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Original Article

Combined treatment of cisplatin and 5-Fluorouracil Induces cell death in ARPE-19 cells through the endoplasmic reticulum stress pathway

Ayten Dogan, Zeynep Banu Doganlar, Oguzhan Doganlar

Department of Medical Biology, Faculty of Medicine, Trakya University, Edirne, Turkey

Abstract

Aim: Cisplatin and 5-Fluorouracil have been used for about 50 years as chemotherapy agents. Combination of these two agents is known as PF therapy and is widely used in the treatment of anal, esophageal cancer, head and neck cancer. One of the problems encountered with antineoplastic agents is the rapid and irreversible side effects on non-target cells and organs. It is common that occourence of ocular toxicity by chemotherapy.

Materials and Methods: ARPE-19 cell viability and toxicity were determined by performing (MTT)-based colorimetric assays and IC50 values calculated by probit analyses options of SPSS 20 Software. Acridine orange/Ethidium bromide (AO/EB) fluorescent staining was performed to determine structural changes such as cell blebbing and chromatin condensation during the apoptosis process on cells. Images examined under a fluorescent microscope (Zeiss Axio Vert.A1) at 40X magnification with FITC filter for AO and Tex Red filter. And Real-time PCR was performed with ABI Step One Plus and Quant Studio 5 Real-Time PCR systems.

Results: PF treatment within 24 hours and 48 hours caused ER stress due to toxic effect and this induced cell death through apoptosis via death receptor signaling and intrinsic pathway in ARPE-19 cells.

Conclusion: PF-induced activation of the ER stress mechanism can be used as a novel therapeutic strategy for the prevention of side effects of non-target cells.

Keywords: ARPE-19; apoptosis; chemotherapy; ER stress; toxicity

INTRODUCTION

Cisplatin and 5-Fluorouracil (5-Fu) have been used for about 50 years as a chemotherapy agent (1,2). Combination of these two agent is known as PF therapy, is usually used for the treatment of anal, head & neck cancer and oesophageal cancer (1,3). It has been known for a long time that chemotherapy agents have different effects in dose-dependent manner on DNA and chromosome. This feature of chemotherapy is used to destruction of cells or induces apoptosis by disrupting the genetic stability of cancer cells. Chemotherapy agents have potential to produce acute and chronic damage in eye tissue by administration of high doses (4,5).

The eye has greater sensitivity than other organs and has potentially high sensitivity to toxic substances. Therefore, it is common that occourence of ocular toxicity by chemotherapy (4). The systemic chemotherapy side effects can cause significant damage to critical structures of vision (5). The use of two or more chemotherapy agent (combination regimens) may produce ophthalmologic side effects and can cause various ocular diseases (6). Retinal pigment epithelium (ARPE) is a highly developed cell which has multiple cellular functions in human eye (7). The ARPE compose the outer blood retinal barrier as a defense mechanisms and in this way it can control the movement of dissolved substances, nutrients and protect the eye (8,9). For this reason, ARPE cells have essential roles in the pathogenesis of ocular diseases (10).

Cisplatin has been used as an antitumor agent for many years. It has been known that nuclear DNA is the critical target of cisplatin (11,12). The cisplatin and DNA adducts formation is an important mechanism which leads to apoptosis on cancer cells and also increased plasma membrane fluidity and ER stress. Cisplatin has the ability to alkylation of DNA, can cause DNA chain breaks, is elemantary cytotoxic action of platinium complexes (4). Ophthalmological toxicity is a side effect of intravenous cisplatin administration and manifests itself in peripheral

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Corresponding Author: Ayten Dogan, Department of Medical Biology, Faculty of Medicine, Trakya University, Edirne, Turkey
E-mail: aytenndogan@gmail.com

neuropathy (13), transient cortical blindness (14), retinopathy (15), transient left homonymous hemianopsia (16), cerebral blindness (17), cortical blindness (18). 5-FU acts as uracil antimetabolites that block cellular proliferation during S-phase of cell division (19,20). Treatment of cells with the 5-FU causes DNA damage, especially double chain breaks which is affecting the proliferation and survival of treated cells (21,22). 5-FU is not active until it converted to its metabolites fluoro-deoxyuridine monophosphate (FdUMP). Several studies showed that 5-FU acts to inhibit DNA proliferation and replication in cancer cells by inhibition of target enzyme thymidylate synthase (1,23,24). Studies have shown that excessive lacrimation, blurred vision, eye irritation (25), tear duct fibrosis (6) and cicatricial ectropion (26) developed in patients after intravenous 5-FU application (27). Other associated symptoms include ocular pain, irritative conjunctivitis, circumorbital edema, and keratitis. Chemotherapy-induced ocular side effects usually occur after the onset of chemotherapy and disappear within 1-2 weeks of termination of treatment (25,28).

The toxic side effects of chemotherapy agents usually cause some problems in the treatment, such as dosage reduction or early termination of treatment. Cancer patients can have many problems due to drug-induced toxicity. The side effects of drugs can be harmfull and effect patients life standarts (29). Reducing the side effects of the therapy might increase the quality of patients life and the survival rate (30).

ER stress mechanism is an important factor in drug-toxicity. Drug-induced ER stress can cause various harmful effects within healty cells. ER stress-induced side effects may occur due to increased intracellular free calcium, ROS overproduction and apoptosis (29, 31). The purpose of this study is to learn novel informations about toxic side effects of chemotherapy agents on ARPE-19 cells. The ER pathway of ocular toxicity is still unclear. For this reason, we investigated the effect of 5-FU and cisplatin combination (PF) treatment on ER stress pathway on ARPE-19 cell line.

MATERIALS and METHODS

Cell Line and Reagents

ARPE-19 (Human retinal pigmented epithelium) cell line obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured at 37°C and 5% CO_2 incubator in DMEM: F-12 Medium (Gibco, Life Technologies) containing 10% heat inactivated FBS (Gibco, Life Technologies), 2 mM glutamine (Gibco-Life Technologies) and 1% penicillin/streptomycin (Invitrogen, Life Technologies). 5-Fluorouracil (5-FU) (1000 mg/20 ml, Koçak Farma, Turkey) and Cisplatin (50 mg/100ml, Koçak Farma, Turkey) were obtained from Trakya University, Medical Oncology Department, Turkey. Cell viability assay solution MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was purchased from Thermo Fisher Scientific (Oregon, USA) and Dimethylsulfoxide (DMSO) was from Merck (KGaA, Darmstadt, Germany). Acridine orange was obtained from Invitrogen (Oregon, USA) and Ethidium Bromide was from Sigma-Aldrich (Steinheim, Germany). The PureLink® RNA Mini Kit, High Capacity cDNA Reverse Transcription Kit, SYBR® Select Master Mix were all purchased from Thermo Fisher Scientific.

Cell Viability Assay (MTT)

Chemotherapy human dose in Cisplatin and 5-Fu were determined using both recommended intravenous (i.v) chemotherapy dose and blood plasma peak level according to previous studies (32-34). For this experiment we determined to use 66.6 µM cisplatin (100 mg/m2) and 1530 µM 5-Fu (1000 mg/m^2) concentration as chemotherapy dose for recommended PF treatment. Cell viability and toxicity were determined by performing 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assays. ARPE-19 cells were placed in microassay culture plates at 2000 cell/well in 96-well plate and grown overnight in a humidified incubator at 37˚C in 5% CO_2. After cells attached, cells were treated with 20 µl three different doses (0.13-2.98 µM, 2.08-47.81 µM and 66.6-1530 µM, Cisplatin-5-Fu respectively) of PF diluted in DNase/RNase-free water and 6 replicates were done for each experiment. Distilled water used as the negative control. After incubation for 12, 24 and 48 hours at 37˚C in 5% CO_2 incubator, 20 µl MTT dye solution (5 mg/ml) was added to each well to a total volume of 200 µl and incubated for 4 h at 37˚C. After incubation, supernatants were removed and 200 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the blue formazan crystals. The optical density of reaction was measured with a microplate reader spectrophotometer (Thermo Scientific, USA) at a wavelength of 490 nm. IC_{50} values were determined by probit analyses options of SPSS 20 software. Experiments were performed using at three independent biological replicates.

Acridine Orange/Ethidium Bromide Fluorescent Staining

In this study we used Acridine orange/Ethidium bromide (AO/EB) fluorescent staining to detect morphological changes such as cell blebbing and chromatin condensation during the apoptosis process on cells. ARPE-19 cells were seeded on 24 well plate at a density of $5x10^4$ cells/well and incubated at 37˚C in 5% CO_2. After 24 h incubation, cells treated with 2.08-47.81 µM low dose (LD) and 66.6-1530 µM chemotherapy dose (ChD) for 24 h and 48 h. After incubation period, 7µl (AO, 100 µg/ ml; EB, 100 µg/ml) dual fluorescent staining solution added to each medium and incubated for 30 min at 37˚C in 5% CO2. The medium was removed and cells were washed with phosphate-buffer saline (PBS). Images examined under a fluorescent microscope (Zeiss Axio Vert.A1) at 40X magnification with FITC filter for AO and Tex Red filter. Procedure was performed three times.

Quantitative Real-Time PCR (qRT-PCR) Analysis

ARPE-19 cells collected from 75 cm^2 tissue culture flasks and total RNA was extracted with PureLink® RNA Mini Kit (Life Tecnologies, USA) according to the manufacturer's

instructions. Total RNA quantified with using Qubit® Fluorometer (Life Tecnologies, USA). For the synthesis of first strand cDNA by reverse transcription was achieved with High Capacity cDNA Reverse Transcription Kit (Life Tecnologies, USA) and thermal cycler Applied Biosystems Veriti. The PCR conditions for the cDNA synthesis were 25°C for 10 min, 37°C for 120 min, 85°C for 5 min (Thermal cycler Applied Biosystems® Veriti®). Real-time PCR was performed after 24 hours and 48 hours of low dose and chemotherapy dose of PF treatment to detect the mRNA expression levels of the ER stress pathway; chemokine growth-regulated oncogene 1 (GRO1), Protein Kinase R/PKR-like ER kinase (PERK), Prostaglandin E synthase (PTGES), Binding immunoglobulin protein (BIP), C/EBP homologous protein (CHOP), ER degradation-enhancing α-mannosidase-like protein (EDEM), Total X-box binding protein (TXBP), Spliced X-box binding protein (XBP1s), Unspliced X-box binding protein (XBP1us), for apoptosis pathway; caspase 8, p53 upregulated modulator of apoptosis (PUMA), Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), death receptors (DR4 and DR5) genes in ARPE-19 cells. Real-time PCR was performed using cDNA samples with SYBR® Select Master Mix (Life Technologies, USA) and PCR primers on an ABI Step One Plus and Quant Studio 5 Real-Time PCR system according to the manufacturer's instructions. Reference genes and primer sequences are given in Table 1. Relative fold change in mRNA expression was calculated by the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method (User Bulletin 2, Applied Biosystems, CA). Gene expressions were determined as the relative fold change compared to the respective control and normalized with β-actin mRNA expressions.

Table 1. Reference genes, primer sequences and PCR conditions used for real time PCR amplification

qRT-PCR Assay											
ER Stress Pathway					**Apoptosis Pathway**						**PCR Conditions**
Genes	**Primer Sequences**				**Genes**	**Primer Sequences**					
GRO1	F	5′	AGGAAGCTCACTGGTGGCTG	3′	Caspase 8	F	5′	AGAGTCTGTGCCCAAATCAAC	3′		
	R	5′	TAGGCACAATCCAGGTGGC	3′		R	5′	GCTGCTTCTCTCTTTGCTGAA	3′		
PERK	F	5′	ATCCCCCATGGAACGACCTG	3′	PUMA	F	5′	CAGACTGTGAATCCTGTGCT	3′		
	R	5′	ACCCGCCAGGGACAAAAATG	3′		R	5′	ACAGTATCTTACAGGCTGGG	3′		
PTGES	F	5′	CCCCCAGTATTGCAGGAGTG	3′	NOXA	F	5′	GTGCCCTTGGAAACGGAAGA	3′		
	R	5′	AGACGAAGCCCAGGAAAAGG	3′		R	5′	CCAGCCGCCCAGTCTAATCA	3′		1 cycle of 50 °C for 2 min and 95 °C for 10 min. 48 cycles were performed, each cycle consisted of 95 °C for 15 s followed by 60 °C for 1 min.
BIP	F	5′	CATGGTTCTCACTAAAATGAAAGG	3′	DR5	F	5′	GGGAAGAAGATTCTCCTGAGATGT	3′		
	R	5′	GCTGGTACAGTAACAACTG	3′		R	5′	ACATTGTCCTCAGCCCCAGGTCG	3′		
CHOP	F	5′	CAGAAGGAAGTGCATCTTCA	3′	DR4	F	5′	CAGAACGTCCTGGAGCCTGTAAC	3′		
	R	5′	TACACTTCCGGAGAGACAGA	3′		R	5′	ATGTCCATTGCCTGATTCTTTGTG	3′		
EDEM	F	5′	CAAGTGTGGGTACGCCACG	3′							
	R	5′	AAAGAAGCTCTCCATCCGGTC	3′	**Homebox Genes**						
XBP1s	F	5′	TTACGAGAGAAAACTCATGGC	3′	β-ACTIN	F	5′	AGAGTCTGTGCCCAAATCAAC	3′		
	R	5′	GGGTCCAAGTTGTCCAGAATGC	3′		R	5′	GCTGCTTCTCTCTTTGCTGAA	3′		
XBP1us	F	5′	CAGCACTCAGACTACGTGCA	3′							
	R	5′	ATCCATGGGGAGATGTTCTGG	3′							

RESULTS

Chemotherapy Treatment with PF Therapy Causes Cytotoxicity and Induces Cell Death on ARPE-19 Cell Line

We have chosen cell viability as a parameter to evaluate the cytotoxic potential of PF treatment on ARPE-19 cell lines. A quantitative assay was performed with 96-well plates. Cell viability was measured after incubating cells with three doses, 0.13-2.98 μM, 2.08-47.81 μM and 66.6-1530 μM, started 512 and 32 times diluated concentration of the PF chemotherapy dose. MTT test was performed after 12, 24 and 48 h incubations and 6 replicates were performed for each dose. After 12 h treatment with 0.13-2.98 μM, 2.08-47.81 μM and 66.6-1530 μM doses, cell viability decreased to 98%, 85%, 74%, respectively.

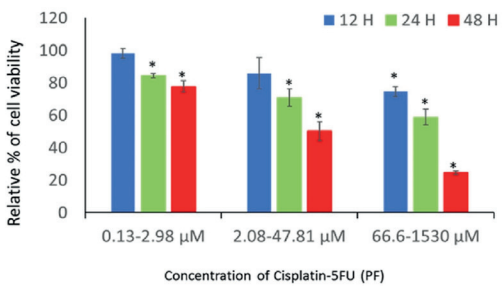

Figure 1. Relative cell viability of ARPE-19 cells after incubation with three different concentration of Cisplatin-5FU therapy. Test performed after 12, 24 and 48 h incubations. All data represent the mean ± SE. * indicates a significant ($P \leq 0.05$) difference between treatment groups compared to control (One-way ANOVA, Duncan test)

These findings indicate that 12 h of incubation of all concentrations have no statistically significant effects compared with respective control. Decreased cell proliferation was determined after 24 h (84%, 70%, 58%, respectively) and 48 h (88%, 50%, 24%, respectively) of treatment. However, cell growth inhibition on ARPE-19 cells became statistically significant at 2.08-47.81 µM and 66.6-1530 µM concentrations after 24 h. MTT data showed that 48 h PF treatment caused obvious cytotoxicity on ARPE-19 cells. According to these informations, we decided to proceed subsequent sections of study with 2.08-47.81 µM (low doses) and 66.6-1530 µM (chemotherapy doses) at 24 h and 48 h time periods (Figure 1).

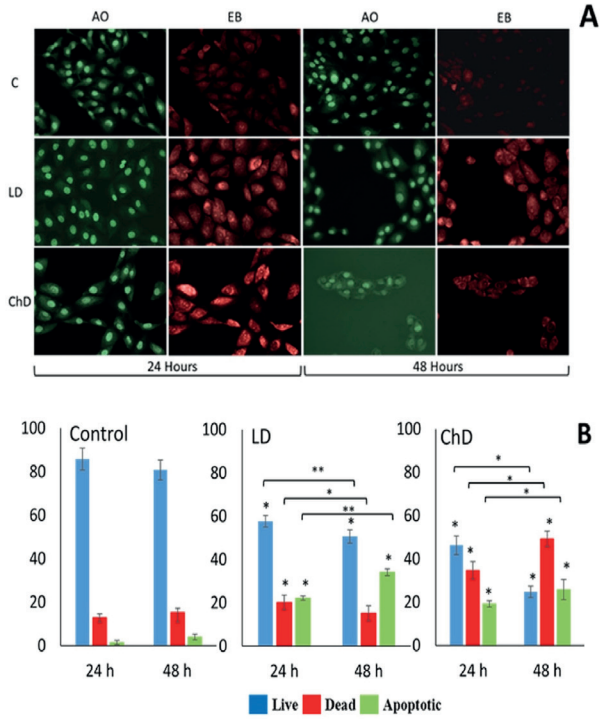

Figure 2. A) Nuclear disintegration and induction of DNA damage in ARPE-19 cells after PF treatment. AO staining in cells were shown in green color. Apoptotic cells showed morphological changes in their nuclei in a manner specific to apoptosis. Red color exhibit DNA damage B) Live, dead and apoptotic cells (%) on ARPE-19 cell line after 24-48 h LD and ChD doses of PF treatment. n =6. * indicates a significant ($P \leq 0.05$) difference between treatment groups compared to control (One-way ANOVA, Duncan test). ** indicates significant ($P \leq 0.05$) difference compared to other treatment time (T-test)

AO/EB Flourescent Dual Staining

ARPE-19 cells were stained with AO/EB after 24 h and 48 h low dose and chemotherapy dose PF treatment. Dual staining was examined under a flourescent microscope (Figure 2A). Apoptotic cells, labeled by green AO/EB nuclear staining, were detected in both experimental group. The number of apoptotic cells, treatment periods and concentrations were determined as follows; LD 24h, 22.16%; LD 48h, 34.6%; ChD 24h, 19.24%; ChD 48h, 25.85%. (Figure 2B). After 24 h and 48 h of chemotherapy dose treatment, the number of apoptotic cells increased significantly. The highest number of apoptotic cells was observed after 48 h low dose treatment. EB can only pass through late apoptotic and dead cell membranes. EB emits orange-red fluorescence when attached to concentrated DNA fragments or apoptotic bodies (35). We also determined that increasing concentrations of PF treatment leading to increase on dead cell number both at 24 h and 48 h (LD 24h, 20.16%; LD 48h, 15.16%; ChD 24h, 34.71%; ChD 48h, 49.28%) (Figure 2B). The highest number of dead cells was observed after 48 h chemotherapy dose treatment.

The Molecular Basis of PF Therapy in ER Stress Formation

In the present study, PF administration on ARPE-19 cell line showed decrease at GRO1, PERK, XBP1s and XBP1us genes after LD 24 h. After 24 h ChD treatment, an increase at PTGES and XBP1us genes were observed while the expression of GRO1 and PERK were significantly decreased. There was a statistically significant increase at DR4 (LD; 8.84- fold, ChD; 43.75- fold) and DR5 (LD 23.92- fold, ChD 35.20- fold) genes after 24 h LD and ChD administration. A significant increase in all genes, except PERK gene, was determined after 48h LD administration. We observed that after 48h administration, LD and ChD doses produced a strong apoptosis signal. Especially for CHOP (LD48h; 69.9- fold, ChD48h; 18.32- fold) and EDEM (LD48h; 15.68- fold, ChD48h; 11.14- fold) genes, the amount of upregulation is quite clear. In the analysis of the heat map, there was a marked x different difference. LD application groups and ChD application groups were clearly included in their own clusters, especially when the 24 h dosing and 48 h dosing provided significant clusters within themselves, and these two main groups were obviously separated from each other in the hierarchical classification. Experiments were performed using at three independent biological replicates (Figure 3).

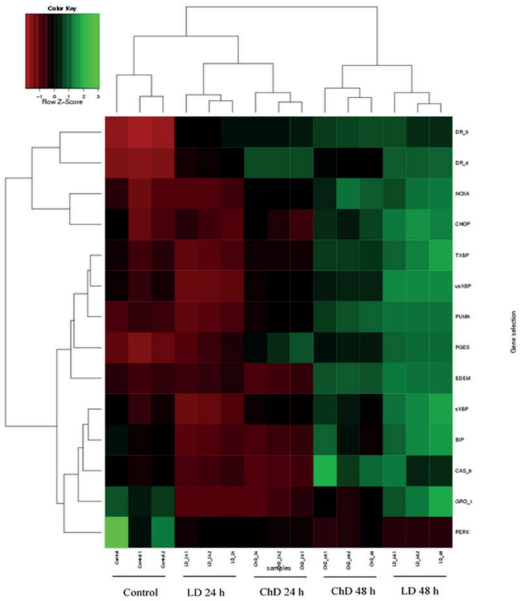

Figure 3. Heat map analysis using gene expressions of ARPE-19 cell line for control, low dose (LD) and Chemotherapy dose (ChD) after 24 h and 48 h of PF treatment. All data were converted to Log10 (x+1) and eBayes and Pearson correlation options were used for hierarchical clustering and correlation analysis, respectively

Table 2. Real Time PCR analysis of ER stress and Apoptosis Pathway

		24 h				48 h				
Gene ID	Control	LD		ChD		LD		ChD		P value
ER Stress Pathway										
GRO1	1.01±0.09^{b}	0.13±0.007^{a}	↓	0.21±0.04^{a}	↓	1.50±0.20^{c}	↑	0.49±0.08^{a}	↓	<0.0001
PERK	1.29±0.61^{b}	0.17±0.05^{a}	↓	0.13±0.006^{a}	↓	0.007±0.002^{a}	↓	0.05±0.04^{a}	↓	0.041
PTGES	1.01±0.13^{a}	1.82±0.21^{a}	→	5.86±0.86^{b}	↑	8.86±0.20^{c}	↑	4.98±0.09^{b}	↑	<0.0001
BIP	1.02±0.16^{ab}	0.23±0.04^{a}	→	0.34±0.03^{a}	→	2.85±0.28^{c}	↑	1.45±0.84^{b}	→	<0.0001
CHOP	2.49±1.94^{a}	1.22±0.46^{a}	→	3.46±1.57^{a}	→	69.90±9.82^{c}	↑	18.32±2.76^{b}	↑	<0.0001
EDEM	1.02±0.15^{a}	1.09±0.14^{a}	→	0.82±0.10^{a}	→	15.68±0.39^{c}	↑	11.14±0.11^{b}	↑	<0.0001
XBP1s	1.01±0.13^{b}	0.43±0.05^{a}	↓	1.14±0.03^{b}	→	3.58±0.28^{d}	↑	1.82±0.16^{c}	↑	<0.0001
XBP1us	1.02±0.14^{b}	0.27±0.02^{a}	↓	1.50±0.12^{c}	↑	6.37±0.08^{e}	↑	2.69±0.07^{d}	↑	<0.0001
Apoptosis Pathway										
DR5	1.03±0.18^{a}	23.92±5.31^{b}	↑	35.20±0.99^{b}	↑	56.67±8.14^{c}	↑	67.18±3.11^{c}	↑	<0.0001
DR4	1.00±0.03^{a}	8.84±1.24^{b}	↑	43.75±0.31^{d}	↑	56.88±1.14^{e}	↑	13.48±1.59^{c}	↑	<0.0001
PUMA	1.00±0.09^{a}	0.70±0.09^{a}	→	2.08±0.20^{b}	↑	7.21±0.04^{d}	↑	5.64±0.46^{c}	↑	<0.0001
NOXA	1.36±0.70^{a}	1.28±0.23^{a}	→	6.58±0.39^{ab}	↑	48.57±9.07^{b}	↑	38.05±11.43^{b}	↑	0.001
CAS8	1.010.13^{a}	0.47±0.51^{a}	→	0.35±0.03^{a}	→	2.68±0.63^{b}	↑	3.85±0.89^{b}	↑	0.002

↑ upregulated → stable (there is no statistically significant change) ↓ downregulated

Expression of Endoplasmic Stress and Apopstosis Genes

We performed qRT-PCR to measure the induction of ER stress-responsive genes mRNAs on ARPE-19 cell line after 24 h and 48 h treatment with low dose and human chemotherapy dose of PF therapy (Table 2). BIP is an ER stress marker gene. Increased expression of BIP gene observed only after 48 h treatment with LD PF therapy. BIP gene expression elevated ~2.85- fold ($P < 0.0001$). When ER stress is triggered by PF treatment, we have seen PERK down-regulated with LD and ChD for both 24h and 48h. The expression of PERK down-regulated approximately 7.5- fold (LD), 9.9- fold (ChD) for 24 h and 184- fold (LD), 25.8- fold (ChD) ($P < 0.041$) for 48 h after PF treatment compared with the control group. Effect of PF therapy on CHOP gene expressions was not statistically significant after 24 h treatment of both doses. However, after 48 h of treatment, ER stress induced a strong upregulation of CHOP gene expressions with LD (~69.90- fold) and ChD (~18.2- fold) treatment compared with the control group ($P < 0.0001$). CHOP induces GRO1 gene expression and activates Ca^{++} release to mitochondria. These circumtances result to cell death. The GRO1 gene showed slight increase ~1.5- fold ($P < 0.0001$) only at LD after 48 h. The mRNA expression levels of PTGES significantly upregulated with chemotherapy dose after 24 h (5.86-fold by LD dose) and low/chemotherapy doses after 48 h treatment (8.86- fold LD and 4.98-fold ChD) ($P < 0.0001$). The expressions of ERAD-related molecule EDEM was not exhibited significant difference for 24 h treatment period. At the same time EDEM gene levels significantly up-regulated with low dose (15.68- fold) and chemotherapy dose (11.14- fold) after 48 h treatment ($P < 0.0001$).

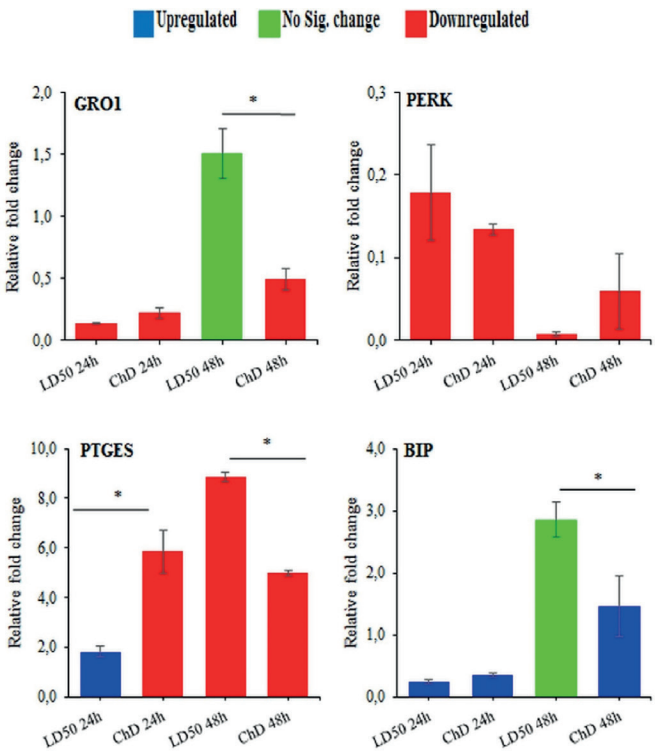

Figure 4. Real Time PCR analysis of ER stress pathway genes GRO1, PERK, PTGES and BIP at low dose and chemotherapy dose of PF therapy in ARPE-19 cells for 24 and 48 h. LD: low dose of PF treatment (2.08 µM cisplatin - 47.81 µM 5-Fu), ChD: Chemotherapy dose of PF treatment (66.6 Cisplatin-1530 µM 5-Fu). The fold increases of mRNA expression levels were determined and normalised with β-actin. Green and red colours indicate significantly different values compared to control. * indicates a significant ($P \leq 0.05$) difference between treatment groups (One-way ANOVA, Duncan test)

The levels of XBP1s and XBP1us genes all increased, when low dose (XBP1s; 3.58- fold, XBP1us; 6.37- fold) and chemotherapy dose (XBP1s; 1.82-fold, XBP1us; 2.69-fold) groups ($P < 0.0001$) compared with the corresponding controls within 48 h, as in the same EDEM gene expressions (Figure 4-5 Table 2). The increase in DR4 and DR5 gene levels observed in ARPE-19 cells following both 24 h and 48 h with low dose and human chemotherapy dose of PF therapy. The expression of DR5 was increased by approximately 23.12- fold (LD), 34.02-fold (ChD) in 24 h and 54.77- fold (LD), 64.93- fold (ChD) in 48 h after PF treatment versus the control group with causing obvious cell death. PUMA and NOXA expressions exhibited an increase after 24 h chemotherapy dose (PUMA, 2.8- fold; NOXA, 4.8- fold) and 48 h low dose (PUMA, 7.21- fold; NOXA, 48.57- fold) and chemotherapy dose (PUMA 5.64-fold, $P < 0.0001$; NOXA 38.05-fold, $P = 0.0001$). We have seen expression of Caspase 8 was not significantly elevated in ARPE-19 cells after incubation for 24 h, but showed a trend to increase (LD 2.68- fold; ChD 3.85- fold, $P = 0.0002$) within 48 h with low doses and chemotherapy doses (Figure 6, Table 2).

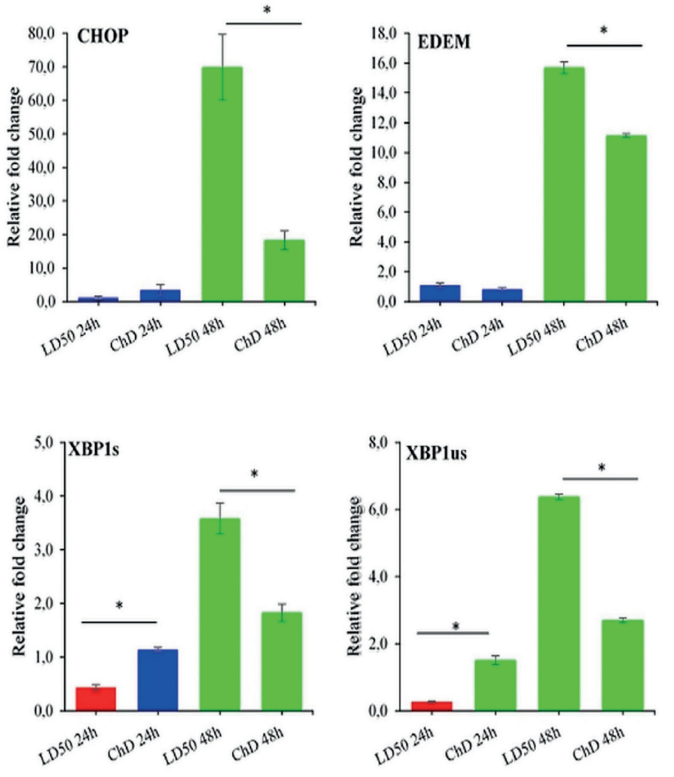

Figure 5. Real Time PCR analysis of ER stress pathway genes CHOP, EDEM, XBP1s, XBP1us at low dose and chemotherapy dose of PF therapy in ARPE-19 cells for 24 and 48 h. LD: low dose of PF treatment (2.08 µM cisplatin - 47.81 µM 5-Fu), ChD: Chemotherapy dose of PF treatment (66.6 Cisplatin-1530 µM 5-Fu). The fold increases of mRNA expression levels were determined and normalised with β-actin. Green and red colours indicate significantly different values compared to control. * indicates a significant ($P \leq 0.05$) difference between treatment groups (One-way ANOVA, Duncan test)

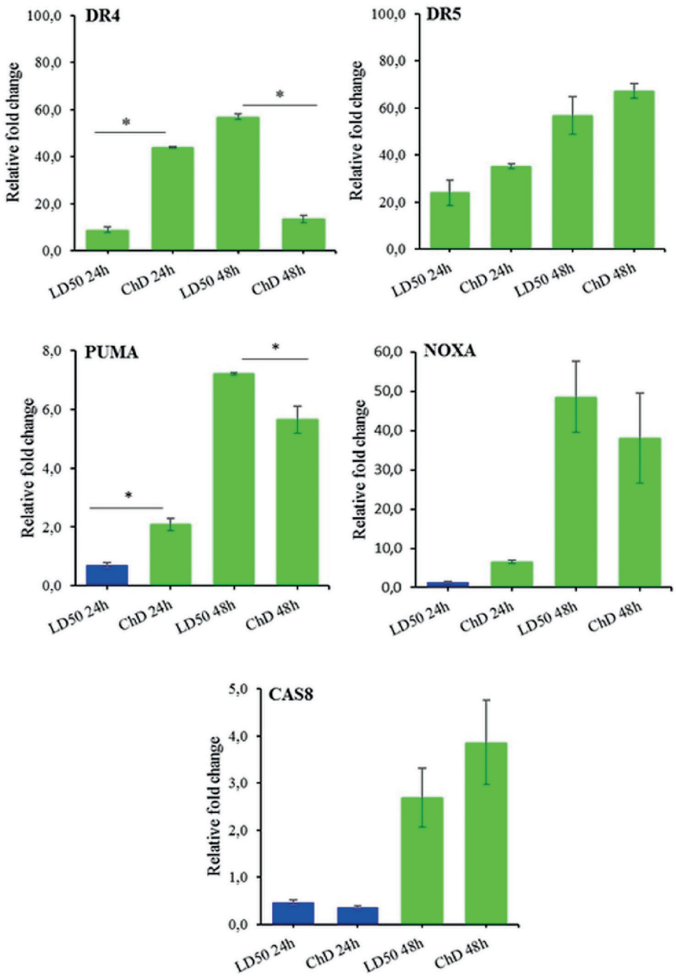

Figure 6. Real Time PCR analysis of apoptosis pathway genes at low dose and chemotherapy dose of PF therapy in ARPE-19 cells for 24 and 48 h. LD: low dose of PF treatment (2.08 µM cisplatin - 47.81 µM 5-Fu), ChD: Chemotherapy dose of PF treatment (66.6 Cisplatin-1530 µM 5-Fu). The fold increases of mRNA expression levels were determined and normalised with β-actin. Green colour indicates significantly different values compared to control and blue color represents there are no statistically significant effects compared with control. *indicates a significant ($P \leq 0.05$) difference between treatment groups (One-way ANOVA, Duncan test)

DISCUSSION

The toxic side effects of chemotherapy agents usually cause some problems in the treatment, such as dosage reduction or early termination of treatment. Cancer patients can have many problems due to drug-induced toxicity. The side effects of drugs can be harmfull and effect patients life standarts (29). Reducing the side effects of the therapy might increase the quality of patients life and the survival rate (30). Cisplatin and 5-Fluorouracil are commonly used as a chemotherapy agent (1,2). Cisplatin and 5FU have various side effects and trigger lots of vital signaling pathways in cells. These chemotherapy agents have potential to produce acute and chronic damage in eye tissue by administration of high doses (4,5). Drug-induced ER stress can cause various harmful effects within cells and tissues (29). For this reason, the main purpose of present studies was to collect new information on the potential

role of drug-induced ER stress on ARPE-19 cell line which is highly developed cell and has multiple cellular functions in human eye (7). By understanding how ARPE-19 cells respond to ER stress under PF treatment at the cellular and molecular levels can be used against these diseases will be facilitated. This study is the first to report the effects of PF treatment on ER stress, apoptosis signalling and PF toxicity on ARPE-19 cell line. We think our results can obtain foresight to possible clinical results for the side effects found in cancer patients treated with Cisplatin-5FU agents. It is common that occourence of ocular toxicity by chemotherapy (4). Combined chemotherapy regimens may produce ophthalmologic side effects and can cause various ocular diseases (6). The ARPE compose the outer blood retinal barrier as a defense mechanisms and in this way it can control the movement of dissolved substances, nutrients and protect the eye (8,9). In the case of ER stress, the signal cascades are regulated by the cellular response involving the activation of some sensors and homeostasis restores again. This adaptive mechanism is a transcriptional program, enhances the expression of genes, contain protein folding and quality control. BIP is one of the ER-resident chaperons and commonly used as a marker of ER stress. When cells are under ER stress, Grp 78 (Glucose-regulatory protein 78, also known as immunoglobulin-binding protein or BIP) separates from PERK, one of the ER signaling transmembrane proteins. PERK is autophosphorylated after dissociation BIP and it leads to activation of unfolded protein response (UPR) arms. In our study the increase in ER stress marker, BIP gene, expressions observed after only 48 h treatment with low dose PF therapy. We observed that PERK down-regulated with low and chemotherapy doses for both 24 h and 48 h compared with the control group. This decline shows us that PERK is consumed in the environment by phosphorylation. In addition to these UPR sensors, there are other signaling nodes which are downstream transcription factors XBP and CHOP. These integral proteins can restore hemostasis by activating the UPR signal pathway after ER stress, as well as necroptosis and cell death by mitochondrial apoptosis through Bcl-2 family proteins (Noxa and Puma). Normal conditions CHOP presents in the cytosol and translocates to nucleus during ER stress and it is a modulator of ER stress induced cell death. The proapoptotic protein CHOP is induced via PERK pathway. CHOP gene expressions observed after 24 h and 48 h treatment of PF therapy and the effect was not statistically significant for 24 h treatment period. However, ER stress induced a strong up-regulation of CHOP by low dose and chemotherapy dose of PF ($P < 0.0001$) with relative to control for 48 h. CHOP induces GRO1 gene expression and activates Ca^{++} release to mitochondria. These circumtances result to cell death. The GRO1 gene showed only slight increase ~1.5- fold ($P < 0.0001$) after 48 h of low dose treatment. The mRNA levels of PTGES significantly upregulated with chemotherapy dose after 24 h (5.86- fold by LD dose) and low/chemotherapy doses after 48 h (8.86- fold LD and 4.98- fold ChD, $P < 0.0001$). When glycoproteins that are not properly folded, they transfered to EDEM (ER-associated degradation enhancing mannosidase-like protein). Its known that overexpression of EDEM increases ERAD mediated protein degradiation. In various studies, it has been reported that EDEM works as an acceptor of misfolded glycoproteins, which will result in degradation process through ERAD sytstem (36). The expressions of ERAD-related molecule EDEM was not exhibited significant difference in 24 h treatment period at both treatment doses. At the same time EDEM gene levels significantly up-regulated at low dose (15.68- fold) and chemotherapy dose (11.14- fold) after 48 h treatment ($P < 0.0001$). It's known that the XBP1us active form is a enhancer for the EDEM gene in ER stress. Our results are compatible with this outlook. The levels of XBP1 and XBP1u mRNA increased ($P < 0.0001$) at both dose groups in the ARPE-19 cells compared with the corresponding controls within 48 h, as in the same EDEM gene. The ability of chemotherapy agents, to activate an ER stress response, evaluated by measuring the splicing of X-box binding protein-1 (XBP1s) genes. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, both of (DR4 and DR5) carry cytoplasmic death domains and mediate apoptosis. After binding to the death receptors, DR4 and DR5 can trigger an cell death signal via caspase dependent apoptosis through caspase 8 pathway (37). The increase at DR4 and DR5 gene levels observed in ARPE-19 cells after both treatment periods with low and human chemotherapy dose of PF therapy. We have seen expression of Caspase 8 was not significantly elevated in ARPE-19 cells after incubation for 24 h, but showed a trend to increase within 48 hours. P53 is a tumor suppressor gene and plays central roles in the organization of stress responses (38). The P53 protein begins to accumulate immediately when the cell is stressed and is activated as a transcription factor. Activated p53 induces stress responses by transcriptional regulation of effector molecules involved in carrying out a specific response. Activation of p53 can lead to elimination of cells by activating the apoptotic pathway (39). Puma and Noxa are p53-inducible BH3- only proapoptotic members of the Bcl-2 family and upregulated by p53 as a response to DNA damage (40). In normal cells, Puma increases the permeability of the mitochondrial outer membrane. This function induces calcium release from the endoplasmic reticulum occurs and triggers subsequent caspase activation. In this study, PUMA and NOXA expressions exhibited an increase after 24 h chemotherapy dose and 48 h low and chemotherapy dose. These findings suggest that PF treatment leads to a strong apoptosis on ARPE-19 cell lines through ER stress signaling.

CONCLUSION

We observed that cisplatin and 5-FU combination therapy is well tolerated at the low dose and chemotherapy dose of 12 h treatment. However, 24 h and 48 h PF treatment caused ER stress due to toxic effect and this induced cell death through apoptosis via death receptor signaling and intrinsic pathway in ARPE-19 cells. According to these results, proapoptotic protein CHOP, ERAD-related

molecule EDEM, death recepors DR4, DR5, Bcl-2 family proteins Noxa and Puma exhibited principal roles in the cellular ER stress and apoptosis from PF chemotherapy. This is the first study to show that PF treatment causes obvious cytotoxicity and induces cell death in ARPE-19 cells through the ER stress pathway in a dose- and time-dependent manner. Our results indicated that PF-induced activation of ER stress mechanism can be used as a novel therapeutic strategy for prevention from side effects of non target cells and organs. However, further studies are required to determine this mechanism in animal models.

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