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

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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
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 (CoCl₂) to trigger hypoxic conditions. 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.

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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 (CoCl₂) 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-hydroxymethylaminomethane, 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-DEVDE-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% CO₂). Group II was the melatonin group and SH-SY5Y cells were incubated with 1 μM melatonin for 24 h (at 37 °C and 5% CO₂) (Olivieri et al., 2001). Group III was the CoCl₂ group and SH-SY5Y cells were incubated with 200 µM CoCl₂ for 24 h, according to the results we obtained from the MTT test (at 37 °C and 5% CO₂), data not shown here. Group IV was the melatonin + CoCl₂ group and SH-SY5Y cells were preincubated with 1 μM melatonin for 12 h and 200 μM CoCl₂ for 24 h, according to the results we obtained from the MTT test (at 37 °C and 5% CO₂) (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 (Uğuz 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 (Uğuz 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 (Uğuz 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 (Uğuz and Naziroglu, 2012).

2.7. Determination of moderate incubation doses of CoCl₂ 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-cm² flasks at a density of 2 × 10⁶/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 (Uğuz 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 (Uğuz 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 (Uğuz 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).

3. Results
The first data that we intended to investigate were the possible effects of melatonin and CoCl₂ on apoptosis levels and cell viability of the groups.

When apoptosis levels in the melatonin group were compared with those of the melatonin + CoCl₂ 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 + CoCl₂ group was significantly increased (P < 0.001) (Figure 1).

![Figure 1. Effects of melatonin (MEL) and cobalt chloride (CoCl₂) on MTT cell viability test in SH-SY5Y cells (mean ± SEM; n = 8). The values are expressed as fold increase over the control level (experimental/control).](image-url)

\(^{a}\) 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 CoCl₂ group.
Compared with the melatonin and melatonin + CoCl₂ groups, there were several significant differences in the control and CoCl₂ 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-SYSY neuroblastoma cells against the harmful effects of CoCl₂ administration (Figure 2).

The ROS levels in the melatonin and melatonin + CoCl₂ groups were significantly lower than those in the control and CoCl₂ groups. Additionally, we observed that the ROS level in the melatonin + CoCl₂ group was significantly higher than that in the CoCl₂ group. It was clearly observed that melatonin has high potency of scavenging CoCl₂ depending on harmful ROS production. Mitochondrial depolarization analyses supported these results (Figure 3).

The mitochondrial depolarization levels in the melatonin and melatonin + CoCl₂ groups were significantly lower than those in the control and CoCl₂ groups (P < 0.001). Moreover, it was determined that the mitochondrial depolarization level in the melatonin + CoCl₂ group was significantly lower than that in the CoCl₂ 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 CoCl₂ groups were significantly higher than those in the melatonin and melatonin + CoCl₂ groups (P < 0.001). It was clearly seen that the caspase-3 level in the melatonin + CoCl₂ group was significantly lower than that in the CoCl₂ group (P < 0.001) (Figure 5A).

The caspase-9 levels in the melatonin and melatonin + CoCl₂ groups were significantly lower than those in the control and CoCl₂ groups (P < 0.001). Furthermore, it was determined that the caspase-9 level in the melatonin + CoCl₂ group was significantly lower than that in the CoCl₂ group (P < 0.001) (Figure 5B).

LP levels were found to be significantly increased in the CoCl₂ group as compared with the other groups (P < 0.001). However, melatonin supplementation significantly decreased LP levels in the CoCl₂ and the melatonin + CoCl₂ 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 CoCl₂ group samples.
as compared with those in the control samples (P < 0.05). However, melatonin supplementation increased the GSH levels in the melatonin + CoCl₂ group. The GSH-Px levels were found to be increased in the melatonin + CoCl₂ group (Table).

### Table. The effects of melatonin (MEL) and cobalt chloride (CoCl₂) on SH-SY5Y neuroblastoma cells lipid peroxidation (LP), reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) values (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>MEL (n = 8)</th>
<th>CoCl₂ (n = 8)</th>
<th>MEL + CoCl₂ (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP (µM/g protein)</td>
<td>43.51 ± 1.99</td>
<td>40.41 ± 6.89 a</td>
<td>46.99 ± 8.53 a,b</td>
<td>45.18 ± 8.56 b,c,d</td>
</tr>
<tr>
<td>GSH (µM/g protein)</td>
<td>29.4 ± 3.82</td>
<td>25.9 ± 2.56 c</td>
<td>27.1 ± 5.28 c</td>
<td>34.41 ± 8.62 a,b,d</td>
</tr>
<tr>
<td>GSH-Px (IU/g protein)</td>
<td>1.88 ± 0.63</td>
<td>1.79 ± 0.13</td>
<td>4.10 ± 3.2 a,b</td>
<td>3.09 ± 0.16 a,b,d</td>
</tr>
</tbody>
</table>

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 CoCl₂ group.

#### 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 (Naziroğlu 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 CoCl₂ group. However, melatonin supplementation reversed the situation by increasing cell viability as compared with the control and CoCl₂ 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\textsuperscript{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\textsubscript{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\textsubscript{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\textsubscript{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).

In conclusion, we observed CoCl\textsubscript{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.

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