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Evaluation of the combined treatment with cisplatin and melatonin on neuroblastoma cell viability and antioxidant capacity

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ABSTRACT

In this study, we investigated the cell viability and antioxidant effects of melatonin both with and without cisplatin (CDDP) on the cultured neuroblastoma cancer cell line. Neuroblastoma cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C with 5% CO_2 to examine the cytotoxic effect of melatonin; cells were cultured both CDDP with and without melatonin and thereafter counted in a 48-well microplate. To examine the effect of melatonin and CDDP, cells were divided into ten groups (control, vehicle, melatonin-5nM and10nM, CDDP-50μM and 100μM, melatonin-5nM+CDDP-50μM, melatonin-5nM+CDDP-100μM, melatonin-10nm+CDDP-50μM and melatonin-10nM+CDDP-100μM) and thereafter cell viability was determined in a 48-well microplate using 3-(4,5-dimetiltriazol-2-il)-2,5- difeniltetrazolium bromid (MTT) assay. In different series, cells were cultured and treated with ethanol, melatonin, CDDP, and combination of melatonin and CDDP. After harvest, TAS and TOS were measured via Elisa assay kits. Melatonin and combination of melatonin and CCDP produced no cytotoxic effect on neuroblastoma cancer cells after 24 hours, but a decrease in the cell viability after 48 hours. Furthermore, CDDP treatment significantly decreased the cell viability both after 24 and 48 hours periods compared to untreated controls. Melatonin enhanced the cytotoxic effects of CDDP after 48 hours neuroblastoma cell lines. Therefore, melatonin may be used as an adjunctive therapy agent to both improve neuroblastoma cancer chemotherapy and depression and for inhibition of CDDP side effects.

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1. Introduction

Cisplatin (CDDP) is a chemotherapeutic agent, which is used for the treatment of human malignancies such as neuroblastoma and osteosarcoma (Karadeniz et al., 2011). However, this drug has many side effects due to severe cytotoxicity against normal cells and tissues. A number of studies have suggested that using a combination of chemotherapeutic agents and natural antioxidant products is a beneficial therapeutic strategy to overcome its cytotoxic effects (Liu et al., 2008; Reiter et al., 2009).

Melatonin, as a hormone, is produced in the pineal gland and several organs, which orchestrate the numerous biological activities in the body such as the modulation of circadian rhythms and regulation of immune systems (Brzezinski, 1997; Dubocovich et al., 2010). It also plays a major role in some psychiatric disorder such as some sleep disorders and insomnias (Bartsch et al., 1989; Colleoni et al., 2000).

Several researchers reported the potential effects of melatonin as a potent combination therapeutic agent to support the efficacy of chemotherapeutic agents and also decrease their side effects in vitro (Futagami et al., 2001) and in vivo (Şener et al., 2000; Parlakpinar et al., 2002). Also, melatonin and melatonin agonists are antidepressant agents, which can be used for clinical treatment of depression

Table 1. Results of MTT (cell viability) analysis after CDDP and/or melatonin treatment for 24- and 48- hour incubation period

Groups	Cell Viability (24 hours)	Cell viability (48 hours)
Control	0.1366±0.023[a]	0.0823±0.016[a]
Vehicle	0.1320±0.035[a]	0.0826±0.032[a]
Mel-5nM	0.1398±0.032[a]	0.0835±0.010[a]
Mel-10nM	0.1412±0.025[a]	0.0880±0.014[a]
CDDP-50μM	0.1119±0.017[b]	0.0700±0.009[b]
CDDP-100μM	0.1068±0.014[b]	0.0690±0.005[b]
Mel-5nM+CDDP-50μM	0.1398±0.013[a]	0.0679±0.003[b]
Mel-10nM+CDDP-50μM	0.1336±0.045[a]	0.0638±0.004[bc]
Mel-5nM+CDDP-100μM	0.1322±0.021[a]	0.0699±0.003[b]
Mel-10nM+CDDP-100μM	0.1395±0.042[a]	0.0589±0.005[c]

Values are expressed as mean ± standard deviation. abc The footnote letters in the same column indicate significant differences between groups (n = 6); P<0.05. For statistical analysis, differences between the groups were tested by analysis of variance followed by the Duncan post hoc test (ANOVA).

(Colleoni et al., 2000; Courtet, 2012). Depression incidence in patients with cancer has been reported as 22-24% (American Psychiatric Association, 2000) Therefore, many neoplastic patients need psychological support, especially after surgery. An interaction between psychological health and anti-cancer therapy has been explained with a possible assumed tendency of depressed patients to be less eager in obtaining health care (Watson et al., 1999). Therefore, treatment of depression might be essential for tailoring the treatment of malignancies.

Reactive Oxygen Species (ROS) are highly reactive molecules with unpaired electrons such as hydrogen peroxide, hydroxyl radicals, and superoxide anion. They are produced in normal physiological processes such as aerobic metabolism or inflammation. The excessive ROS production shows detrimental effects on cells or tissues, which are inhibited by the antioxidant defence system in the cellular defence system. Chemotherapeutic agents can cause a disproportional increase in intracellular ROS, cell cycle arrest and apoptosis activation via death signalling pathways. The advantage of this therapeutic strategy is that normal cells are not affected by the chemotherapeutics. However, if a threshold of toxicity in these cancer cells is reached and an additional increase in the ROS level occurs these can cause more mutations and invasion in cancer cells (Storz et al., 2009; Trachootham et al., 2009). Therefore, a combination of chemotherapeutics with antioxidant substances increases the ROS level in cancer cells and protects normal cells from cytotoxic effects of chemotherapeutics (Trachootham et al., 2009). Nevertheless, ROS mediated signalling pathways selectively killing cancer cells and overcoming drug resistance are unknown.

Understanding the detailed effects on living cells during cisplatin treatment would aid to the development of new treatment strategies to improve the therapeutic roles. The first aim of the study was to investigate the cytotoxic effect of melatonin alone on the neuroblastoma cell line. The second aim was to evaluate the interaction between melatonin and cisplatin treatment on the neuroblastoma cell line. The third purpose was to investigate the antioxidant capacity when neuroblastoma cell line was treated with melatonin, CDDP, or melatonin plus CDDP.

2. Materials and methods

Chemicals

Melatonin was purchased from Sigma Chemical Company (St. Louis, MO) and CDDP were purchased from Koçak Farma, Turkey. Melatonin was dissolved in ethanol to a concentration of 0.1M. The final concentration of solvents in the culture was less than 0.1%, such a concentration that exhibits no effect on cell growth and viability, as was experimentally confirmed.

Cell line

The neuroblastoma cell line used was NA/An1 (mouse neuroblastoma cancer), which was obtained from Sap Institute, Turkey. The cell line was subcultured once a week at 37°C atmosphere of 5% CO_2 and 100% relative humidity, and maintained at a low passage number (Hrushesky, 1985; Lissoni et al., 1997). The culture medium was Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum, 2mM L-glutamineand 1mM penicillin–streptomycin-amphotericin (Sigma Chemical Company).

Cell growth and viability assays

Adherent cells at a logarithmic growth phase were detached by the addition of 3mL of a trypsin-EDTA mixture (Gibco-BRL) and incubated for 5 minutes at 37°C. Cells were plated (200mL per well) in 48-well micro-plates at a density of 5,000 cells per well. Cells were left for 24 hours at 37°C to resume exponential growth. Six replicate wells for each concentration were used. Cell growth was measured 24 and 48 hours later with MTT assays. All experimental measurements were performed at least twice.

Table 2. Total antioxidant status (TAS) and total oxidant (TOS) status analysis results after CDDP and/or melatonin treatment for 24- and 48- hour incubation period

Groups	TAS 24h	TAS 48h	TOS 24h	TOS 48h
Control	0.2810±0.168[a]	0.3472±0.117[a]	2.9038±0.080[a]	2.8606±0.048[a]
Vehicle	0.2781±0.103[a]	0.3384±0.094[a]	2.9241±0.105[a]	2.8732±0.124[a]
Mel-5nM	0.2957±0.069[a]	0.4012±0.024[b]	2.9567±0.182[a]	3.0385±0.221[a]
Mel-10nM	0.3207±0.033[b]	0.5747±0.065[c]	2.8269±0.145[a]	3.0769±0.117[a]
CDDP-50μM	0.1572±0.083[c]	0.2775±0.131[c]	2.8654±0.142[a]	3.0769±0.135[a]
CDDP-100μM	0.2470±0.149[c]	0.2292±0.091[c]	2.9038±0.219[a]	3.0192±0.160[a]
Mel-5nM+CDDP-50μM	0.1665±0.038[c]	0.2552±0.099[c]	2.8846±0.135[a]	3.0000±0.291[a]
Mel-10nM+CDDP-50μM	0.1792±0.021[c]	0.2860±0.084[c]	3.2308±0.602[b]	3.0962±0.299[a]
Mel-5nM+CDDP-100μM	0.1774±0.086[c]	0.2216±0.084[c]	3.0385±0.174[a]	2.8269±0.109[a]
Mel-10nM+CDDP-100μM	0.1798±0.082[c]	0.2644±0.040[c]	2.9808±0.152[a]	2.6923±0.067[b]

Values are expressed as means ± standard deviation. abc The footnote letters in the same column indicate significant differences between groups (n=6); P<0.05. For statistical analysis, differences between the groups were tested by analysis of variance followed by the Duncan post hoc test (ANOVA).

Effects of CDDP and/or melatonin on cell viability

The experiment was designed to examine the effects of melatonin (5 and 10nM) (Kim et al., 2012) and/or CDDP (50 and 100μM) (Spano et al., 2008). The concentration of melatonin and CDDP was selected in accordance with the IC50 of the neuroblastoma cancer cell line used and previous studies (Spano et al., 2008; Kim et al., 2012). Test substances (CDDP and melatonin) were placed in tubes filled with 10mL of the medium, and 100mL of that medium was used for experiments. Cells were plated (200mL per well) in 48-well micro-plates and left for 24 hours. The control, melatonin and/or CDDP combination groups were divided into ten subgroups:

Control, in which the medium was replaced with a fresh medium and incubated for 24 and 48 hours;

Vehicle, in which the medium was replaced with a vehicle solution (ethanol) including the medium and incubated for 24 and 48 hours.

Mel-5nM, in which the medium was replaced with a 5nM concentration of melatonin including the medium and incubated for 24 and 48 hours.

Mel-10nM, in which the medium was replaced with a 10nM concentration of melatonin including the medium and incubated for 24 and 48 hours.

CDDP-50μM, in which the medium was replaced with a 50μM concentration of CDDP including the medium and incubated for 24 and 48 hours.

CDDP-100μM, in which the medium was replaced with a 100μM concentration of CDDP including the medium and incubated for 24 and 48 hours.

Mel-5nM+CDDP-50μM, in which the medium was replaced with a 5nM concentration of melatonin 50μM concentration of CDDP including the medium and incubated for 24 and 48 hours.

Mel-10nM+CDDP-50μM, in which the medium was replaced with a 10nM concentration of melatonin 50μM concentration of CDDP including the medium and incubated for 24 and 48 hours.

Mel-5nM+CDDP-100μM, in which the medium was replaced with a 5nM concentration of melatonin 100μM concentration of CDDP including the medium and incubated for 24 and 48 hours.

Mel-10nM+CDDP-100μM, in which the medium was replaced with a 10nM concentration of melatonin 100μM concentration of CDDP including the medium and incubated for 24 and 48 hours.

Cell viability assay

The cytotoxic effects of melatonin and/or CDDP on neuroblastoma cell line (NA/An1-mouse neuroblastoma) were assessed using an MTT cell proliferation kit (Sigma Chemical Company) according to the manufacturer's instructions. Briefly, 5,000 cells/well in a 200mL medium were seeded to attach type 48-well plates and after overnight incubation at 37°C media were changed with melatonin and CDDP containing media. The first and sixth rows of a plate were designated as the medium control that contained the medium only and cell control that included cells in the medium, respectively. Starting from the second row, serial dilutions of melatonin and CDDP containing medium were added to wells at a 2:3 dilution ratio up to the last row. Four identical plates were prepared, and they were incubated at 37°C for 24 and 48 hours. After 24- and 48- hour incubation periods, an MTT reaction solution containing an activation reagent (2%) was added to all of the wells containing the medium. Following three hours of incubation at 37°C, the absorbance was measured with an ELISA plate reader (μ-Quant, BioTek Instrument, USA) at 570nm. The Microsoft Excel programme was utilised to generate the percentage of cell viability versus dose curves.

Determination of TAS and TOS

Measurement of the Total Antioxidant Status (TAS): TAS concentrations were measured using an ELISA kit TAS assays Kits (Rel Assay Diagnostics, Turkey) according to the manufacturer's protocols. TAS levels of the cell medium for all groups was determined using a novel automated measurement method developed by Erel (Erel, 2004). The total antioxidant capacity method is based on the bleaching of the characteristic color of a more stable 2,2'-azino-bis (3-ethylbenz-thiazo-line-6-sulfonic acid) (ABTS) radical cation by antioxidants. The medium samples were measured with an ELISA plate reader (μ-Quant, BioTek Instrument, USA) at 660nm and the results were expressed as mmol Trolox Equiv/L.

Measurement of the Total Oxidant Status (TOS): TOS concentrations were measured using an ELISA kit TAS assays Kits (Rel Assay Diagnostics) according to the manufacturer's protocols. TOS levels were determined using a novel automated measurement method developed by Erel (Erel, 2005). The total oxidant status method is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the ferric ion by xylenol orange. The medium samples were read with an Elisa plate reader (μ-Quant, BioTek Instrument) at 560nm. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micro-molar hydrogen peroxide equivalent per litre (μmol H_2O_2 Equiv./L).

3. Results

Cell viability results

An MTT assay was used to investigate whether or not melatonin has cytotoxic effects on neuroblastoma cell line both with and without CDDP treatment. Cells were treated with melatonin (5 and 10nM) and/or CDDP (50 and 100μM) for 24- and 48-hour periods.

For the 24-hour period, while melatonin treatment did not show a significant effect on the cell viability, CDDP treatment significantly reduced the cell viability compared to the control groups ($P<0.05$). In addition, melatonin and CDDP treatments had no significant effect on the viability, even at the higher concentration of CDDP compared with the untreated control ($P<0.05$) (Table 1).

For the 48-hour period, CDDP reduced the cell proliferation or viability, but melatonin did not affect the cell proliferation or viability. Conversely and interestingly, combined treatment with cisplatin and melatonin significantly suppressed the cell viability compared with the untreated control ($P<0.05$). These results were confirmed by morphological analysis under an inverted microscope (Fig. 1, 2) (Table 2).

TAS and TOS analysis results

In analysis of TAS levels, after the 24- and 48- hour periods, TAS levels were increased with the melatonin treatment, but decreased with CDDP and a combination of CDDP with melatonin treatment compared to the untreated and vehicle treated controls ($P<0.05$).

Analysis of TOS levels revealed that in group 7 after 24 hours the TOS level was significantly increased, while in group 10 after 48 hours TOS level was significantly decreased when compared with other groups ($P<0.05$). However, there were no significant differences between the remaining groups after 24 and 48 hours ($P>0.05$).

4. Discussion

Neuroblastoma is the most common cancer type and has shown a higher mortality rate. For the treatment of neuroblastoma cancer, surgical and chemotherapeutic applications were used for neoplastic patients. However, a surgery and platinum-based chemotherapy combination were recommended for advanced neuroblastoma cancer at stage IIb–IV (Evans et al., 1971). CDDP is a platinum-based chemotherapy agent, although it has shown limited therapeutic efficacy due to serious side effects. Several studies have reported that combined chemotherapy of CDDP and natural products may be useful for the treatment of many cancer types with improved therapeutic efficacy and ameliorated side effects (Choi et al., 2011; Su et al., 2011; Yunos et al., 2011). Therefore, in this study, we focused on the cell viability and antioxidant effects of melatonin both with and without CDDP treatment.

The present studies demonstrated that melatonin showed the synergetic cytotoxic effects with CDDP treatment on the neuroblastoma cell line. Contrary to CDDP, melatonin treatment induced the cell growth in 24 hours and a higher dose of melatonin (10nM) showed the synergic effect on cisplatin-induced cell death for 48 hours. Furthermore, previous studies have suggested the cytotoxic effect of melatonin on many cancer types of human (Futagami et al., 2001).

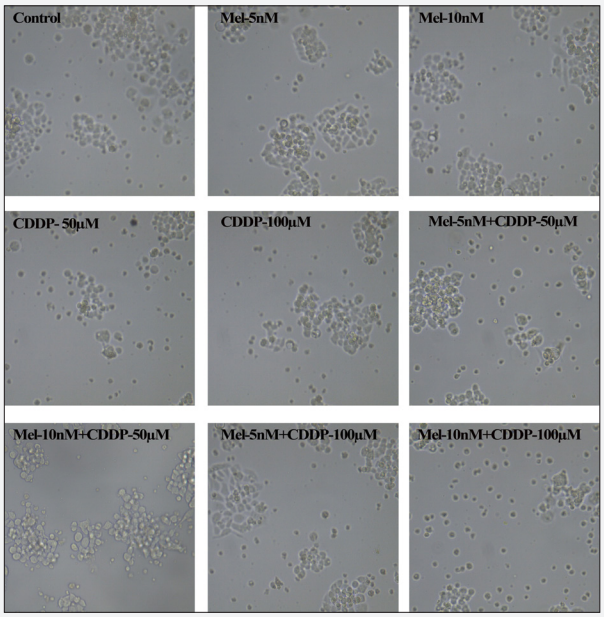

Fig. 1. Morphologic analysis of cell proliferation under inverted microscope for 24-hour incubation period groups

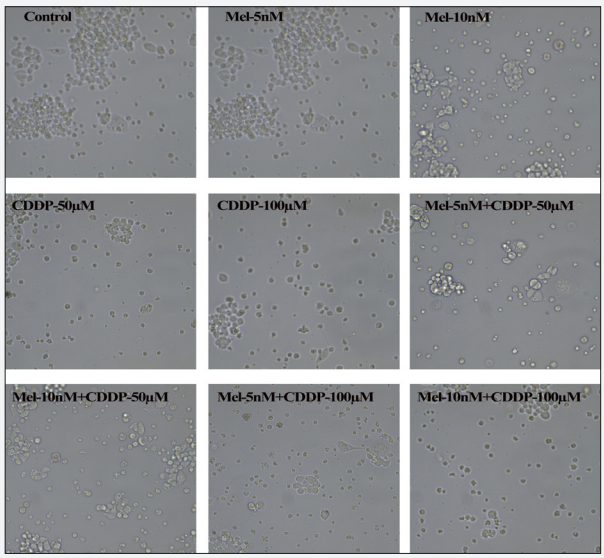

Fig. 2. Morphologic analysis of cell proliferation under inverted microscope for 48- hour incubation period

Under normal conditions, the physiological levels of ROS protect the cells from death. However, in cancer cell lines, growth factors or cytokines stimulate the production of ROS to induce cell death (Lissoni et al., 1999; Shiu et al., 2010). Therefore, melatonin can enhance the production of ROS and accelerate the cell death in neuroblastoma cell line, which is consistent with other studies (Hara et al., 2001; Reiter et al., 2002).

In an attempt to protect body function from CP toxicity several antioxidants have been analysed (Karadeniz et al., 2011, Kim et al., 2012). Melatonin is a powerful antioxidant agent that potentially attenuates the cytotoxicity of CDDP and inhibits the cell growth and proliferation (Lissoni et al., 1997; Kim et al., 2012). Also, pro-apoptotic, anti-tumour, and anti-angiogenetic effects of melatonin were reported by some researches (Hermann et al., 2002, Wenzel et al., 2005). However, many scientists argue the relationship between the plasma concentration and anti-tumour effect of melatonin (Blask et al., 1999; Hill et al., 2009). Actually, inhibition of cell proliferation by high concentration of melatonin is strongly associated with the antioxidant properties (Garcia-Navarro et al., 2007; Hill et al., 2009). Also, high plasma level of melatonin can promote the effect of chemotherapeutic drugs, suggesting that melatonin can be cytotoxic for malignant cells in particular conditions.

In the biochemical analysis, the total antioxidant and oxidants capacities were changed during melatonin and/or CDDP treatment owing to an increase in the antioxidant and a decrease in the oxidant status levels. In the proliferation of cancer cells, activation of the MAPK (mitogen-activated protein kinase)/Erk1/2 (extracellular-regulated kinase 1/2) pathways due to the effect of ROS inhibitors functionally regulates the cancer cell survival (Liou and Storz, 2010). In cancer treatment strategies, many different chemotherapeutic agents are designed to increase cellular ROS levels to induce cell damage, resulting in tumour cell apoptosis (Trachootham et al., 2009), dependent on the tumour type (Bairati et al., 2005; Alexandre et al., 2006; Llobet et al., 2008). Furthermore, contrary to cancer cells, which have high ROS levels during all malignancy stages, cancer stem cells have a

higher antioxidant capacity (Trachootham et al., 2009). This suggests that the antioxidant and oxidants' capacity may be changed by melatonin and/or CDDP treatment depending on cell proliferation activity.

In conclusion, treatment with melatonin may be attenuated to the toxic effects of ROS in neoplastic patients with CDDP treatment and can be useful in a combination with chemotherapeutic agents to improve the therapeutic effects.

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