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Original Article

The effects of endosulfan on the expression levels of DNA damage and apoptotic genes in HT22 cells: First preliminary study

Sibel Demir Ozturk[1], Sadegul Tuncer[1], Husniye Rustemoglu[1], Aydin Rustemoglu[2]

[1]Department of Medical Biology, Faculty of Medical, Tokat Gaziosmanpasa University, Tokat, Turkey
[2]Department of Medical Biology, Faculty of Medical, Aksaray University, Aksaray, Turkey

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Abstract

Aim: The aim of this study was to determine the IC50 dose of endosulfan in the hippocampal HT22 cell line, and also to elucidate the effect of expression of DNA-PK, Bax, Bcl2 and Casp-3 genes involved in DNA repair and apoptotic pathway.

Material and Methods: Cytotoxic effect of endosulfan and IC50 dose were determined by using XTT method after 24 hours of culture by applying endosulfan at 5 different concentrations (10, 25, 50, 75 and 100 µM) to HT22 cell lines.

HT22 cells were then seeded into 6 sterile plates and treated with Endosulfan at IC50 for 12 hours. The expression changes of DNA-Pk, Bax, Bcl2 and Casp-3 genes, after total RNA isolation and cDNA formation, were determined by RT-PCR. Expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

Results: After 24 hours of endosulfan treatment at different doses in HT22 cell lines, a significant loss of viability was observed in all endosulfan treated groups. It was determined by XTT test, that the IC50 dose of endosulfan was 50 µM in 24 hours treatment. After 12 hours administration of IC50 endosulfan dose in HT22 cells following the examinations of DNA-PK and some apoptotic genes we observed different amounts of increases in expression as follows; 5-fold for DNA-PK, 18-fold for Bax, and 4-fold for Casp-3. On the other hand, approximately 2-fold decrease was detected in Bcl-2 gene.

Conclusion: The IC50 dose of 24-hour endosulfan administration in HT22 cell lines was found to be 50 µM. Expression changes in the proapoptotic and antiapoptotic genes have shown that apoptosis is induced in endosulfan-administrated cells. In addition, the increase in DNA-PK gene expression suggests that endosulfan causes DNA damage in cells and triggers DNA repair mechanisms.

Keywords: Apoptosis; DNA-PK; endosulfan; HT22

INTRODUCTION

Organochlorinated pesticides (OCP) are one of the classes of pesticides known to cause toxic and various health effects on non-target organisms, including humans (1,2). Endosulfan (ES) (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide), is an cyclodien OCP member with α and β isomers (3: 1 ratio), and an environmental pollutant pesticide with negative effects on human health (3,4). ES, which has been used as a pesticide since the 1960s, has been banned in 2007 in Europe as well as in Turkey.

It is known that ES has adverse health effects such as reproductive defects, neuro-behavioral disorders, endocrine and immunological toxicity (4,5). In addition, ES has been shown to block GABA-mediated chlorine channel operation, impair brain cortex function, and cause neurotoxicity (6). The mechanisms of action of ES in causing these diseases include mitochondrial dysfunction, induction of oxidative stress, and modulation of stress-sensitive signal transduction pathways (5).

Recently, in vitro, ex vivo and in vivo studies have shown that ES induces cellular Reactive Oxygen Derivatives (ROS) which caused double strand breaks (DSBs) in DNA (7). DSBs are a rare form of mutation with very serious negative consequences. DSBs can be repaired by two types of pathways: Homologous recombination (HR) and non-homologous end joining (NHEJ) (8,9). HR is a typical genetic recombination and requires the presence of homologous DNA sequences. NHEJ, the other repair mechanism of DSBs, is a repair mechanism characterized by direct binding at the broken ends, without directing by homologous sequences (10). In the first stage of NHEJ, the Ku70 and Ku80 heterodimeric protein complex binds directly to the double chain break site and protects it from degradation. This heterodimer Ku complex also interacts

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Corresponding Author: Aydin Rustemoglu, Department of Medical Biology, Faculty of Medical, Aksaray University, Aksaray, Turkey
E-mail: arustamov@yahoo.com

with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to trigger the intracellular repair pathway (11).

Although several studies investigating the molecular mechanism of cytotoxicity effect of endosulfan and the relationship with apoptosis have been reported in the literature, there are no studies investigating the effect of DNA-PK expression, which plays role particularly in DNA double chain breakage repair and apoptosis. In addition, there is no study investigating the cytotoxic effect of the endosulfan on the HT22 cell line and its IC50 dose. In this study, we were investigated the IC50 concentration of the ES by XTT method after 24 hours of administration of different ES doses in HT22 cell lines. Than after were investigated relationships of the IC50 dose of ES with the expression of the DNA-PK which effective in the NHEJ repair pathway, and Caspase 3, Bcl-2 and Bax genes which effective in the apoptosis.

MATERIAL and METHODS

The study was conducted in Tokat Gaziosmanpaşa University Faculty of Medicine Cell Culture Laboratory and Medical Biology Department Laboratory. In our study, cytotoxic effect of ES in HT22 cell lines and DNA repair were investigated. For cytotoxicity the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl) -2H-Tetrazolium-5-Carboxanilide) method was used. The expression levels of targeted genes were found by RT-PCR.

Cell culture

In this study; hippocampal neuronal HT22 cell line was used. The HT22 cell line is a cell line developed as a sub-line from the HT4 trunk line, which is immortalized mouse hippocampal neuronal precursor cells (12-14). Because of their tissue origin, HT22 cells have been used as hippocampal neuronal cell models in many studies (15-19).

1 vial frozen (approximately 1,000,000 cells) HT22 cells used in our study were thawed rapidly in a water bath set at 37 °C, and then seeded in cell T25 flask with DMEM-HA (Capricorn) medium, which admixtured 10% FBS (Capricorn), 1% penicillin/streptomycine (Capricorn), and 1% L-glutamine. Cells were incubated at 37 °C growns of 95% humidity and 5% CO_2. The density of HT22 cells in the flask held in a CO_2 incubator for 48-72 hours was examined by invert microscopy and cells were passage from the flask that reached 80-90% density (Confluence) to new flasks. The number and viability of the grown cells were determined with Trypan Blue. After the third passage, for the stock the Freezing protocol with dimethylsulfoxide (DMSO) was done and cells were stored. After the fourth passage, HT22 cells were seeded in 12 well plates with an average of 100,000 cells added in each well to see the morphological effect of different doses of ES. Simultaneously an average of 10000 cells seeded in each well of 96 well plates for the detection of IC50 dose of ES with the XTT method. Sowing well plates were allowed to incubate in a CO2 incubator at 37 ° C for 24 hours in a medium.

In vitro cytotoxicity assays

When the density of the cells was 70% in each of the wells of the 96 well plate which seeded, ES were applied this wells as 10, 25, 50, 75 and 100 µM concentrations. For the observation of morphological changes, same ES concentrations were applied the 12 well plates. Table 1 shows the experimental scheme for the cytotoxicity test performed on a 96 well plate.

XTT Cell Viability Test (Biotium) was applied to the cells after 24 hours of ES application. The absorbance signal of the cells was measured at 492 nm wavelength by spectrophotometer (Heales MB-530) and cytotoxicity level was determined according to the absorbance value read. Cytotoxicity levels were calculated by the following formula:

(Absorbance of the test well - Absorbance of the medium well / Absorbance of the control well - Absorbance of the medium well)

A dose of 50 µM was determined as the 24-hour ES IC50 dose for HT22 cells after XTT test.

Table 1. Experimental scheme of ES for XTT cell viability test in HT22 cell line in 96 well plate

X	X	X	X	X	X	X	X	X	X	X	X
X	M	C	10µM	25µM	50µM	75µM	100µM	C	C	M	X
X	M	C	10µM	25µM	50µM	75µM	100µM	C	C	M	X
X	M	C	10µM	25µM	50µM	75µM	100µM	C	C	M	X
X	M	C	10µM	25µM	50µM	75µM	100µM	C	C	M	X
X	M	C	10µM	25µM	50µM	75µM	100µM	C	C	M	X
X	X	X	X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X

X: Free, C: Control (Mediun+HT22 cells), M: Medium (Only); Applied Endosulfan concentrations: 10, 25, 50, 75 and 100 µM

Total Ribonucleic Acid Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

After determining the IC50 dose of ES, the HT22 cells obtained after the fourth passage for the main experiment were seeded into six-well culture dishes with approximately 300,000 cells per well. When the HT22 cells reached a concentration of 70% in a CO2 incubator at 37°C for 24 hours incubation in 5% CO2 medium, ES solution was added to in each well so that a final concentration of 50µM. ES solution was added to each well, as to a final concentration of 50µM. To control wells were added medium which containing DMSO at equivalent concentration with ES-treated wells. For both endosulfan and control groups' three wells were used. 12 hours after ES administration, HT22 cells were collected separately for the isolation of total ribonucleic acid (RNA) for each group (ES and control). Total RNA was isolated for each experimental group using a commercial kit (Hybrid-RTM RNA, GeneAll, Korea). cDNAs was obtained with a ready-made commercial kit (GeneAll 2X HyperScript TM master mix, GeneAll, Korea), from the purified total RNA. Using these cDNAs, mRNA expression levels of Bax, Bcl2, Caspase 3 and DNA-PK genes were determined. The application was performed on the StepOnePlus RT-PCR instrument (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). The results were checked by performing three RT-PCR reactions for each sample. $2^{-\Delta\Delta Ct}$ was calculated for each target gene using mean CT values obtained from PCR reactions. Actin Beta gene was used as internal control and expression levels of other genes were normalized. This value calculated for each gene represents the mRNA level of the control samples relative to the control samples. Results were evaluated using the StepOne™ Software on the StepOnePlus RT-PCR instrument.

RESULTS

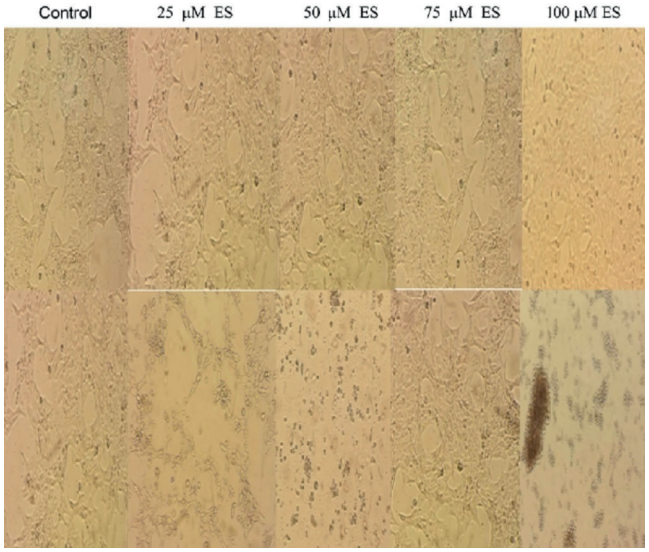

Figure 1. Invert Microscope (10X) Image of Endosulfan Doses (Control, 25, 50, 75 and 100 µM) in HT22 Cell Series

Table 2. ELISA Results and Cytotoxicity Ratios of Endosulfan Concentrations in HT 22 Cell Series

		Control	Medium	1.Dose (10)	2.Dose (25)	3.Dose (50)	4.Dose (75)	5.Dose (100)
ELİSA (Raw Data)	1. experiment	0.4801	0.2729	0.4434	0.4314	0.3884	0.3009	0.2984
	2. experiment	0.5748	0.3482	0.5542	0.5360	0.4550	0.4167	0.4093
ELİSA (Calculated)	1. experiment			0.1705	0.1585	0.1155	0.028	0.0255
	2. experiment			0.2060	0.1878	0.1068	0.0685	0.0611
Cytotoxicity (%)	1. experiment			18	24	45	86	88
	2. experiment			10	18	53	70	74

The microscope views of the 12 well plates are given in Figure 1 to observe the morphological effect of ES on the cells. After 24 hours of ES administration, dose-dependent changes (Control, 25, 50, 75 and 100 µM) were observed in the HT22 cell line before and after administration.

The experiment was repeated twice to determine the IC50 value of ES in our HT22 cells. Table 2 shows the results of ELISA and calculated cytotoxicity ratio by means of microplate reader after XTT test.

The comparison of the results of the two experiments we performed in our study with the cytotoxicity ratio after 24 hours of ES administration at different doses is shown graphically in Figure 2.

In Table 3 shows the mean CT's and its $2^{-\Delta\Delta Ct}$ values calculated from the average CT's of the amplification curves obtained from RT-PCR application, after 12 hours treatment of HT22 cells with of 50 µM ES.

The graphic shown in Figure 3 shows that after 12 hours of ES treating, Bax gene expression is increased 18-fold and Bcl 2 gene expression is suppressed (compared to the control). A 4-fold increase of Casp3 gene expression is observed. Similarly, the DNA-PK gene expression in the NHEJ pathway was found to be 5-fold higher in ES treated cells than the control.

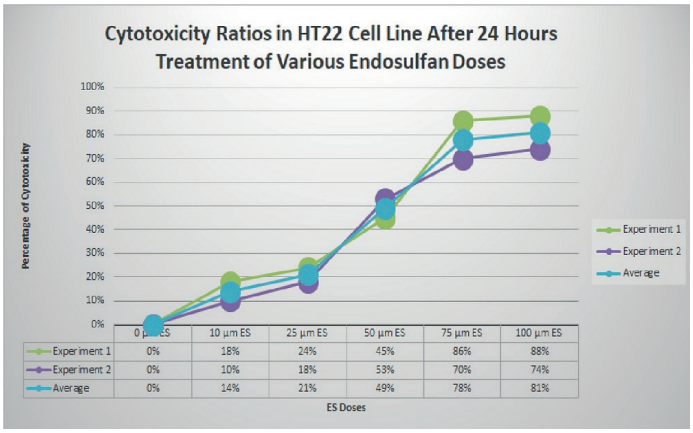

Figure 2. Comparison of Cytotoxicity Ratios of Endosulfan Doses (10, 25, 50, 75 and 100 µM) Applied in HT 22 Cell Line

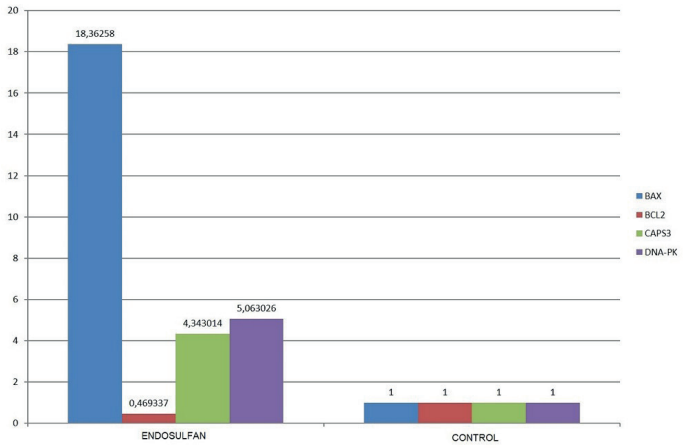

Figure 3. Comparison of BAX, BCL2, CASP3 and DNA-PK gene expression data according to RT-PCR results between endosulfan administration and control groups

Table 3. Average RT-PCR Ct ve $2^{-\Delta\Delta Ct}$ Values of Analized Genes

C_t	BAX	BCL2	CASP3	DNA-PK
Endosulfan	29.35	29.79	24.73	31.69
Control	31.10	26.25	24.40	33.65
$2^{-\Delta\Delta Ct}$				
Endosülfan	18.36258	0.469337	4.343014	5.063026
Control	1	1	1	1

DISCUSSION

One of the important classes of pesticides is organochlorinated pesticides. It is known to cause toxic and various health effects on non-target organisms including humans (2). One of member of this group, Endosulfan, it is known that has adverse health effects such as endocrine, immunological toxicity, reproductive defects and neurobehavioral disorders (4-6). The mechanisms of action that ES plays a role in the development of these disorders include mitochondrial dysfunction, signal transduction pathways and oxidative stress induction (5). In various studies to determine the cytotoxic effect of ES in the literature, it was observed that IC50 doses may be different in different cells. In a study by Sinha et al., using rat testicular cells in culture (Sertoli-germ cell culture), the cytotoxic effects of ES was evaluated. The researchers emphasized that doses between 20 and 80 µM were effective and the toxicity was higher after 48 hours in the experiment performed with 24 and 48 hours treatment using 2, 20, 40 and 80 µM doses of ES (20).

In a study by Sohn et al., the toxic effect of ES in human cell lines HeLa and HepG2 cells was investigated. In a 48-hour experiment conducted with 50, 100 and 200 µM doses of ES by the researchers, HepG2 cells was found much more susceptible to ES than HeLa, which IC50 doses were 49.37 µM for HepG2 and 86 µM for HeLa. As a result, it has been shown that the sensitivity of different cell lines to ES may be different (21).

In a study by Sebastian et al., after 48 and 72 hours of treatment with different doses of ES (1, 5, 10, 25 and 50 µM) for Reh and K562 cells, the IC50 value of ES toxicity in 48 hours treatment 29.5 µM for Reh and 43.2 µM for K562, were found (7).

In the literature, there are no studies investigating the effect of ES on HT22 cell lines, which used in our study. Therefore, we aimed to determine the IC50 dose after 24 hours of treatment at different doses of ES. In this context, our experience was repeated twice to find the effective dose. In the first experiment, the cytotoxicity of ES were found 18% for 10 µM, 24% for 25 µM, 45% for 50 µM, 86% for 75 µM, and 88% for 100 µM. In our second experience cytotoxicity were found 10%, 18%, 53%, 70% and 74%, respectively (Figure 2, Table 2). In our results, IC50 value was determined to be 50 µM after 24 hours of treatment in HT22 cell line.

There are some studies in the literature to understand the mechanisms of cytotoxicity and genotoxicity caused by ES. Xu et al. were studied in mice using spermatogenic cell lines (GC-1 spg) to investigate reproductive toxicity of ES at doses of 0, 6, 12 and 24 µg / ml for 24 hours, and shows that, ES inducted apoptosis via oxidative stress and mitochondrial dysfunction. Western blot and immunohistochemically analysis of the mitochondria in the study of expression of apoptosis-related proteins in the pathway, the results showed that ES increased cytochrome c expression in GC-1 spg cells, significantly increased the activity of caspases 9 and 3 as well as suppressing Bcl-2 expression (22).

In another study, caspase-3 activity of K562 cells was investigated to understanding the effects of ES on apoptosis, and was found that the 50 and 75 µM ES caused a significantly increasing of apoptosis and the relative activity of caspase-3. In the same study, Western Blot results showed that ES reduced Bcl-2 expression, but increased BAX protein expression, and as a result higher BAX / Bcl-2 ratio (23).

In our study, BAX, BCL2 and CASP3 gene expression levels were determined in ES treated HT22 cells with IC50 value of 50 µM. In endosulfan-treated cells, gene expression of BAX increased 18-fold, whereas BCL2 gene expression was suppressed. The gene expression of CASP3 has been increased about four times (Figure 3, Table 3). Our results showed that ES induces apoptosis with a possible increase in intracellular ROS and mitochondrial dysfunction.

ROSs are one of the main pathophysiological factors responsible for DNA damage in cells. It has been observed that ES induces ROS in cell lines in a concentration and time dependent manner. Induction of ROS in both human and mouse cells leads to double chain fractures in DNA. ES-induced DNA damage leads to DNA damage responses and results in elevated levels of NHEJ (7). In the present study, we first examined the expression of DNA-PK gene in the NHEJ pathway for the first time in ES, which was first applied to HT22 cells. According to our results, DNA-PK gene expression was increased about 5 times compared to control (Figure 3, Table 3).

CONCLUSION

In this study, the dose and time dependent effects and 24 hours IC50 dose of ES on a neuronal cell line HT22, was demonstrated. For the first time in the literature, we demonstrated the effects of ES on the expression of DNA-PK, Bax, Bcl-2 and CASP3 genes. It is a deficiency that the expression results obtained in this study, which is a limited preliminary study, are not supported by protein levels. However, our results show that induction of apoptosis in HT22 cells depends on both ES concentration and time applied in accordance with similar studies in the literature. We also found that ES, which was first applied to HT22 cells in the literature, caused an increase in DNA-PK gene expression in the NHEJ pathway. Although our results need to be tested in a similar direction in the future, it is clear that it is an important preliminary data for such studies. These data shed light on the future studies to explain the effects of molecular mechanism of endosulfan on DNA damage and cell apoptosis at the level of both protein and gene expression. It will also shed light on the future studies which investigate the mechanisms of action of certain proteins and chemicals to reduce these negative effects of endosulfan.

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Competing interests: The authors declare that they have no competing interest.

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Ethical approval: Because of the study was carried out with commercial cell lines, the Ethics Committee approval is not required.

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