Turkish Journal of Biology

Volume 39 | Number 6 Article 11

1-1-2015

Melatonin attenuates apoptosis and mitochondrial depolarization levels in hypoxic conditions of SH-SY5Y neuronal cells induced by cobalt chloride (CoCl2)

ABDÜLHADİ CİHANGİR UĞUZ

AHMİ ÖZ

BÜŞRA YILMAZ

SEDA ALTUNBAŞ

ÖMER ÇELİK

TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2015) 39: 896-903

doi:10.3906/biy-1505-100

Melatonin attenuates apoptosis and mitochondrial depolarization levels in hypoxic conditions of SH-SY5Y neuronal cells induced by cobalt chloride ($CoCl_2$)

Abdülhadi Cihangir UĞUZ[1,2,*], Ahmi ÖZ[1], Büşra YILMAZ[3], Seda ALTUNBAŞ[3], Ömer ÇELİK[1,2]

[1]Department of Biophysics, School of Medicine, Süleyman Demirel University, Isparta, Turkey
[2]Neuroscience Research Center, Süleyman Demirel University, Isparta, Turkey
[3]School of Medicine, Süleyman Demirel University, Isparta, Turkey

Received: 28.05.2015 • **Accepted/Published Online:** 23.07.2015 • **Printed:** 31.12.2015

Abstract: Melatonin (MEL) and its metabolites serve as endogenous reactive oxygen species (ROS) scavengers and have a wide spectrum of antioxidant activity. Cobalt chloride is one of the commonly used hypoxia-mimetic agents, due to blocking the degradation of and triggering the accumulation of hypoxia-inducible factor-1 alpha (HIF-1α) protein, which is very well known as a critical regulator of the cellular response against hypoxia. In the current study we aimed to determine the possible protective effects of melatonin on a cobalt chloride-induced hypoxia model of SH-SY5Y neuronal cells. Group I was the control group and SH-SY5Y cells were incubated in normal culture media without any chemical administration. In Group II, SH-SY5Y cells were incubated with 1 μM MEL for 24 h. In Group III cells were incubated with 200 μM cobalt chloride for 24 h. The last group, Group 4, was a combination group of cobalt chloride and MEL. Cells were preincubated with 1 μM for 12 h and then 200 μM cobalt chloride for 24 h. We performed the cell viability test (MTT) and checked the caspase-3 and -9 activities and the lipid peroxidation (LP), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), mitochondrial depolarization, and intracellular ROS levels. We observed that cobalt chloride increased intracellular ROS, caspase-3 and -9, lipid peroxidation, and mitochondrial membrane depolarization values, while decreasing GSH levels and GSH-Px activity. However, GSH and GSH-Px values were increased by melatonin treatment although lipid peroxidation level, intracellular ROS production, and caspase-3 and -9 activities were decreased by the treatment. In conclusion, we observed that melatonin incubation protects neuronal cells against hypoxia-induced oxidative stress.

Key words: SH-SY5Y neuroblastoma cells, cobalt chloride, oxidative stress, melatonin, mitochondrial membrane depolarization

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a highly preserved antioxidant molecule that can be found in organisms from unicells to vertebrates (Tan et al., 2010). Due to its chemical structure, melatonin and its metabolites serve as endogenous free radical scavengers and wide-spectrum antioxidants (Galano et al., 2011, 2013). Regarding melatonin's therapeutic action, it modulates the immune reactions (Carrillo-Vico et al., 2003), displays antiproliferative effects on tumor cells (Bejarano et al., 2009; Hill et al., 2009), and has cytoprotective effects in healthy cells (Martin et al., 2002). One of the most beneficial effects of melatonin administration is its regulator effect on mitochondrial physiology (Martin et al., 2000; Espino et al., 2011a). However, melatonin may also have antiapoptotic function in neuronal cells. Its antiapoptotic actions result from binding some specific receptors/proteins and plasma membrane proteins (Das et al., 2010), thereby eliciting some specific intracellular signal transduction pathways. Nowadays, an increasing number of studies are emerging with strong evidence demonstrating that the control of cellular apoptosis is one of the most remarkable effects of melatonin on the immune system (Espino et al., 2011b). Furthermore, melatonin levels were found to be extremely high in bone marrow (cells) (Tan et al., 1999); this cornerstone discovery is consistent with the necessity for higher levels of melatonin to control apoptosis and support neuroendocrine functions (Barrett and Bolborea, 2012).

The SH-SY5Y (human neuroblastoma) cell line has become very popular for different neurological disease models including Parkinson and Alzheimer diseases because it possesses many characteristics of dopaminergic neurons. SH-SY5Y cells express dopamine-beta-hydroxylase and tyrosine hydroxylase, and they can easily differentiate into functionally mature neurons.

Under physiological conditions, aerobic organisms continuously generate reactive oxygen species (ROS) due

* Correspondence: cihangiruguz@sdu.edu.tr

to mitochondrial respiration, which is called 'the main source' of intracellular ROS. This electron transport chain reaction occurs in the inner membrane of the mitochondria. It has been well known that ROS may act as one of the second messengers in the cellular mechanisms. Cells are well equipped with scavenging mechanisms against ROS, but when oxidants overcome these mechanisms, the result is an overproduction of oxygen-derived molecules. The unbalanced situation between increased ROS levels and decreased antioxidant enzymes is called oxidative stress.

Apoptosis is a vital, complex, biological process that enables an organism to remove or terminate the life of unwanted cells during development. In the current literature, two main apoptotic pathways were indicated: intrinsic and extrinsic pathways. Caspase family members are crucial mediators of apoptosis, or programmed cell death. Among the family members, caspase-3 and -9 are catalyzing cleavage of many other cellular key proteins. Caspase-3 and -9 are essential for normal neuronal cell development, such as hippocampal neurons (D'Amelio et al., 2010).

The aim of this study was to investigate the role of melatonin on intracellular cell death mechanisms. Because ROS activates not only cell death signals but also triggers intracellular cascades that are mediated by cellular defense systems, we used cobalt chloride ($CoCl_2$) to trigger hypoxic conditions. We found that melatonin, besides protecting against ROS generation, one of its most known antioxidant properties, also requires caspase interaction to counteract ROS-generated neuronal cell apoptosis.

2. Materials and methods

2.1. Chemicals

All chemicals (cumene hydroperoxide, KOH, NaOH, thiobarbituric acid, 1,1,3,3-tetraethoxypropane,5,5-dithiobis-2 nitrobenzoic acid, Tris-hydroxymethyl-aminomethane, glutathione, butyl hydroxy toluene, Triton X-100 and ethylene glycol-bis[2-aminoethyl-ether]-N,N,N,N-tetraacetic acid [EGTA]) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all organic solvents (n-hexane, ethyl alcohol) were purchased from Merck (Darmstadt, Germany). Fura-2 AM (acetoxymethyl ester) was purchased from Invitrogen (Carlsbad, CA, USA). All reagents were of analytical grade. All reagents except the phosphate buffers were prepared daily and stored at 4 °C. Reagents were equilibrated at room temperature for 30 min before an analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at 4 °C for 1 month. The APOPercentage assay kit was purchased from Biocolor (Belfast, UK). JC-1 was obtained from Life Technologies (New York, NY, USA). Dihydrorhodamine-123 (DHR 123) was obtained from Molecular Probes (Eugene, OR, USA). For caspase-3 and -9 substrate, AC-DEVD-AMC and AC-LEHD-AMC were purchased from Bachem (Bubendorf, Switzerland). Melatonin was purchased from Sigma-Aldrich.

2.2. Study groups

Group I was the control group and SH-SY5Y cells were incubated for 24 h with their medium (at 37 °C and 5% CO_2).

Group II was the melatonin group and SH-SY5Y cells were incubated with 1 μM melatonin for 24 h (at 37 °C and 5% CO_2) (Olivieri et al., 2001).

Group III was the $CoCl_2$ group and SH-SY5Y cells were incubated with 200 μM $CoCl_2$ for 24 h, according to the results we obtained from the MTT test (at 37 °C and 5% CO_2), data not shown here.

Group IV was the melatonin + $CoCl_2$ group and SH-SY5Y cells were preincubated with 1 μM melatonin for 12 h and 200 μM $CoCl_2$ for 24 h, according to the results we obtained from the MTT test (at 37 °C and 5% CO_2) (Olivieri et al., 2001).

2.3. Cell culture

The SH-SY5Y human-derived cell line was purchased from ATCC (USA) (Dunn et al., 1996). The cells were grown in a mixed medium containing 1:1 ratio of Dulbecco's modified eagle medium (DMEM) and Ham's F12 medium supplemented with 10% fetal bovine serum (Biochrom, Germany) and 1% penicillin-streptomycin combination (Biochrom), according to the supplier's instructions. Cells were used at passages 4 to 11.

2.4. Measurement of ROS-sensitive fluorescence

Cells were loaded with 2 μm DHR-123 by incubation at 37 °C for 30 min as previously described (Uguz et al., 2012). This probe is a nonfluorescent cell-permeable compound. Once inside the cell, it turns fluorescent upon oxidation to yield rhodamine-123 (Rh-123), with the fluorescence being proportional to ROS generation. The fluorescence intensity of Rh-123 was measured in an automatic microplate reader (Tecan Infinite pro200). Excitation was set at 488 nm and emission at 543 nm. Treatments were carried out in triplicate. Data are presented as fold increase over the pretreatment level (experimental/control).

2.5. Measurement of mitochondrial membrane potential (MMP)

Cells were incubated with 1 μm JC-1 for 15 min at 37 °C as previously described (Uguz et al., 2012). The cationic dye, JC-1, exhibits potential-dependent accumulation in mitochondria. It indicates mitochondria depolarization by a decrease in the red-to-green fluorescence intensity ratio. After incubation with JC-1, the dye was removed, and the cells were washed in PBS. The green JC-1 signal was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, and the red signal was at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Fluorescence changes were analyzed using a fluorescence spectrophotometer (Tecan Infinite pro200). Treatments were carried out in triplicate.

Data are presented as emission ratios (590/535). Changes in mitochondrial membrane potential were quantified as the integral of the decrease in JC-1 fluorescence ratio.

2.6. Apoptosis assay

The APOPercentage assay (Biocolor Ltd.) was performed according to the instructions provided by Biocolor Ltd. and elsewhere (Uguz and Naziroglu, 2012). The APOPercentage assay is a dye-uptake assay that stains only the apoptotic cells with a red dye. When the membrane of an apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into the cells, staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometer (Uguz and Naziroglu, 2012).

2.7. Determination of moderate incubation doses of $CoCl_2$ and melatonin by cell viability (MTT) assay

Cell viability was evaluated by the MTT assay based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. The enzymatic reduction of the tetrazolium salt happens only in living, metabolically active cells, not in dead ones. SH-SY5Y cells were seeded in 25-cm^2 flasks at a density of 2×10^6/tube and subsequently exposed to several concentrations of cobalt chloride (100 to 500 µM) at 37 °C. After the treatments, the medium was removed and MTT was added to each tube and then incubated for 90 min at 37 °C in a shaking water bath. The supernatant was discarded and DMSO was added to dissolve the formazan crystals. Treatments were carried out in duplicate. Optical density was measured in an automatic microplate reader (Tecan Infinite pro200) at 490 and 650 nm (as reference wavelength) and presented as the fold increase over the pretreatment level (experimental/control) as described elsewhere (Uguz et al., 2009).

2.8. Assay for caspase activities

To determine caspase-3 and -9 activities, SH-SY5Y cells were sonicated and cell lysates were incubated with 2 mL of substrate solution (20 mM HEPES [pH 7.4], 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 8.25 µM of caspase substrate) for 1 h at 37 °C as previously described (Uguz et al., 2009). The activities of caspase-3 and -9 were calculated from the cleavage of the respective specific fluorogenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AMC for caspase-9). Substrate cleavage was measured with a fluorescence spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary experiments confirmed that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK, or z-LEHD-FMK, respectively. The data were calculated as fluorescence units per milligram of protein (Uguz et al., 2009).

2.9. Measurement of lipid peroxidation level (LP)

LP levels in the SH-SY5Y cells were measured with the thiobarbituric acid reaction by the method published by Placer et al. (1966). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption with the standard curve of malondialdehyde (MDA) equivalents generated by acid catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane.

2.10. Reduced glutathione (GSH), glutathione peroxidase (GSH-Px), and protein assay

The GSH content of the SH-SY5Y cells was measured at 412 nM using the method published by Sedlak and Lindsay (1968). GSH-Px activities of SH-SY5Y cells were measured spectrophotometrically at 37 °C and 412 nM according to the method published by Lawrence and Burk (1976). The protein content in the SH-SY5Y cells was measured by the method of Lowry et al. with bovine serum albumin as the standard (1951).

2.11. Statistical analysis

Data are expressed as means ± SEM of the number of determinations. Statistical significance was analyzed using SPSS 9.05 (SPSS, Chicago, IL, USA). To compare the effects of different treatments, statistical significance was calculated by Mann–Whitney U test. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

The first data that we intended to investigate were the possible effects of melatonin and $CoCl_2$ on apoptosis levels and cell viability of the groups.

When apoptosis levels in the melatonin group were compared with those of the melatonin + $CoCl_2$ group, they were significantly lower in the melatonin group ($P < 0.005$). On the other hand, MTT assay results showed that the cell viability of the melatonin + $CoCl_2$ group was significantly increased ($P < 0.001$) (Figure 1).

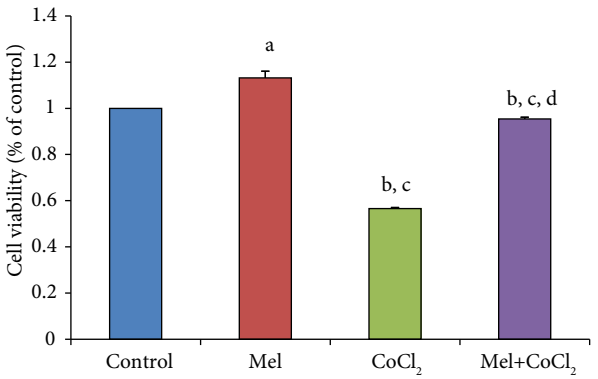

Figure 1. Effects of melatonin (MEL) and cobalt chloride ($CoCl_2$) on MTT cell viability test in SH-SY5Y cells (mean ± SD; n = 8). The values are expressed as fold increase over the control level (experimental/control).

[a]: $P < 0.05$ vs. the control group, [b]: $P < 0.001$ vs. the control group, [c]: $P < 0.001$ vs. the melatonin group, [d]: $P < 0.001$ vs. the $CoCl_2$ group.

Compared with the melatonin and melatonin + $CoCl_2$ groups, there were several significant differences in the control and $CoCl_2$ groups ($P < 0.001$). It was clearly demonstrated that melatonin supplementation has positive effects on cell viability via decreasing apoptosis levels. Melatonin preincubation protects SH-SY5Y neuroblastoma cells against the harmful effects of $CoCl_2$ administration (Figure 2).

The ROS levels in the melatonin and melatonin + $CoCl_2$ groups were significantly lower than those in the control and $CoCl_2$ groups. Additionally, we observed that the ROS level in the melatonin + $CoCl_2$ group was significantly higher than that in the $CoCl_2$ group. It was clearly observed that melatonin has high potency of scavenging $CoCl_2$ depending on harmful ROS production. Mitochondrial depolarization analyses supported these results (Figure 3).

The mitochondrial depolarization levels in the melatonin and melatonin + $CoCl_2$ groups were significantly lower than those in the control and $CoCl_2$ groups ($P < 0.001$). Moreover, it was determined that the mitochondrial depolarization level in the melatonin + $CoCl_2$ group was significantly lower than that in the $CoCl_2$ group ($P < 0.001$) (Figure 4).

We investigated two precursor enzymes of the apoptosis pathway, caspase-3 and -9. Their levels showed statistical variations. The caspase-3 levels in the control and $CoCl_2$ groups were significantly higher than those in the melatonin and melatonin + $CoCl_2$ groups ($P < 0.001$). It was clearly seen that the caspase-3 level in the melatonin + $CoCl_2$ group was significantly lower than that in the $CoCl_2$ group ($P < 0.001$) (Figure 5A).

The caspase-9 levels in the melatonin and melatonin + $CoCl_2$ groups were significantly lower than those in the control and $CoCl_2$ groups ($P < 0.001$). Furthermore, it was determined that the caspase-9 level in the melatonin + $CoCl_2$ group was significantly lower than that in the $CoCl_2$ group ($P < 0.001$) (Figure 5B).

LP levels were found to be significantly increased in the $CoCl_2$ group as compared with the other groups ($P < 0.001$). However, melatonin supplementation significantly decreased LP levels in the $CoCl_2$ and the melatonin + $CoCl_2$ groups. Moreover, when we compared the melatonin group with the control group, we also determined decreased LP levels ($P < 0.001$; Table).

We also studied GSH and GSH-Px values. GSH levels were determined to be lower in the $CoCl_2$ group samples

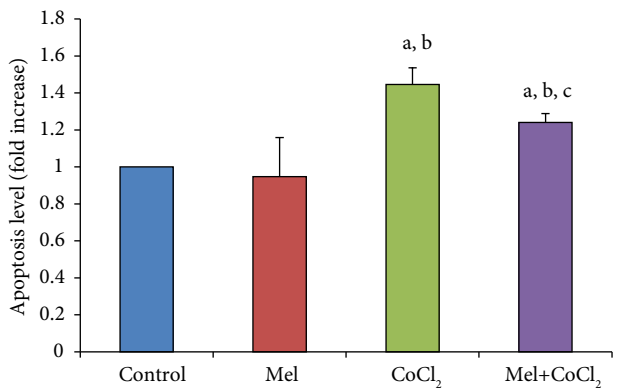

Figure 2. Effects of melatonin (MEL) and cobalt chloride ($CoCl_2$) on apoptosis levels in SH-SY5Y cells (mean ± SD; n = 8). The values are expressed as fold increase over the control level (experimental/control).
[a]: $P < 0.001$ vs. the control group, [b]: $P < 0.001$ vs. the melatonin group, [c]: $P < 0.05$ vs. the $CoCl_2$ group.

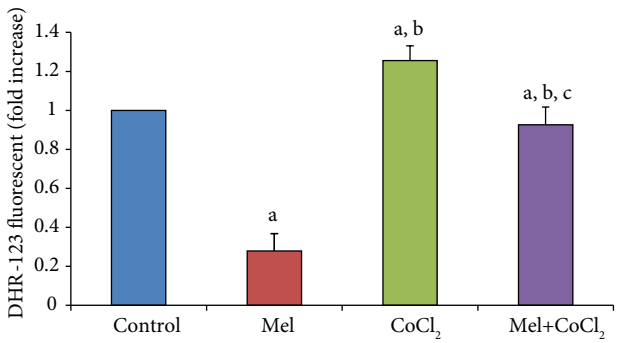

Figure 3. Effects of melatonin (MEL) and cobalt chloride ($CoCl_2$) on reactive oxygen species levels in SH-SY5Y cells (mean ± SD; n = 8). The values are expressed as fold increase over the control level (experimental/control).
[a]: $P < 0.001$ vs. the control group, [b]: $P < 0.001$ vs. the melatonin group, [c]: $P < 0.001$ vs. the $CoCl_2$ group.

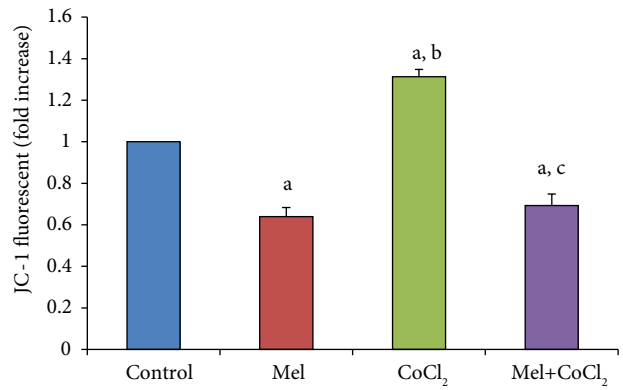

Figure 4. Effects of melatonin (MEL) and cobalt chloride ($CoCl_2$) on mitochondrial depolarization levels in SH-SY5Y cells (mean ± SD; n = 8). The values are expressed as fold increase over the control level (experimental/control).
[a]: $P < 0.001$ vs. the control group, [b]: $P < 0.001$ vs. the melatonin group, [c]: $P < 0.05$ vs. the $CoCl_2$ group.

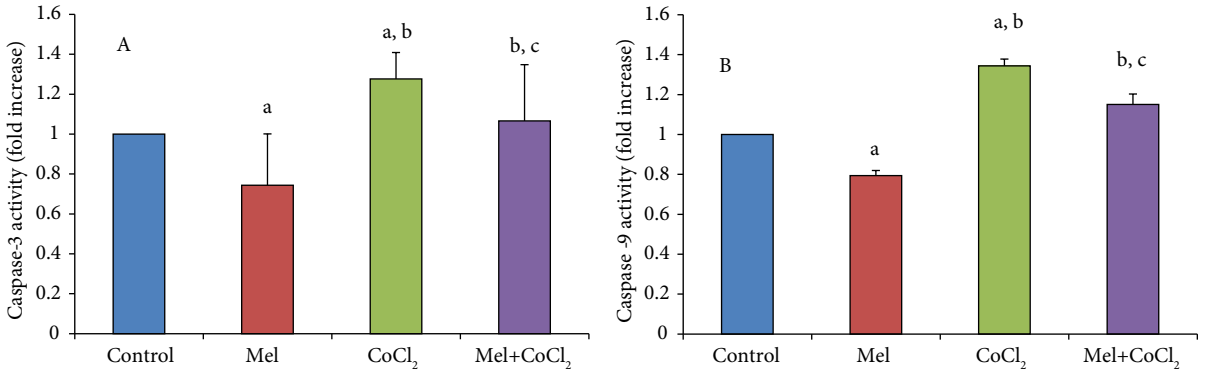

Figure 5. Effects of melatonin (MEL) and cobalt chloride ($CoCl_2$) on caspase-3 (**A**) and caspase-9 (**B**) in SH-SY5Y cells (mean ± SD; n = 8). The values are expressed as fold increase over the control level (experimental/control).
[a]: P < 0.05 vs. the control group, [b]: P < 0.001 vs. the melatonin group, [c]: P < 0.05 vs. the $CoCl_2$ group.

Table. The effects of melatonin (MEL) and cobalt chloride ($CoCl_2$) on SH-SY5Y neuroblastoma cells lipid peroxidation (LP), reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) values (mean ± SD).

	Control (n = 8)	MEL (n = 8)	$CoCl_2$ (n = 8)	MEL + $CoCl_2$ (n = 8)
LP (μM/g protein)	43.51 ± 1.99	40.41 ± 6.89 [a]	46.99 ± 8.53 [a, b]	45.18 ± 8.56 [b,c,d]
GSH (μM/g protein)	29.4 ± 3.82	25.9 ± 2.56 [c]	27.1 ± 5.28 [c]	34.41 ± 8.62 [a,b,d]
GSH-Px (IU/g protein)	1.88 ± 0.63	1.79 ± 0.13	4.10 ± 3.2 [a,b]	3.09 ± 0.16 [a,b,d]

[a]: P < 0.001 vs. the control group, [b]: P < 0.001 vs. the melatonin (MEL) group, [c]: P < 0.05 vs. the control group, [d]: P < 0.001 vs. the $CoCl_2$ group.

as compared with those in the control samples (P < 0.05). However, melatonin supplementation increased the GSH levels in the melatonin + $CoCl_2$ group. The GSH-Px levels were found to be increased in the melatonin + $CoCl_2$ group (Table).

4. Discussion

Oxidative stress can be addressed as the main source of many disorders, including some central nervous system diseases. Most of them are caused by an irregular failure of the antioxidant defense system or by increased levels of environmental stress factors (Naziroglu et al., 2014). This unbalanced situation promotes cellular death, and in addition triggers tissue damage by disrupting cellular structures. Particularly in neuronal cells, oxidative stress and oxidative stress-caused DNA damage have been identified as the general features in multiple sclerosis, Alzheimer disease, or Parkinson disease (Chang et al., 2014). Elevated levels of DNA fragmentation due to increased oxidative stress levels have been determined to be the main source of the apoptotic pathway in neuronal cells (Paredes Royano and Reiter, 2010). The subsidence of mitochondrial membrane potential results in triggering proapoptotic proteins that activate apoptosis. For these reasons, it is widely considered that oxidative stress increases the apoptosis levels in the central nervous system. It is very well known that hypoxia is one of the main reasons of neuronal damage (Lan et al., 2011).

The results of the current study demonstrated that in a neuronal cell type, like the SH-SY5Y human neuroblastoma cells, increased levels of oxidative stress are strongly involved in the cell death mechanism. Cell viability (MTT test) showed a significant reduction in the $CoCl_2$ group. However, melatonin supplementation reversed the situation by increasing cell viability as compared with the control and $CoCl_2$ groups (P < 0.001). The hypothesis that free radical production is responsible for an increase in mitochondrial depolarization and ROS production is supported by the ability of the peroxyl scavenging properties of melatonin to prevent an increase in mitochondrial depolarization levels through the scavenging of ROS.

Amoroso et al. (1999) used *tert*-butylhydroperoxide (*t*-BOOH) to induce oxidative stress in SH-SY5Y neuronal

cells. They determined that *t*-BOOH induced oxidative damage and also elevated intracellular Ca^{2+} levels and participated in cell injury (Amoroso et al., 1999). Moreover, Sakaida et al. (1991) reported that this damage happens in a dose-dependent manner. In addition to these results regarding oxidative stress findings, apoptosis seemed to occur concomitantly. After these studies, Amoroso et al. (2002) determined that *t*-BOOH, an oxidative stress inducer, triggered caspase-2 and -3 activation in SH-SY5Y neuronal cells. Our findings also support these data; we determined that oxidative stress triggered caspase-3 and -9 activation in SH-SY5Y neuronal cells.

Mitochondria are vital organelles for sustaining and/or terminating life. They are the key leading players in the energy mechanisms of the cell. Impaired mitochondrial function was determined in many neurological disorders (Yong-Kee et al., 2012). One of the most important ways in which mitochondrial dysfunction is manifested is through increased ROS levels (Chen et al., 2014). The highest percentages of oxidative stress products are produced in the mitochondria. Overproduction of ROS in the mitochondria is in a loop, which takes a role in cell depolarization, then causes impaired function. For this reason we wanted to determine the effects of $CoCl_2$ on mitochondrial depolarization levels. Cell viability (MTT test) results also contributed to our mitochondrial depolarization findings that incubation of SH-SY5Y cells with $CoCl_2$ showed significant reduction in cell viability ($P < 0.001$). The cell injury mechanism, supported by our mitochondrial depolarization level results, can be attributed to the overproduction of reactive oxygen species. These results are supported by the LP levels, which we determined to be increased in the $CoCl_2$ group. Yurekli et al. (2013) reported increased caspase-3 levels in a Parkinson disease model of a cell culture study. Moreover, they correlated the caspase result with increased LP levels in their cell culture model. Our results are in agreement with the results mentioned above. We also determined decreased GSH levels while LP levels were increasing. However, melatonin supplementation reversed the situation. Moreover, Lan et al. (2011) determined increased ROS levels, activated caspase-3, and a loss of MMP, as well as increased apoptotic cells with flow cytometry. They administered N-acetyl-cysteine (NAC) as a ROS scavenger and determined that NAC administration decreased all the parameters mentioned above. We also found that melatonin pretreatment decreased caspase-3, intracellular ROS, and MMP levels. Increased ROS levels and the alteration in the MMP interaction were well documented in previous studies. Sudden changes in the MMP result in a high amount of ion influx to the cytosol. As a consequence of the buffering deficiency, cells will undergo apoptosis (Özdemir et al., 2015).

Our findings are comparable with the current evidence that melatonin scavenges hydrogen peroxide (H_2O_2)-induced ROS production and inhibits apoptosis of PC12 neuronal cells induced by H_2O_2 (Chen et al., 2014). Liu et al. (2009) reported that hypoxia-induced apoptosis is related to p38MAPK activity in cultured rat cerebellar neurons. However, MAPK members can be activated by ROS products generated intracellularly, as well as by an exterior substance, such as H_2O_2 administered (Banno et al., 2001; Gaitanaki et al., 2007; Chen et al., 2009, 2014). Our findings are consistent with those of the previous studies mentioned above and comparable with the recent evidence that the members of different proteins such as ERK1/2, p38MAPK, and MAPKs mediate neuronal apoptosis.

In conclusion, we observed $CoCl_2$-induced cell membrane damages, evidenced by a decrease in cell viability and an increase in caspase-3 activation, apoptotic cells, ROS production, and MMP. However, the values were modulated by melatonin treatment. On the basis of our findings and those in the literature mentioned above, it is easily hypothesized that during oxidative stress in SH-SY5Y neuronal cells the production of ROS triggers a mechanism, through the release of caspase-3 and -9, that causes cellular apoptosis and mitochondrial depolarization. Hence, we determined a protective role of melatonin on the hypoxia-induced oxidative stress model in neuronal cells. Our results demonstrated that melatonin has a strong regulator effect on mitochondrial membrane potential and is a powerful antioxidant.

Acknowledgments

This study was presented in the 13th 'National Neuroscience Congress' organized by the Neuroscience Society of Turkey, between 30 April and 3 May 2015 in Konya, Turkey, as a poster presentation. This study was also partially supported by the Scientific and Technological Research Council of Turkey.

References

Amoroso S, D'Alessio A, Sirabella R, Di Renzo G, Annunziato L (2002). Ca(2+)-independent caspase-3 but not Ca(2+)-dependent caspase-2 activation induced by oxidative stress leads to SH-SY5Y human neuroblastoma cell apoptosis. J Neurosci Res 68: 454–462.

Amoroso S, Gioielli A, Cataldi M, Di Renzo G, Annunziato L (1999). In the neuronal cell line SH-SY5Y, oxidative stress-induced free radical overproduction causes cell death without any participation of intracellular Ca(2+) increase. Biochim Biophys Acta 1452: 151–160.

Banno Y, Wang S, Ito Y, Izumi T, Nakashima S, Shimizu T, Nozawa Y (2001). Involvement of ERK and p38 MAP kinase in oxidative stress-induced phospholipase D activation in PC12 cells. Neuroreport 12: 2271–2275.

Barrett P, Bolborea M (2012). Molecular pathways involved in seasonal body weight and reproductive responses governed by melatonin. J Pineal Res 52: 376–388.

Bejarano I, Redondo PC, Espino J, Rosado JA, Paredes SD, Barriga C, Reiter RJ, Pariente JA, Rodriguez AB (2009). Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. J Pineal Res 46: 392–400.

Carrillo-Vico A, Garcia-Maurino S, Calvo JR, Guerrero JM (2003). Melatonin counteracts the inhibitory effect of PGE2 on IL-2 production in human lymphocytes via its mt1 membrane receptor. FASEB J 17: 755–757.

Chang CY, Lui TN, Lin JW, Lin YL, Hsing CH, Wang JJ, Chen RM (2014). Roles of microRNA-1 in hypoxia-induced apoptotic insults to neuronal cells. Arch Toxicol (in press).

Chen L, Liu L, Yin J, Luo Y, Huang S (2009). Hydrogen peroxide-induced neuronal apoptosis is associated with inhibition of protein phosphatase 2A and 5, leading to activation of MAPK pathway. Int J Biochem Cell Biol 41: 1284–1295.

Chen TI, Chiu HW, Pan YC, Hsu ST, Lin JH, Yang KT (2014). Intermittent hypoxia-induced protein phosphatase 2A activation reduces PC12 cell proliferation and differentiation. J Biomed Sci 21: 46.

D'Amelio M, Cavallucci V, Cecconi F (2010). Neuronal caspase-3 signaling: not only cell death. Cell Death Differ 17: 1104–1114.

Das A, McDowell M, Pava MJ, Smith JA, Reiter RJ, Woodward JJ, Varma AK, Ray SK, Banik NL (2010). The inhibition of apoptosis by melatonin in VSC4.1 motoneurons exposed to oxidative stress, glutamate excitotoxicity, or TNF-alpha toxicity involves membrane melatonin receptors. J Pineal Res 48: 157–169.

Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM (1996). ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res 62: 155–169.

Espino J, Bejarano I, Paredes SD, Barriga C, Reiter RJ, Pariente JA, Rodriguez AB (2011a). Melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in leukocytes from elderly humans. Age (Dordr) 33: 497–507.

Espino J, Bejarano I, Paredes SD, Barriga C, Rodriguez AB, Pariente JA (2011b). Protective effect of melatonin against human leukocyte apoptosis induced by intracellular calcium overload: relation with its antioxidant actions. J Pineal Res 51: 195–206.

Gaitanaki C, Kalpachidou T, Aggeli IK, Papazafiri P, Beis I (2007). $CoCl_2$ induces protective events via the p38-MAPK signaling pathway and ANP in the perfused amphibian heart. J Exp Biol 210: 2267–2277.

Galano A, Tan DX, Reiter RJ (2011). Melatonin as a natural ally against oxidative stress: a physicochemical examination. J Pineal Res 51: 1–16.

Galano A, Tan DX, Reiter RJ (2013). On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. J Pineal Res 54: 245–257.

Hill SM, Frasch T, Xiang S, Yuan L, Duplessis T, Mao L (2009). Molecular mechanisms of melatonin anticancer effects. Integr Cancer Ther 8: 337–346.

Lan A, Liao X, Mo L, Yang C, Yang Z, Wang X, Hu F, Chen P, Feng J, Zheng D et al. (2011). Hydrogen sulfide protects against chemical hypoxia-induced injury by inhibiting ROS-activated ERK1/2 and p38MAPK signaling pathways in PC12 cells. PLoS One 6: e25921.

Lawrence RA, Burk RF (1976). Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 71: 952–958.

Liu AL, Wang XW, Liu AH, Su XW, Jiang WJ, Qiu PX, Yan GM (2009). JNK and p38 were involved in hypoxia and reoxygenation-induced apoptosis of cultured rat cerebellar granule neurons. Exp Toxicol Pathol 61: 137–143.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Martin M, Macias M, Escames G, Reiter RJ, Agapito MT, Ortiz GG, Acuna-Castroviejo D (2000). Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. J Pineal Res 28: 242–248.

Martin V, Sainz RM, Antolin I, Mayo JC, Herrera F, Rodriguez C (2002). Several antioxidant pathways are involved in astrocyte protection by melatonin. J Pineal Res 33: 204–212.

Naziroglu M, Cig B, Ozgul C (2014). Modulation of oxidative stress and Ca(2+) mobilization through TRPM2 channels in rat dorsal root ganglion neuron by *Hypericum perforatum*. Neuroscience 263: 27–35.

Olivieri G, Hess C, Savaskan E, Ly C, Meier F, Baysang G, Brockhaus M, Müller-Spahn F (2001). Melatonin protects SHSY5Y neuroblastoma cells from cobalt-induced oxidative stress, neurotoxicity, and increased beta-amyloid secretion. J Pineal Res. 31: 320–325.

Özdemir ÜS, Nazıroğlu M, Şenol N, Ghazizadeh V (2015). *Hypericum perforatum* attenuates spinal cord injury-induced oxidative stress and apoptosis in the dorsal root ganglion of rats: involvement of TRPM2 and TRPV1 channels. Mol Neurobiol (in press).

Paredes Royano SD, Reiter RJ (2010). Melatonin: helping cells cope with oxidative disaster. Cell Membr Free Radic Res 2: 99–111.

Placer ZA, Cushman LL, Johnson BC (1966). Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal Biochem 16: 359–364.

Sakaida I, Thomas AP, Farber JL (1991). Increases in cytosolic calcium ion concentration can be dissociated from the killing of cultured hepatocytes by tert-butyl hydroperoxide. J Biol Chem 266: 717–722.

Sedlak J, Lindsay RH (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25: 192–205.

Tan DX, Hardeland R, Manchester LC, Paredes SD, Korkmaz A, Sainz RM, Mayo JC, Fuentes-Broto L, Reiter RJ (2010). The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. Biol Rev Camb Philos Soc 85: 607–623.

Tan DX, Manchester LC, Reiter RJ, Qi WB, Zhang M, Weintraub ST, Cabrera J, Sainz RM, Mayo JC (1999). Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta 1472: 206–214.

Uguz AC, Cig B, Espino J, Bejarano I, Naziroglu M, Rodriguez AB, Pariente JA (2012). Melatonin potentiates chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor cells. J Pineal Res 53: 91–98.

Uguz AC, Naziroglu M (2012). Effects of selenium on calcium signaling and apoptosis in rat dorsal root ganglion neurons induced by oxidative stress. Neurochem Res 37: 1631–1638.

Uguz AC, Naziroglu M, Espino J, Bejarano I, Gonzalez D, Rodriguez AB, Pariente JA (2009). Selenium modulates oxidative stress-induced cell apoptosis in human myeloid HL-60 cells through regulation of calcium release and caspase-3 and -9 activities. J Membr Biol 232: 15–23.

Yong-Kee CJ, Sidorova E, Hanif A, Perera G, Nash JE (2012). Mitochondrial dysfunction precedes other subcellular abnormalities in an in vitro model linked with cell death in Parkinson's disease. Neurotox Res 21: 185–194.

Yurekli VA, Gurler S, Naziroglu M, Uguz AC, Koyuncuoglu HR (2013). Zonisamide attenuates MPP+-induced oxidative toxicity through modulation of Ca2+ signaling and caspase-3 activity in neuronal PC12 cells. Cell Mol Neurobiol 33: 205–212.