed, and the ventilator in the hood was turned off). After UV-C irradiation, the cells were incubated with the MTT solution (5 mg/ml MTT dissolved in PBS) for 4 h (37°C, 5%CO_2). After centrifugation (1000 rpm, 5 min) and removal of the supernatant, cells were resuspended with 200µl DMSO (Sigma Chemical, ST. Lois, Mo.) per well and incubated for 30 min on a shaker in the dark at room temperature. The absorbance was measured at 595 nm wavelength (multimode micro plate detector, Zenith 3100). The percentage of viable cells was calculated using the formula: mean optical density (OD) treatment/mean OD control *100= % of viable cells.

Morphological changes

Following UV-C radiation for 30, 60 and 90 min, cell morphology was examined by phase-contrast microscopy.

Staining procedure by acridine orange/ethidium bromide

In order to visualize early and late apoptotic changes for both control and treated cells 0.01% acridine orange/ethidium bromide (AO/EB) was performed. AO/EB

was used to stain ThESC cells grown on coverslips in 12 well plates. After different times of exposure (30, 60 and 90 min) to UV irradiation, cells were fixed in 95% methanol and 5% PBS at 32°C for 10 min and then stained with 0.01% AO/EB (Sigma Aldrich, A8097) in PBS at room temperature for 5 min. Coverslips with cells were then mounted onto slides and examined using Olympus BX51microscope.

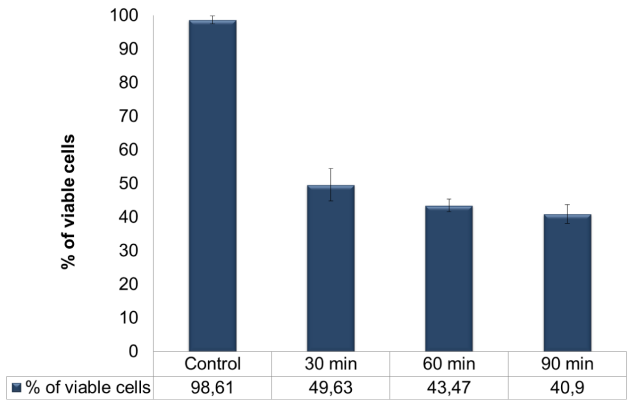

Figure 1. Determination of the percentage of the viable ThESC cells was evaluated using MTT assay after UV exposure. The percentage of the viable cells after UV exposure was significantly lower in every experimental group compared to the viability of the control group of cells. Statistical evaluation was performed using Student's t-test for paired observations, or one-way ANOVA depending on data distribution. Significant differences were observed between control and UV-treated cells ($p<0.05$).

Statistics

Statistical evaluation was performed using Student's t-test for paired observations, or one-way ANOVA depending on data distribution. P values that were less than 0.05 were considered significant. The data were evaluated using SPSS for Windows software package.

Results

MTT assay results showed that the viability of the cells exposed to UV treatment decreased significantly ($p<0.05$) compared to the control cells (Figure 1). The group of cells that were exposed to UV treatment for 90 min had 40.9% viable cells, the group of cells treated for 60 min showed 43.47% viable cells, while the group of cells treated for the shortest time (30 min) showed 49.63% viable cells. In the experimental groups of cells, UV exposure lowered the percentage of the viable cells in a time-dependent manner, however, without statistical significance ($p>0.05$). The percentage of control untreated viable cells was 98.61%, which was higher by 2.4, 2.2 and 1.9 times compared to the percentage of the viable cells exposed UV radiation for 30, 60 and 90 min, respectively ($p<0.05$). Based on the results of MTT assay, UV treatment significantly decreased the percentage of the viable ThESC cells compared to the viability of the control cells.

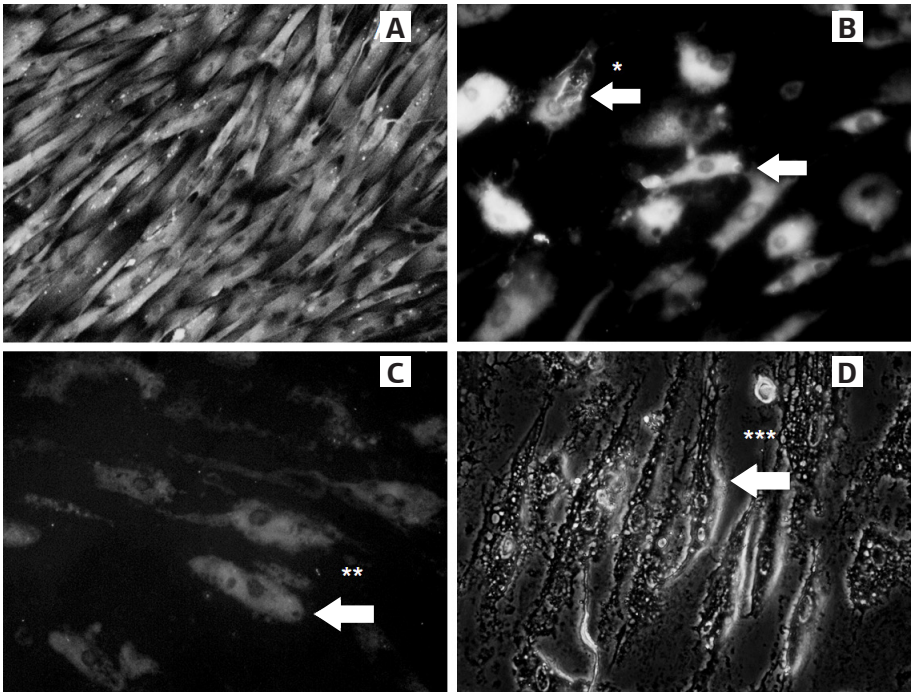

Figure 2. Morphological features of human endometrial stromal cells ThESC exposed to UV irradiation for 30, 60 and 90 min **(B, C and D)** compared to control cells **(A)**. In cells exposed to UV irradiation disrupted morphological features could be observed. UV-treated cells lost their shape*; cell shrinkage was visible*; with complete disruption of the cell morphology***. The most visible morphological damage was present in cells that were exposed to UV irradiation for 90 min.

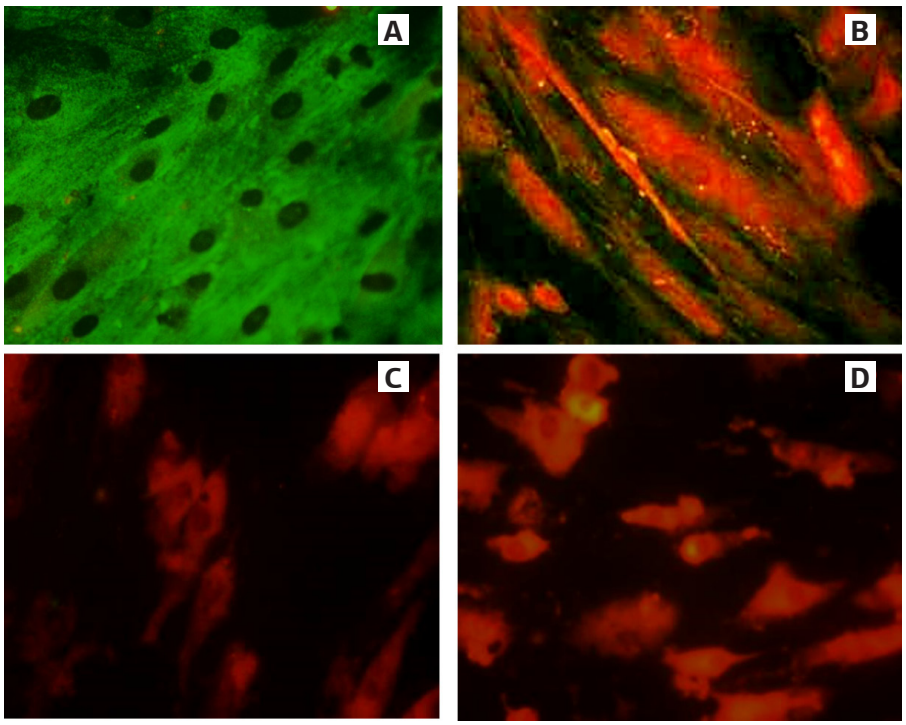

Figure 3. AO/EB staining of both control and experimental (ThESC) cells. **A:** Control cells are seen as normal, viable cells with intensive green color. **B:** Endometrial stromal cells after exposure to UV radiation for 30 min. Normal morphology of the cell is disrupted, with presence of green/orange red spectrum corresponding to early apoptotic cell. **C** and **D:** Endometrial stromal cells after 60 and 90 min of exposure to UV radiation. Complete disappearance of morphology of the cells is seen with clear loss of cell shape and higher red color intensity. Orange red color intensity corresponds to late apoptotic changes.

Changes in the cell morphology between control and UV-treated cells where determined using phase microscopy. Following UV-C radiation of the cells for 30, 60 and 90 min, morphological changes of the UV-treated cells clearly indicated significant disturbance of cell morphology compared to the morphology of untreated cells ($p<0.05$; Figure 2). Exposure of ThESC cells to UV radiation clearly resulted in cytoskeletal blebbing, loss of the cell shape, membrane disruption and shrinkage of the whole cell, indicating severe cell damage (Figure 2). The alterations of actin filaments that support the cell cytoskeleton morphology are shown in Figure 2. All these morphological changes in the UV-treated cells clearly contributed to the cell detachments and death.

The apoptotic changes in the UV-treated cells were investigated using AO/EB staining (Figure 3). The control group of cells showed normal morphology and appeared green after AO/EB staining (Figure 3A). However, the experimental cells exposed to 30 min of UV radiation presented some disturbance in the cell morphology and stained with orange/red color (Figure 3B). Cells exposed to UV radiation for 60 and 90 min (Figure 3 C, D) expressed high morphology disruption followed with loss of both the cells shape and the presence of defragmented debris stained with intense red color.

The difference in cell morphology between the experimental cells that were exposed to UV radiation for 60 and 90 min showed no statistical significance. These results suggest that exposure of ThESC cells to UV radiation for different time points induced changes in cell morphology. These changes were in direct correlation with the UV exposure times and ranged from mild to severe.

Discussion

UV radiation can be described as a double-edged sword; on the one hand it represents a powerful carcinogen (sunlight) causing sunburn inflammation (erythema) and local or systemic immunosuppression, photocarcinogenesis, and photoaging, while on the other hand usage of the artificial lamps emitting UV radiation is used in the therapy of many skin diseases including psoriasis and vitiligo [16,17]. Treatment of skin diseases using UV radiation (mostly UV-B) is also followed with vitamin D production in the skin [17] generating nitric oxide (NO) that may reduce blood pressure, have antimicrobial effects, act as a neurotransmitter and may improve the mood through the release of endorphins. Besides the positive effect of UV therapy, the second blade of the sword arises in the form of undesirable side effects. One of the most undesir-

able effects of UV radiation is the high rate of mutagenesis of the cell caused as a direct effect of UV ray on DNA. Thus, as a result of prolonged exposure to UV radiation, cells undergo malignant transformation resulting in the development of malignant tumor. This most common side effect of UV radiation is being used in many *in vitro* studies where investigators researched the effect of UV exposure on different types of human tumor cell lines. The aim of modern therapies is selectivity to positively transforming tumor cell without negative consequences on normal cell. This selectiveness and array of different methods are used in *in vitro* studies to induce apoptosis in tumor cells. In experiments conducted by Miller et al and Lin et al [18,19] the effect of UV radiation on HeLa cells and seven types of human non-small cell lung carcinoma lines (CL1-0, CL1-1, CL1-5, CL1-5–F4, NCI-H358, NCI-H1437 and A549) was mediated via induction of apoptosis that was realized via caspase 3 and cytochrome C release from mitochondria to cytosol (activation of inner mitochondrial apoptotic pathway). Combination of both UV-C radiation and two caspase inhibitors (ICE and CPP32) did not abolish UV-C induced apoptosis in HeLa cells, but it altered its incidence and progression [20]. Similar to these previous researches, here we investigated the effect of different time exposure of ThESC cells to UV radiation on both cell viability and induction of apoptosis. Based on our results, we demonstrated that the different time points of 90, 60 and 30 min of UV exposure of cells significantly decreased the percentage of the viable ThESC cells compared to the control group. Not all experimental groups of cells showed statistically significant difference in the percentage of the viable cells. Our results obtained by MTT assay are in positive correlation with the available literature data. After we had determined significant decrease in the percentage of the viable cells that were exposed to UV radiation, our next step was to determine the type of the cell death caused by this type of radiation. The main difference between the two key types of cell death, apoptosis and necrosis are: necrosis represents premature death of cells caused by external factors, such as infection, toxins or trauma. During necrosis membrane disruption occurs, hypoxia causing ATP depletion, metabolic collapse, cell swelling and rupture of the cell that is leading to inflammation [21], while during apoptosis cells undergo apoptotic changes including cell blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation. In order to differentiate necrosis from apoptosis AO/EB staining of the cells was conducted after UV radiation. Here, we stained UV-treated ThESC cells with AO/EB and our results demonstrated specific apoptotic changes in cells exposed to UV radiation. In the experimental group of the UV exposed cells, AO/EB staining showed both presence of ongoing apoptosis and different stages of cell death (from early to late stages of apoptosis). Our study, for the suggests first time that UV radiation causes decrease in the percentage of the viable ThESC cells by induction of apoptosis.

Conclusion

Numerous studies have shown that the UV light can be a potent cytotoxic agent in inducing apoptosis in various cells systems. In our investigation, for the first time, we have examined the effect of UV exposure on ThESC cells viability and the possible type of cell death caused by UV light. The obtained results by applying MTT assay and AO/EB staining indicated efficient UV radiation-induced cytotoxicity and apoptosis in ThESC cells. These results suggest the potential role of UV light for nonsurgical treatment option for benign changes of endometrial hyperlasia, alone or in the combination with other treatment modalities.

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Conflict of interests

The authors declare no conflict of interests.

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