Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Apr 23; 10(B):1079-1088.
https://doi.org/10.3889/oamjms.2022.8885
eISSN: 1857-9655
Category: *B - Clinical Sciences*
Section: *Gynecology and Obstetrics*

The Effect of Melatonin and Cisplatin Combination Using Copper-Transporting ATPase-1, P-Glycoprotein, and Gamma-Glutamylcysteinylglycine on Ovarian Cancer Biological Cell SKOV3

Cut Adeya Adella[1]*, M. Fidel Ganis Siregar[2], Imam Budi Putra[3], Poppy Anjelisa Hasibuan[4], Andrijono Andrijono[5], Adang Bachtiar[6], Sarma N. Lumbanraja[7], Iqbal Pahlevi Nasution[8]

[1]Department of Obstetrics and Gynecology, Oncology Gynecology Division, Faculty of Medicine, Universitas Sumatera Utara, Sumatra, Indonesia; [2]Department of Obstetrics and Gynecology, Fertility Endocrinology Reproduction Division, Faculty of Medicine, Universitas Sumatera Utara, Sumatra, Indonesia; [3]Department of Dermatology and Venerology, Faculty of Medicine, Universitas Sumatera Utara, Sumatra, Indonesia; [4]Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Sumatra, Indonesia; [5]Department of Obstetrics and Gynecology, Oncology Gynecology Division, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [6]Department of Public Health, Faculty of Public Health, Universitas Indonesia, Jakarta, Indonesia; [7]Department of Obstetrics and Gynecology, Feto Maternal Division, Faculty of Medicine, Universitas Sumatera Utara, Sumatra, Indonesia; [8]Department of Surgery, Faculty of Medicine, Universitas Sumatera Utara, Sumatra, Indonesia

Edited by: Ksenija Bogoeva-Kostovska
Citation: Adella CA, Siregar MFG, Putra IB, Hasibuan PA, Andrijono A, Bachtiar A, Lumbanraja SN, Nasution IP. The Effect of Melatonin and Cisplatin Combination Using Copper-Transporting ATPase-1, P-Glycoprotein, and Gamma-Glutamylcysteinylglycine on Ovarian Cancer Biological Cell SKOV3. Open Access Maced J Med Sci. 2022 Apr 23; 10(B):1079-1088. https://doi.org/10.3889/oamjms.2022.8885
Keywords: Melatonin; Cisplatin; Copper-Transporting ATPase-1; p-Glycoprotein; Glutamylcysteinylglycine; SKOV3 cells
***Correspondence:** Cut Adeya Adella, Jl. Dr. T. Mansyur No. 5, Medan, North Sumatera, 20155. E-mail: cutadeya@usu.ac.id
Received: 04-Feb-2022
Revised: 10-Apr-2022
Accepted: 13-Apr-2022

Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Ovarian cancer is the fifth-most common female cancer and third-most common cancer in Indonesia, but most are advanced-stage patients that experiencing recurrence, which indicates resistance to treatment, especially to cisplatin. Melatonin appears as an alternative that can support the apoptotic effect of cisplatin as a chemotherapy regimen.

AIM: To determine the effect of the combination of melatonin and cisplatin compared with cisplatin only chemotherapy on chemotherapy resistance with Copper-Transporting ATPase-1 (CTR-1), P-glycoprotein (P-Gp), and gamma-glutamylcysteinylglycine (GSH) biomarkers in ovarian cancer biological cells SKOV3.

METHODS: This research design was an experimental laboratory, posttest only control group design, using SKOV3 cell culture. This study was performed in the Stem Cells and Tissues Engineering Research Cluster IMERI FKUI laboratory and Integrated Laboratory FKUI. MTS assay was used to calculate the IC50 of each material. The materials used were melatonin (concentration was 25, 50, 100, 200, 300 nM), cisplatin (concentration was 0.1, 0.5, 1, 2, 5 mM), and doxorubicin (concentration 10, 20, 40, 50, 80, 100, 200 μM). IC50 melatonin was 1841 mM, IC50 cisplatin was 117,5 μM, and IC50 doxorubicin was 14,72 μM. Samples were control negative group, IC50 doxorubicin as control positive, IC50 cisplatin, IC50 melatonin, combination group of melatonin and cisplatin were 1xIC50, ¾ × IC50, ½ × IC50, and ¼ x IC50. ANOVA and the Bonferroni test were used for the statistical test.

RESULTS: Based on data processing, IC50 of melatonin was 1841 mM, IC50 of doxorubicin was 14.72 μM, whereas IC50 of cisplatin was 117.5 μM. The mean expression of CTR-1 in the IC50 melatonin group was 15.77 ± 0.21 and in the IC50 cisplatin group was 10.87 ± 0.91, mean expression in the IC50 doxorubicin group was 30.33 ± 0.4. Meanwhile, the mean expression of CTR-1 in IC50 cisplatin was 7.37 ± 0.7, and in combination 1 group (1 x IC50 melatonin and 1 x IC50 cisplatin) was 19. 73 ± 1.0. 49. For P glycoprotein, mean expression in IC50 cisplatin was 16 ± 1.59, in IC50 melatonin group was 7.37 ± 0.21, in IC50 doxorubicin was 0, and in combination 1 group (1 × IC50 melatonin and 1 × IC50 cisplatin) was 6.7 ± 0.17. Last, in GSH, mean expression in the IC50 cisplatin group was 33.2 ± 0.87, in IC50 melatonin group was 12.57 ± 0.12, in the IC50 doxorubicin group was 1.33 ± 0.66, and in combination 1 group (1 × IC50 melatonin and 1 × IC50 cisplatin) was 11.7 3± 0.67. There was a significant difference of CTR-1 expression in combination 1 group, which was higher (19.73%), P-Gp expression in combination 1 group, which was lower (6.7%), and also GSH expression in combination 1 group was lower (11.73%) compared to other groups.

CONCLUSION: The group 1 combination of 1 × IC50 melatonin and 1 × IC50 cislatin with 1.841 mM and cisplatin 117.5 uM was able to reduce cisplatin chemotherapy resistance by increasing drug influx activity by increasing CTR-1 expression, decreasing drug efflux through decreasing P-Gp expression, and decreased DNA repair activity through decreased GSH expression.

Introduction

According to the Global Burden Cancer 2020 data, ovarian cancer incidence reached 313,959 new cases worldwide with a mortality rate 207,252 [1]. The ovarian cancer incidence in Indonesia is not known with certainty, but based on national data from the Indonesian Gynecological Oncology Association from 2016 to 2020, ovarian cancer ranks third after breast cancer and cervical cancer with 3398 new cases and at RSUP H. Adam Malik Medan found 387 new cases consisting of 119 early-stage cases and 268 advanced-stage cases [2].

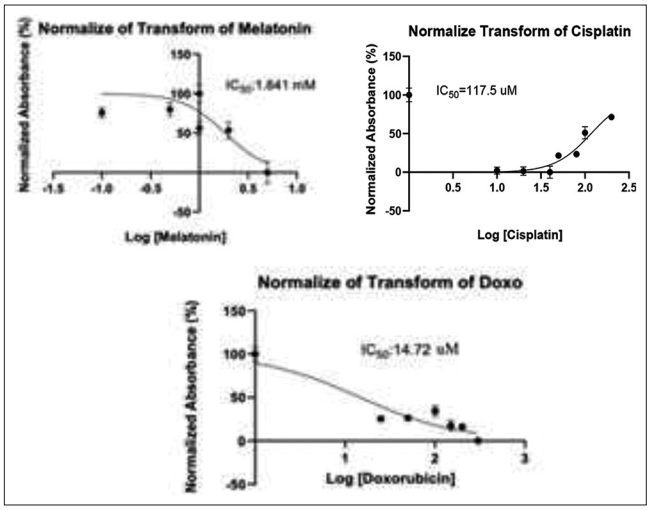

Figure 1: IC50 graphics of doxorubicin, melatonin, and cisplatin

Standard treatment for ovarian cancer in both early and advanced stages is debulking surgery, followed by chemotherapy, generally using platinum and taxane agents. Initial response in patients receiving chemotherapy is quite high (70%–80%), but mostly patients with advanced stages will experience a relapse within 2 years. If there is a recurrence, it is very difficult and even impossible to cure because of the frequent occurrence of cisplatin chemotherapy resistance [3]. The mechanism of chemotherapy resistance begins with cancer stem cells (). These cells have the ability to self-renew, and make up a small part of tumor [4].

Decreased chemotherapy drugs influx into resistant cells is the main resistance mechanism in vitro. Mechanisms that contribute to decreasing chemotherapy drugs accumulation in resistant cells can be inhibition of drug influx, increased drug efflux, or both [3]. Metallic transporters such as copper transporters, for example, Copper-Transporting ATPase-1 (CTR-1), ATP7A, and ATP7B, have a particular role in platinum-derived

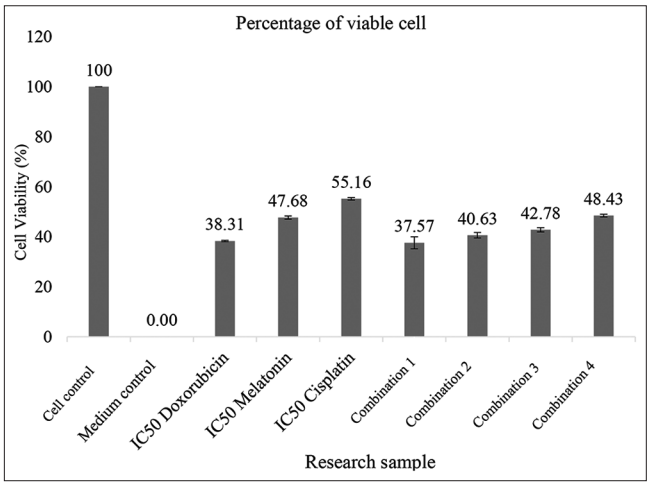

Figure 2: Percentage of SKOV3 cell viability after incubated for 48 hours with materials. Combination 1 (1 × IC50 melatonin and 1 × IC50 cisplatin), combination 2 (¾× IC50 melatonin and ¾ × IC50 cisplatin), combination 3 (½× IC50 melatonin and ½ × IC50 cisplatin), and combination 4 (¼× IC50 melatonin and ¼ × IC50 cisplatin)

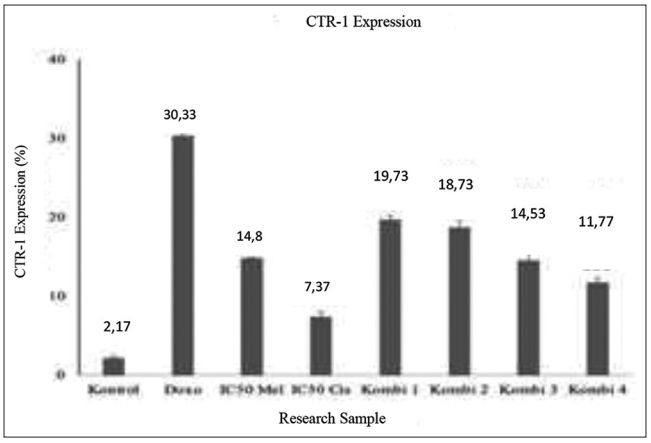

*Figure 3: Percentage of CTR-1 expression in each research group. ***p < 0,001, compared to control; $^{++}$p < 0,001, compared to IC50 doxorubicin; $^{\#}$p < 0,01, $^{\#\#}$p < 0,001, compared to IC50 melatonin; $^{\Phi\Phi}$p < 0,001, compared to IC50 cisplatin; ††p < 0,001 compared to combination 1; $^{\ni\ni}$p < 0,001, compared to combination 2; $^{\Psi}$p < 0,01 compared to combination*

drugs [5]. Platinum and its analogs accumulate into cells by passive diffusion or via facilitated transport. CTR-1 regulates platinum influx and its analogs into cells, leading to increased platinum resistance and decreased platinum intracellular accumulation in number of cancer culture cells, including ovarian cancer cells [3].

Multi-drug resistance protein (MRP) is part of the adenosine triphosphate-binding cassette family which plays a role in anti-cancer drugs efflux. Therefore, it has been speculated that deregulation of MRP components may affect cisplatin-platinum chemotherapy resistance. The multi-drug resistance gene (MDR1) encodes an integral membrane protein named P-glycoprotein (P-Gp) or ATP-binding cassette subfamily B that acts as drug efflux pump. P-Gp recognizes the large number of anti-cancer drugs and can decrease intracellular cytotoxic drugs concentration, such as platinum agents [3].

The involvement of both Glutathione-S-Transferase (GST)π and γ -glutamylcysteine synthetase (γ-GCS) in Gamma-Glutamylcysteinylglycine (GSH) synthesis is associated with platinum resistance in ovarian, cervical, and lung cancer cultured cell [3]. There are three mechanisms of GSH's role in cisplatin sensitivity, namely: (i) GSH can function as a cofactor in facilitating MRP2-mediated cisplatin efflux in mammalian cells because MRP2 transfected cells have been shown to be associated with cisplatin resistance; (ii) GSH can function as a redox regulatory cytoprotector, wherein many cisplatin-resistant cells express more GSH and γ-GCS, a rate-limiting enzyme for GSH biosynthesis; (iii) GSH can function as a copper (Cu) chelator. The increased expression of GSH reduces copper levels resulting in increase of high-affinity Cu transporter (hCTR-1), which is also a cisplatin transporter. This suggests that overexpression of GSH via γ -GCS transfection leads to decreased cisplatin sensitization [6].

Melatonin (N-acetyl-5-methoxytryptamine) is a small lipophilic indolamin produced by pineal gland and extrapineal tissue (ovaries, retina, gastrointestinal tract). In healthy cells, melatonin inhibits apoptosis

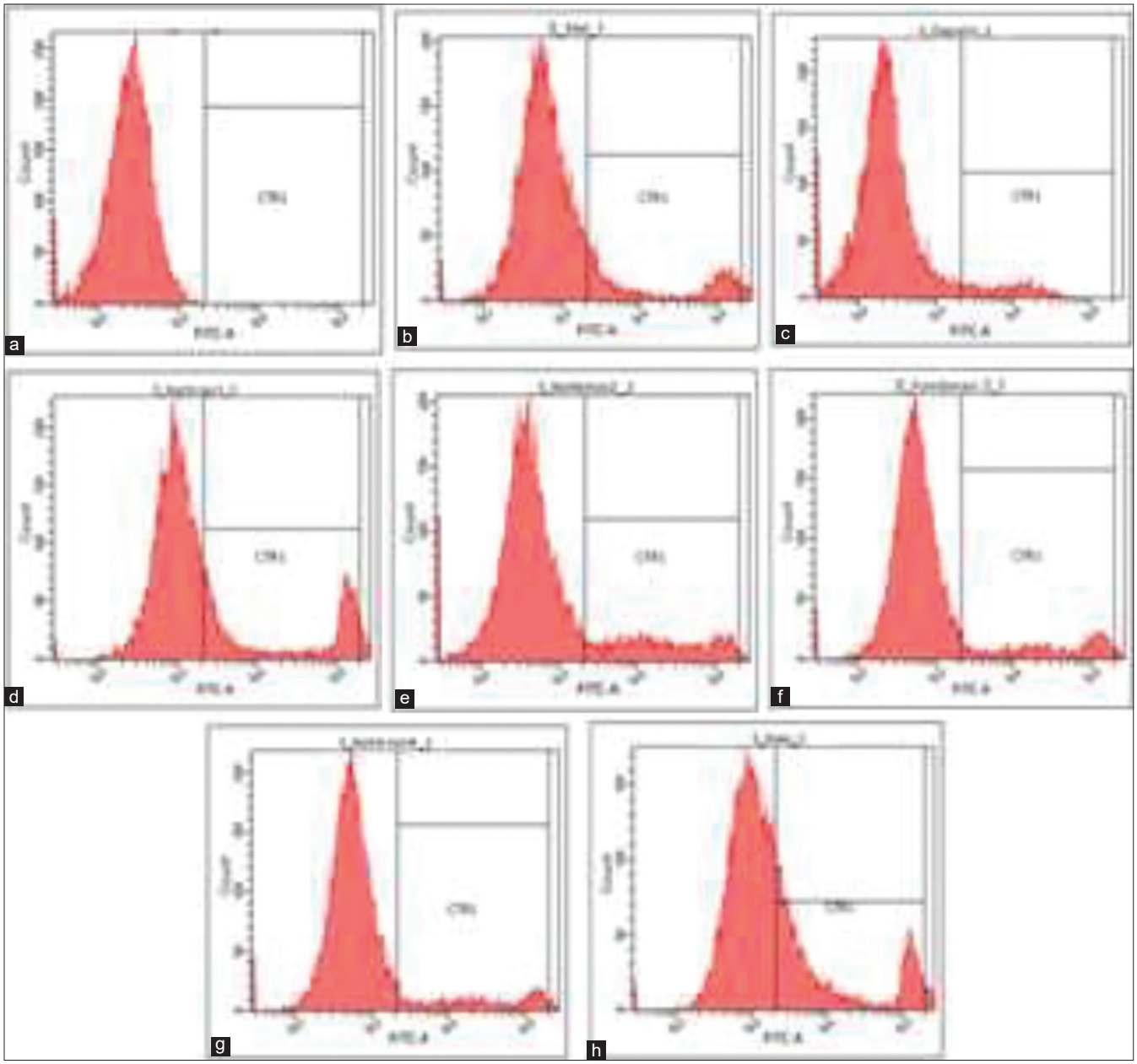

Figure 4: Representative flowcytometry of CTR-1 expression results in group (a) (Control); (b) (IC50 Melatonin); (c) (IC50 Cisplatin); (d) (Combination 1); (e) (Combination 2); (f) (Combination 3); (g) (Combination 4), and (h) (IC50 Doxorubicin)

process through several mechanisms. In contrast, melatonin has often been reported to be antiproliferative, antiangiogenic, pro-apoptotic, and immunomodulatory in various types of cancer, including ovarian cancer [7], [8]. In SKOV3 cultured cells, the combination of cisplatin and melatonin can increase the apoptotic process. Melatonin upregulates pro-apoptotic proteins such as p53, BAX, and activates caspase-3 [9], [10].

Melatonin has been reported to contribute for better clinical outcomes in several types of cancer, including ovarian cancer, both *in vitro* and *in vivo*. However, research on melatonin related to its effect on cisplatin chemotherapy resistance incidence is still very limited. Therefore, it is important to conduct further research on the melatonin effect to improving cisplatin chemotherapy resistance pathways in SKOV3 ovarian cancer biological cells, so in the future, melatonin can be used as an additional therapeutic modality in ovarian cancer, especially in cases of platinum resistance.

Table 1: Variations of research material concentration for determining the IC50 value

Research material	Concentration variant
Dox (μM)	25, 50, 100, 150, 200, 300
Mel (mM)	0.1, 0.5, 1, 2, 5
Cis (μM)	10, 20, 40, 50, 80, 100, 200

Dox: Doxorubicin, Mel: Melatonin, Cis: Cisplatin, Combi: Combination, Half Maximal Inhibitory Concentration

Methods

This research is a laboratory exploratory, experimental research using posttest only control group

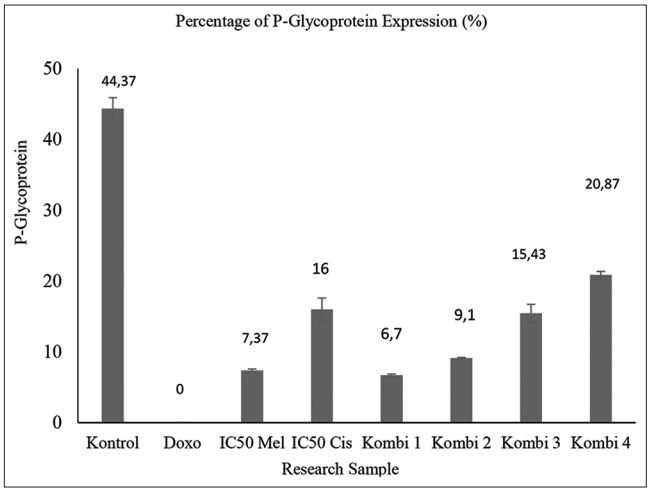

*Figure 5: Percentage of p-glycoprotein expression in each test group. ***p < 0,001, compared to control; ++p < 0,001, compared to IC50 doxorubicin; #p < 0,01, ###p < 0,001, compared to IC50 melatonin; ΦΦp < 0,001, compared to IC50 cisplatin; ††p < 0,001 compared to combination 1; ∋∋p < 0,001, compared to combination 2; Ψp < 0,01 compared to combination 3*

design to prove that a combination of melatonin and cisplatin can affect cisplatin-resistant chemotherapy through various mechanisms, including drug influx, drug efflux, and DNA damage repair, compared to cisplatin only chemotherapy. SKOV3 culture and treatment with melatonin in this research were carried out in the Stem Cells and Tissues Engineering Research Cluster (SCTE) laboratory of Institute of Medical Research Indonesia (IMERI), Faculty of Medicine, Universitas Indonesia, and flow cytometry researching was carried out in the Integrated Laboratory of FKUI. This research was conducted from September 2020 to November 2021.

This research used an in vitro research, in which ovarian cancer cell line SKOV3 was cultured on a suitable and controlled medium to grow these cells. SKOV3-American Type Culture Collection (ATCC) biological cells were derived from ATCC Catalog no HTB-77, which is a cell line from adenocarcinoma type ovarian epithelial cancer tissue in human ovaries.

Calculation with Mead's formula, the minimum sample used in this research was 16 with three repetitions (triplo) in each research group; the total sample used was 24 samples and eight research groups. Flowcytometry examination required a larger number of cells; cells were grown on well plate 6 with a diameter of 34.8 mm. Most of the cells can be harvested in 100% confluence in each well 6 is 9.5×10^5.

This research used several combinations; combination 1 was 1xIC50 melatonin and 1xIC50 cisplatin, combination 2 was ¾x IC50 melatonin and ¾x IC50 cisplatin, combination 3 was ½x IC50 melatonin and ½x IC50 cisplatin, and combination 4 was ¼x IC melatonin and ¼x IC50 cisplatin.

IC50 value determination

The research solution concentration required to reduce cell viability is expressed by IC50 value. The decrease in cell viability is 50%. Determination of IC50 value is done by making research solutions at various concentrations to be research on cells.

In this research, the IC50 value for research materials will be determined, namely doxorubicin, melatonin, and cisplatin. The concentration of each research material used to determine the IC50 value is illustrated in the following Table.1

From this calculation, the results of each IC50 value were IC50 melatonin was 1841 mM, IC50 cisplatin was 117.5 μM, and IC50 doxorubicin was 14.72 μM.

Cytotoxic activity test using MTS assay

MTS assay was conducted to count the percentage of cell viability after incubation with material (melatonin and a combination of melatonin and cisplatin). In color intensity which formed by the reduction of tetrazolium salt into formazan crystal that indicates the percentage of cell viability. Viable cells will induce more formazan crystal than nonviable cells. SKOV3 cell was harvested in well plate 96 with 25×10^4 cell/*well* and incubated in 37°C, and CO_2 concentration was 5% for 24 hours before conducting the test. MTS solution (*CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay* Promega) was added into each well and incubated for 3 hours, and then, it was read in spectrophotometry using 490 nm wavelength.

CTR-1, P glycoprotein, dan GSH examination using flowcytometer

The treated cells were harvested by adding trypsin-EDTA into culture container of as much as 1 mL. Cells were then collected and rotated at 2000 rpm for 5 min. The supernatant was discarded, and the cell pellet was resuspended with 1x PBS solution. The cells were put into a flow cytometry tube and redissolved

Table 2: Copper-transporting ATPase 1 expression in test group

Group	Mean ± SD	p[a]	*Post-hoc*[b]						
			Dox	Mel	Cis	Combi 1	Combi 2	Combi 3	Combi 4
Control	2.17 ± 0.21	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IC50 Dox	30.33 ± 0.4			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IC50 Mel	14.8 ± 0.1				<0.001	<0.001	<0.001	1.000	0.001
IC50 Cis	7.37 ± 0.7					<0.001	<0.001	<0.001	<0.001
Combi (1) IC50 1 ×	19.73 ± 0.49						1.000	<0.001	<0.001
Combi (2) IC50 ¾ ×	18.73 ± 0.84							<0.001	<0.001
Combi (3) IC50 ½ ×	14.53 ± 1.14								0.002
Combi (4) IC50 ¼ ×	11.77 ± 0.55								

[a]ANOVA one-way, [b]Bonferroni. SD: Standard deviation, Dox: Doxorubicin, Mel: Melatonin, Cis: Cisplatin, Combi: Combination, Half Maximal Inhibitory Concentration.

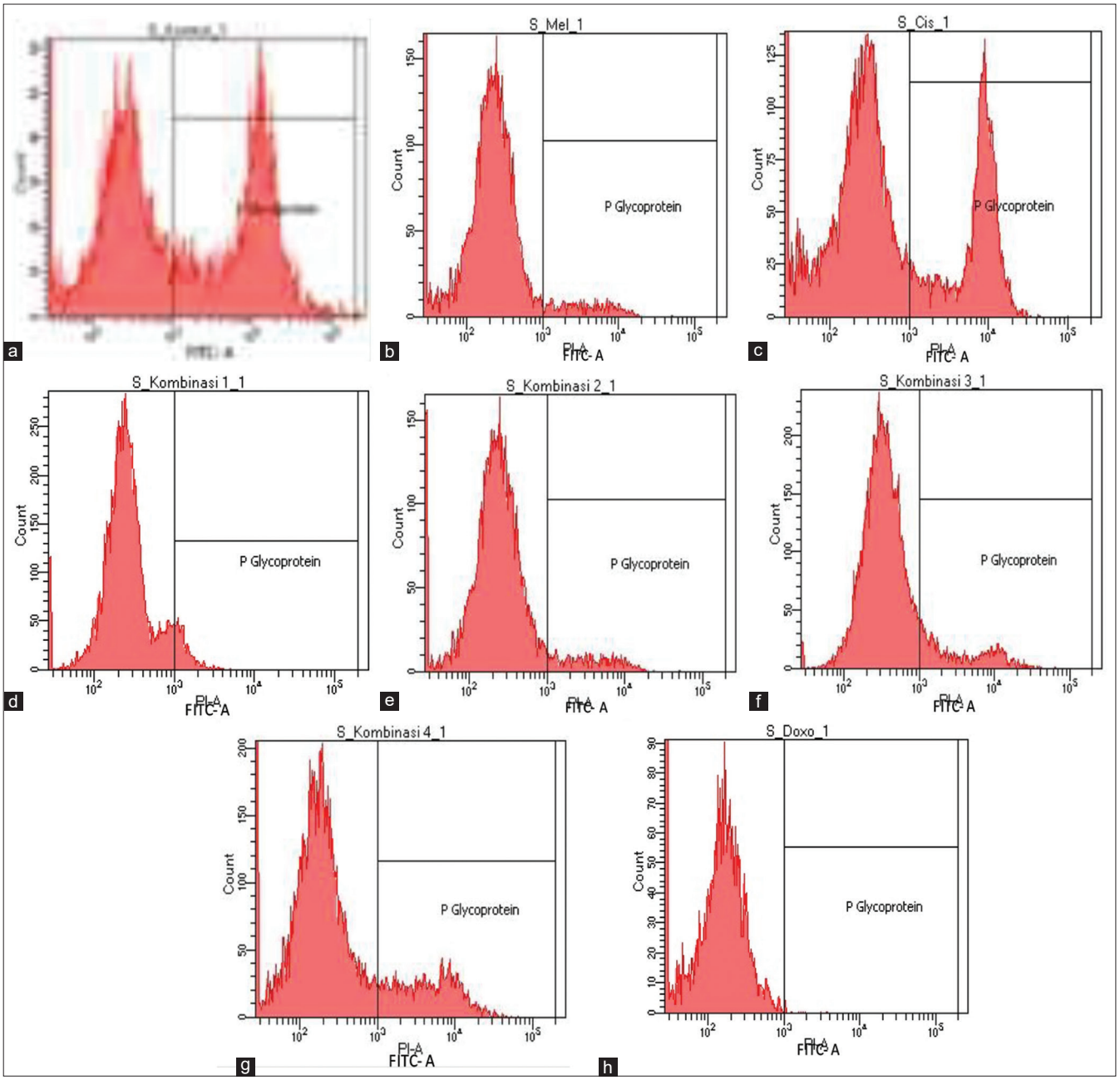

Figure 6: Representative flowcytometry results of p-glycoprotein expression. On Group (a) (Control); (b) (IC50 Melatonin); (c) (IC50 Cisplatin); (d) (Combination 1); (e) (Combination 2); (f) (Combination 3); (g) (Combination 4), dan (h) (IC50 Doxorubicin)

AQ1

with stain buffer solution, and rotated at 2100 rpm for 5 min. The supernatant was discarded, and the cell pellet was added 0.1 mL of binding buffer solution and CTR-1, P Glycoprotein, dan GSH antibody each according to examination type. Cells were vortexed for homogeneity, and then incubated at room temperature and dark conditions for 20 min. After incubation, PI solution was added to the cells and incubated again at room temperature and dark conditions for 10 min. After incubation, cells were washed with 1 mL of stain buffer solution, and then rotated at 2100 rpm for 5 min. The supernatant was discarded, and cell pellet was resuspended or redissolved with stain buffer solution. Cells are ready to be read on flowcytometer.

Table 3: P-glycoprotein expression in the test group

Group	Rerata ± SD	p[a]	*Post-hoc*[b]						
			Dox	Mel	Cis	Combi 1	Combi 2	Combi 3	Combi 4
Control	44.37 ± 1.55	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IC50 Dox	0			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IC50 Mel	7.37 ± 0.21				<0.001	1.000	0.986	<0.001	<0.001
IC50 Cis	16 ± 1.59					<0.001	<0.001	1.000	<0.001
Combi (1) IC50 1 ×	6.7 ± 0.17						0.161	<0.001	<0.001
Combi (2) IC50 ¾ ×	9.1 ± 0.1							<0.001	<0.001
Combi (3) IC50 ½ ×	15.43 ± 1.25								<0.001
Combi (4) IC50 ¼ ×	20.87 ± 0.49								

[a]ANOVA one-way, [b]Bonferroni. SD: Standard deviation, Dox: Doxorubicin, Mel: Melatonin, Cis: Cisplatin, Combi: Combination, Half Maximal Inhibitory Concentration.

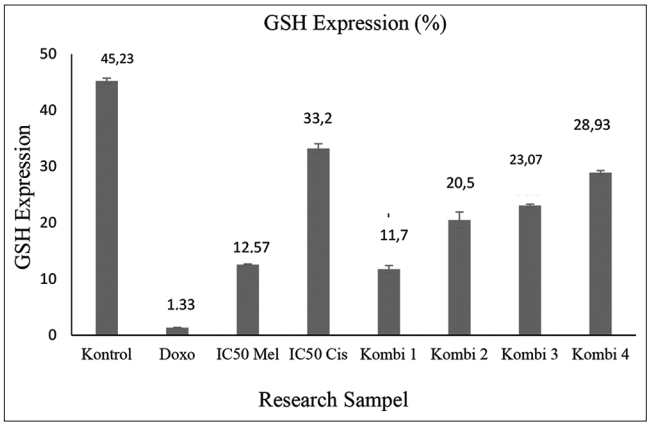

*Figure 7: The percentage of GSH expression in each test group. *** p < 0,001, compared to control; ++p < 0,001, compared to IC50 doxorubicin; #p < 0,01, ##p < 0,001, compared to IC50 melatonin; ϕϕp < 0,001, compared to IC50 cisplatin; ††p < 0,001 compared to combination 1; ∋∋p < 0,001, compared to combination 2; Ψp < 0,01 compared to combination 3*

Results

The IC50 melatonin value was obtained using an MTS assay at various concentrations, which were 0.1 mM, 0.5 mM, 1 mM, 2 mM, and 5 mM. The purpose of this stratified concentration was to determine the IC50 value and concentration of melatonin which could reduce SKOV3 cells viability by 50%. Assessment of absorbance values for all samples using a spectrophotometer with wavelength 490 nm.

The IC50 value was obtained by analyzing the absorbance value at each concentration using GraphPad software. Based on the data processing, the IC50 doxorubicin value was 14.72 μM. The IC50 melatonin value was 1841 mM. These results indicated that 1841 mM melatonin was required to decrease SKOV3 cells viability by 50%. The IC50 cisplatin value was 117.5 μM. This indicates that to reduce SKOV3 cells viability by 50%, 117.5 μM of cisplatin was needed. The IC50 doxorubicin value was 14.72 μM.

The IC50 cisplatin group obtained a mean decrease in cell viability was 55.16%. In all combination groups, there was a decrease in cell viability which was better than IC50 cisplatin ($p < 0.001$; one-way ANOVA). In the IC50 melatonin group, when compared to other groups, the mean decrease in SKOV3 cell viability was 47.68 %. It was seen that in all combination groups, there was a decrease in cell viability that was better than IC50 melatonin ($p < 0.001$; one-way ANOVA). However, when compared to group 4 (¼ IC50 melatonin and IC50 cisplatin) with a mean 48.43%, the IC50 melatonin group more decreased cell viability ($p < 0.001$; one-way ANOVA) Figures 1 and 2.

CTR-1 analysis

Examination using cell-specific markers was carried out using BD FACS ARIA III flowcytometry device. The groups used for this flowcytometry examination were the control group and the research group. The control group was cell control group and the positive control group. The cells control group was SKOV3 culture with a standard complete medium without additional research material. The positive control group was doxorubicin. The research sample groups used in this research were melatonin, cisplatin, and a combination of melatonin and cisplatin. The combination group of melatonin and cisplatin was divided into four combinations; combination 1 (1 × IC50), combination 2 (¾× IC50), combination 3 (½× IC50), and combination 4 (¼× IC50). The higher CTR-1 expression value, the higher drug influx into cells, lower cancer cell resistance to chemotherapy incidence.

Mean CTR-1 expression in the IC50 doxorubicin group was 30.33 ± 0.4, IC50 melatonin group was 14,8 ± 0,1, IC50 cisplatin group was 7.37 ± 0.7. While mean expression in combination group 1 (IC50 melatonin and IC50 cisplatin) was 19.73 ± 0.49, in combination group 2 (¾ IC50 melatonin and ¾ IC50 cisplatin) was 18.73 ± 0.84, in combination group 3 (½IC50 melatonin and ½ IC50 cisplatin) was 14.53 ± 1.14, in combination group 4 (¼ IC50 melatonin and ¼ IC50 cisplatin) was 11.77 ± 0.55.

There was no significant difference in CTR-1 expression in combination 1 group (IC50 melatonin and IC50 cisplatin) was 19.73% and combination group 2 (¾ IC50 melatonin and IC50 cisplatin) was 18.73% ($p > 0.05$; ANOVA one way). Combinations 1 and 2 were group with the highest CTR-1 expression significantly compared to the test treatment group ($p < 0.001$; one-way ANOVA), in addition to positive control. This indicates that the administration of combinations 1 and 2 has a higher incidence of therapeutic agents influx.

The CTR-1 expression percentage in combination 1, combination 2, combination 3, and combination 4 group was higher than the cisplatin

Table 4: Gamma-Glutamylcysteinylglycine expression in the test group

Group	Rerata ± SD	p[a]	*Post-hoc*[b]						
			Dox	Mel	Cis	Combi 1	Combi 2	Combi 3	Combi 4
Control	45.23 ± 0.5	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IC50 Dox	1.33 ± 0.06			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IC50 Mel	12.57 ± 0.12				<0.001	1.000	<0.001	<0.001	<0.001
IC50 Cis	33.2 ± 0.87					<0.001	<0.001	<0.001	<0.001
Combi (1) IC50 1 ×	11.73 ± 0.67						<0.001	<0.001	<0.001
Combi (2) IC50 — ×	20.5 ± 1.42							0.008	<0.001
Combi (3) IC50 ½ ×	23.07 ± 0.23								<0.001
Combi (4) IC50 ¼ ×	28.93 ± 0.38								

[a]ANOVA one-way, [b]Bonferroni. SD: Standard deviation, Dox: Doxorubicin, Mel: Melatonin, Cis: Cisplatin, Combi: Combination, Half Maximal Inhibitory Concentration.

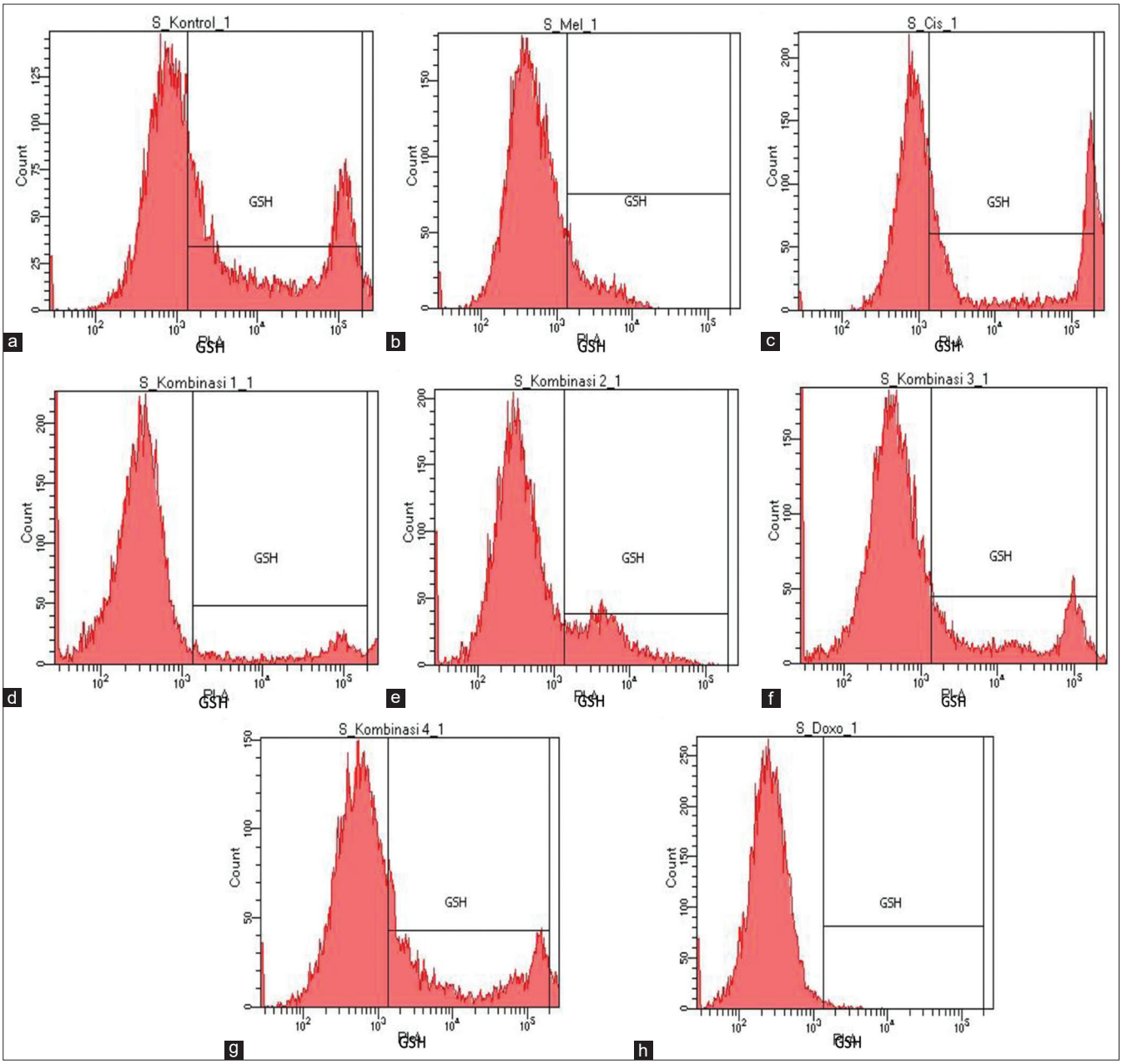

Figure 8: Representative flowcytometry results of GSH expression. On Group (a) (Control); (b) (IC50 Melatonin); (c) (IC50 Cisplatin); (d) (Combination 1); (e) (Combination 2); (f) (Combination 3); (g) (Combination 4), dan (h) (IC50 Doxorubicin)

group ($p < 0.001$; one-way ANOVA). This indicates that therapeutic agents influx incidence after administration of combination 1, combination 2, combination 3, and combination 4 is higher than cisplatin administration or in other words, higher therapeutic agents influx, the incidence of resistance chemotherapy will decrease.

In melatonin group, CTR-1 expression was lower than in combination 1 and combination 2, meaning that in combination 1 and combination 2 group, chemotherapy drugs influx was better than the single melatonin group. The intervention of administration melatonin did not result in a significant difference with combination 3 group ($p > 0.05$; one-way ANOVA). This shows that there is no difference in melatonin and combination 3 group in SKOV3 cancer cell influx against therapeutic agents (Table 2, Figures 3 and 4).

P-Glycoprotein analysis

Higher P-Gp percentage indicates cells ability to resist the response of cytotoxic compounds, with higher resistant chemotherapy incidence in cancer cells. In addition, it can be stated that if P-Gp expression percentage is low, then drug cytotoxic ability has a positive effect on cells.

Mean P-Gp expression in the IC50 doxorubicin group was 0, IC50 melatonin group was 7.37 ± 0.21, and IC50 cisplatin group was 16 ± 1.59. While mean expression in combination group 1 (C50 melatonin and IC50 cisplatin) was 6.7 ± 0.17, in combination group 2 (¾ IC50 melatonin and ¾ IC50 cisplatin) was 9.1 ± 0,1, in combination group 3 (½ IC50 melatonin and ½ IC50 cisplatin) was 15.43 ± 1,25, and in combination

group 4 (¼ IC50 melatonin and ¼ IC50 cisplatin) was 20.87 ± 0.49.

Combination 1 group could reduce more P-Gp expression with 6,7 % compared to other groups The administration of test material for the cisplatin group when compared with combination 1, combination 2, combination 3, and combination 4 group had a significant difference ($p < 0.001$; one-way ANOVA). It was seen that combination group 1 and combination 2 were able to lower P-Gp expression more than the cisplatin group. However, when compared with combination 4, the cisplatin group had the ability to lower P-Gp expression more. The lower drug efflux activity, the lower chemotherapy resistance incidence, so that the treatment outcome will be better.

The melatonin group had P-Gp expression percentage 7.37%, where when compared with other test groups, there was a significant difference between melatonin and cisplatin test group, combination 2 and combination 3 ($p < 0.001$). When compared with combination 1 and combination 2 groups, there was no significant difference between the melatonin group, combination 1 and combination 2 group. However, the combination 1 group was still better at reducing P-Gp expression. This indicates that a combination of IC50 melatonin and IC50 cisplatin can reduce the efflux activity of therapeutic agents; therefore, the incidence of chemotherapy resistance will decrease (Table 3, Figures 5 and 6).

GSH analysis

GSH is a protein commonly used as drug inactivation marker. The mean GSH expression in the control group was 45.23 ± 0.5, IC50 doxorubicin group was 1.33 ± 0.06, IC50 melatonin group was 12.57 ± 0.12, and IC50 cisplatin group was 33.2 ± 0.87. While mean expression in combination group 1 (IC50 melatonin and IC50 cisplatin) was 11.73 ± 0.67, in combination group 2 (¾ IC50 melatonin and ¾ IC50 cisplatin) was 20.5 ± 1.42, in combination group 3 (½ IC50 melatonin and ½ IC50 cisplatin) was 23.07 ± 0.23, and in combination group 4 (¼ IC50 melatonin and ¼ IC50 cisplatin) was 28.93 ± 0.38.

It was found that combination 1 group had the lowest GSH expression percentage compared to other combination groups, which was 11.73%. The analysis results showed that there was a significant difference in GSH expression between combination 1 and other combination groups ($p < 0.001$; one-way ANOVA).

The cisplatin group had GSH expression percentage with 33.2%. The administration of test material for the cisplatin group when compared to combination 1, combination 2, combination 3, and combination 4 groups had a significant difference ($p<0.001$; one-way ANOVA). It was seen that combination 1, combination 2, combination 3, and combination 4 groups were able to lower GSH expression more than the cisplatin group. The lower drug inactivation activity, the lower chemotherapy resistance incidence; therefore, the treatment outcome will be better.

The melatonin group had GSH expression percentage of 12.57%. GSH expression in melatonin group was significantly lower when compared to the cisplatin group, combination 2, combination 3, and combination 4 ($p < 0.001$; one-way ANOVA). However, when compared with the combination 1 group, it was seen that combination 1 was able to reduce GSH expression more than melatonin group, but not statistically significant (Table 4, Figures 7 and 8).

Discussions

CTR-1 is a marker for drug influx, while P-Glycoprotein (P-Gp) is a marker for drug efflux. Measurement of CTR-1 and P-gp expression was performed by flowcytometry analysis. This influx and efflux occur due to passive diffusion or facilitated transport. Chemotherapeutic drug substances enter the cell through the Cu2+ transporter CTR1. After that, it is actively removed from the cell by ATP7A and ATP7B copper transporters [3].

The value of CTR-1 and P-gp is closely related to the nature of cell resistance to drug substances. The higher CTR-1 expression value, it means greater drug influx, this clinically has a positive effect on drug activity. On the other hand, if the P-gp expression value is higher, it means that drug efflux is getting bigger; this clinically has a negative effect on drug action. In addition, CTR-1 gene deletion in yeast results in resistance increase of drug substance, in this case, cisplatin. Similarly, mouse cells lacking CTR-1 had higher cisplatin resistance. High-CTR-1 expression correlates with a better prognosis in patients receiving platinum-based therapy [11].

The higher incidence of influx or selective entry of chemotherapy drugs into cells will cause cells to undergo SKOV3 cell apoptosis. However, if a high incidence of influx is followed by the high incidence of efflux without involving SKOV3 cell apoptosis, it can be said that these cells are resistant to chemotherapy drugs [12]. In addition, this is due to efflux occurrence, which causes chemotherapy drug ingredients to be pumped back out of the cell; therefore, drug concentration in cell becomes less. Therefore, overexpression of P-gp in SKOV3 cells indicates that cells tend to pump chemotherapy drugs out of the cells. If chemotherapeutic drug material is removed or effluxed, the effect of the chemotherapy drug is reduced so that it can cause resistance [13].

Overexpression of γ-GCS, which catalyzes cysteine ligase with glutamate, increases glutathione

production. This glutathione increase acts as a Cu depletor, as evidenced by Cytochrome C Oxidase and Superoxide Dismutase (SOD) activity decrease as well as the component of holo-ceruloplasmin (CuCpm). Intracellular Cu deficiency increases hCTR-1 expression. Increased hCTR-1 transporter results in increased cisplatin uptake, which results in increased cisplatin administration sensitivity [14], [15], [16].

Epirubicin-induced increased expression of P-gp protein may be associated with the activation NF-kB pathway [17]. In several publications, it has been reported that upregulation of the NF-kB pathway is a possible mechanism for the development of MDR in resistant cancers [18]. In that in vitro experiment, with increasing epirubicin concentration, intracellular DNA damage gradually accumulated, and P-gp and P65 expression increased. The Western blot data further confirm that downregulation of P-gp are associated with inhibition of the NF-κB pathway. Kim et al. conducted a similar in vitro study and noted that inhibition of the NF-kB pathway can sensitize lymphoma cells to cytostatics [19].

In Liu's (2021) study, it was found that a combination of melatonin and epirubicin increased DLBCL cells sensitivity to epirubicin, including increasing suppression of cell viability and induction of apoptosis. Furthermore, molecular mechanisms underlying the enhanced action may be related to mitochondrial and P-gp-mediated apoptotic pathways. The IHC study showed that P-gp expression was positively correlated with NF-κB P65 expression. Epirubicin, a DNA-damaging cytotoxic, induces increased P-gp expression by activating the NF-κB pathway. Melatonin co-treatment was found to inhibit P-gp function and P-gp expression through suppression of the NF-κB pathway [17].

GSH functions to inactivate chemotherapy drugs by undergoing conjugation reaction mediated by GST through detoxification so that drug effectiveness decreases. Increased activity of GSH and GST has been reported to contribute cisplatin resistance [3], [20]. Giving melatonin to oxidative model mice for 6 days can reduce GSH levels and increase GSH-Peroxidase activity, an antioxidant enzyme that reduces hydroxylation radicals formation [21].

Fernandez (2019) reported that melatonin can increase the apoptotic effect of irradiation and cisplatin. In addition, melatonin can reduce oxidative stress by reducing GSH levels, as evidenced by the increase in GSSG/GSH ratio. High doses of melatonin increase glutathione synthesis. A concomitant decrease in GSH-Peroxidase activity was observed at the highest melatonin concentrations, especially in cisplatin-treated cells. However, at 100 μM melatonin combined with irradiation, they observed an increase in GSH-peroxidase activity. These results are consistent with lower ROS levels observed at 100 μM melatonin compared to 1500 μM. These data suggest that mitochondria induce melatonin-induced ROS response in cancer cells that enhances the cytotoxic effect of irradiation and cisplatin [22].

Melatonin enhances intramitochondrial antioxidant defenses by increasing reduced glutathione levels and inducing glutathione peroxidase and Mn-superoxide dismutase (Mn-SOD) in matrix and Cu, Zn-SOD in intermembrane space [23]. Glutathione plays a role in the regulation of intracellular copper pools that affect cisplatin uptake in cells. Hypersensitivity to cisplatin toxic effects was associated with increased uptake of cisplatin in these transfected cells. The cisplatin transporter was subsequently identified as a high-affinity Cu transporter (hCTR-1), and the mechanism of this hypersensitivity was due to the upregulation of hCTR-1 in these transfected cells. The expression of hCTR-1 is upregulated under low copper conditions and downregulated under adequate copper levels [6].

Conclusion

Melatonin plays an anticarcinogenic role through estrogen receptors in cancer cells. The combination of melatonin and cisplatin was able to increase drug influx activity by increasing CTR-1 expression, reduce drug efflux through decreasing P-Gp expression; and also reduce DNA repair activity by decreasing GSH expression.

Acknowledgment

The authors would like to give regard to all who participated and contributed to this research.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre AL, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worlwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424. https://doi.org/10.3322/caac.21492 PMid:30207593
2. Indonesia Society of Gynecologic Oncology. National Data, 2000-2016. Available from: http://inasgo.org [Last accessed on 2021 Aug 20].
3. Tapia G, Padilla I. Molecular mechanism of platinum resistance in ovarian cancer. In: Ovarian Cancer: A Clinical and Translational Update. Ch. 10. London: InTech DTP Team; 2013. p. 205-9. https://doi.org/10.5772/55562
4. Damia G, Broggini M. Platinum resistance in ovarian cancer:

Role of DNA repair. Cancers. 2019;11(1):119. https://doi.org/10.3390/cancers11010119
PMid:30669514

5. Sousa GF, Wlodarczyk S, Monteiro G. Carboplatin: molecular mechanisms of action associated with chemoresistance. Braz J Pharm Sci. 2014;50:694-7. https://doi.org/10.1590/s1984-82502014000400004
6. Chen HW, Kuo MT. Role of glutathione in the regulation of cisplatin resistance in cancer chemotherapy. Metal Based Drugs 2010;2010:430939. https://doi.org/10.1155/2010/430939
PMid:20885916
7. Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre M, Qin L. Melatonin as an antioxidant: Under promises but over delivers. J Pineal Res. 2016;61(3):253-78. https://doi.org/10.1111/jpi.12360
PMid:27500468
8. Favero G, Moretti E, Bonomini F, Reiter RJ, Rodella RF, Rezzani R. Promising antineoplastic actions of melatonin. Front Pharmacol. 2018:9:1086. https://doi.org/10.3389/fphar.2018.01086
PMid:30386235
9. Chuffa L, Reiter R, Lupi L. Melatonin as a promising agent to treat ovarian cancer: molecular mechanisms. Carcinogenesis. 2017;38(10):945-52. https://doi.org/10.1093/carcin/bgx054
PMid:28575150
10. Li Y, Li S, Zhou Y, Meng X, Zhang JJ, Xu DP, *et al*. Melatonin for the prevention and treatment of cancer. Oncotarget. 2017;8(24):39896-921. https://doi.org/10.18632/oncotarget.16379
PMid:28415828
11. Yang T, Chen M, Chen T, Thakur A. Expression of the copper transporters hCTR-1, ATP7A and ATP7B is associated with the response to chemotherapy and survival time in patients with resected non-small cell lung cancer. Oncol Lett. 2015;10(4):2584-90. https://doi.org/10.3892/ol.2015.3531
PMid:26622894
12. Zhang X, Hou G, Liu A, Xu H, Guan Y, Wu Y, *et al*. Matrine inhibits the development and progression of ovarian cancer by repressing cancer associated phosphorylation signaling pathways. Cell Death Dis. 2019;10(10):770. https://doi.org/10.1038/s41419-019-2013-3
PMid:31601793
13. He C, Sun Z, Hoffman R, Yang Z, Jiang Y, Wang L. P-glycoprotein overexpression is associated with cisplatin resistance in human osteosarcoma. Anti Cancer Res. 2019;39(4):1711-8. https://doi.org/10.21873/anticanres.13277
PMid:30952710
14. Kilari D, Guancial E, Kim ES. Role of copper transporters in platinum resistance. World J Clin Oncol. 2016;7(1):106-13. https://doi.org/10.5306/wjco.v7.i1.106
15. Silva MM, Rocha CR, Kinker GS, Pelegrini AL, Menck CF. The balance between NRF2/GSH antioxidant mediated pathway and DNA repair modulates cisplatin resistance in lung cancer cells. Sci Rep. 2019;9:17639. https://doi.org/10.1038/s41598-019-54065-6
16. Kalayda GV, Wagner CH, Jaehde U. Relevance of copper transporter 1 for cisplatin resistance in human ovarian carcinoma cells. J Inorg Biochem. 2012;116:1-10. https://doi.org/10.1016/j.jinorgbio.2012.07.010
PMid:23010323
17. Liu K, Song J, Yan T, Zou K, Che Y, Wang B, *et al*. Melatonin increases the chemosensitivity of diffuse large B-cell lymphoma cells to epirubicin by inhibiting P-glycoprotein expression via the NF-κB pathway. Transl Oncol. 2021;14(1):100876. https://doi.org/10.1016/j.tranon.2020.100876
PMid:33007707
18. Yang X, Ding Y, Xiao M, Liu X, Ruan J, Xue P. Anti-tumor compound RY10-4 suppresses multidrug resistance in MCF-7/ADR cells by inhibiting PI3K/Akt/NF-κB signaling. Chem Biol Interact. 2017;278:22-31. https://doi.org/10.1016/j.cbi.2017.10.008
PMid:28987325
19. Kim JH, Kim SW, Hong JY, Ryu KJ, Kim SJ, Park C. Epstein-Barr virus EBNA2 directs doxorubicin resistance of B cell lymphoma through CCL3 and CCL4-mediated activation of NF-κB and Btk, Oncotarget. 2017;8(3):5361-70. https://doi.org/10.18632/oncotarget.14243
PMid:28036258
20. Jamali B, Nakhjavani M, Hosseinzadeh L, Amidi S, Nikounezhad N, Shirazi FH. Intracellular GSH alterations and its relationship to level of resistance following exposure to cisplatin in cancer cells. Iran J Pharm Res. 2015;14(2):513-9.
PMid:25901159
21. Medina-Leendertz S, Mora M, Vielma JR, Bravo Y, Atencio-Bracho L, Leal-Yépez A, *et al*. Melatonin decreases oxidative stress in *Drosophila melanogaster* exposed to manganese. Investig Clín. 2018;59(3):230-41. https://doi.org/10.22506/ti/2017/v24/i1/149037
22. Fernandez-Gil BI, Guerra-Librero A, Shen YQ, Florido J, Martinez-Ruiz L, García-López S, *et al*. Melatonin enhances cisplatin and radiation cytotoxicity in head and neck squamous cell carcinoma by stimulating mitochondrial ROS generation, apoptosis, and autophagy. Oxid Med Cell Longev. 2019;2019:7187128. https://doi.org/10.1155/2019/7187128
23. Harderland R. Melatonin and the electron transport chain. Cell Mol Life Sci. 2019;74(21):3883-96. https://doi.org/10.1007/s00018-017-2615-9