Effects of LED irradiation on the expression of apoptosis-related molecules in human SH-SY5Y neuroblastoma cells

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Abstract

To verify the inhibitory or protective effects of light-emitting diode(LED) irradiation on apoptotic cell death induced by $CoCl_2$, human SH-SY5Y cells were treated with $CoCl_2$ and LED were used to irradiate the cells. In the cell viability assay, cells were died slowly from 50 μM to 250 μM and about 50% of cells died after 12 hours at 400 μM of $CoCl_2$. The Diff-Quik staining revealed that cells showed condensation of DNA and blebbing of the cell membrane. The DNA fragmentation assay revealed the DNA fragmentation, which is another apoptosis marker, occurred in cells treated with 400 μM $CoCl_2$ for 16 hours. In the western blot for HIF-1α, HIF-1α was expressed after 3 hours from induction and peaked maximally at 16 hours. In the cell viability assay of the effects of LED irradiation (at 590 nm for 1 hour 20 minutes), the cells showed more proliferation (about 20%) than the control group. The RPA assay of various apoptosis-related molecules showed that pro-apoptosis molecules such as Bax, Bak, and Bid were upregulated in the $CoCl_2$ treatment group. This means that the apoptotic cell population was increased. However there was some significant changes in LED irradiated cells. In the $CoCl_2$-treated LED irradiation group, those molecules were down-regulated more than in the only $CoCl_2$-treated group. These results have shown that $CoCl_2$ may induce apoptotic cell death in human SH-SY5Y neuroblastoma cells. And LED irradiation has a positive effect on apoptotic cells by down-regulation of pro-apoptotic molecules.

Key words
LED irradiation, Apoptotic cell death, Human SH-SY5Y cells

INTRODUCTION

Low-energy stimulation of tissues by lasers or light-emitting diode(LED) have shown to increase cellular activity during wound healing by increasing collagen production and angiogenesis. The previous reports suggest that biostimulation of near-infrared(NIR) lasers or LED influenced cell proliferation by increasing mitochondria respiration through stimulation of cytochrome oxidase. In other experiments, photo-irradiation by NIR-light-emitting diode increased the production of cytochrome oxidase in cultured primary neurons and reversed the reduction of cytochrome oxidase activity produced by metabolic inhibitors[1]. In this report, there are significant upregulation of gene expression in pathways involved in mitochondrial energy production and antioxidant cellular protection. In connection with those results, we hypothesized LED can be used as a non-invasive therapeutic approach for cell death by modulating cellular signalling mechanisms[2-4].

Apoptosis is a gene-regulated mechanism of cell death, playing a pivotal role in physiological and pathological processes[5]. Hypoxic / ischemic condition has been recognized for a long time as an important mediator or modulator of apoptosis because this condition is accompanied by the production of reactive oxygen species (ROS) which can attack nucleic acids, proteins and membrane phospholipids[6-8]. Hypoxia / ischemia - induced apoptosis is a major concern in various clinical entities such as ischemic diseases, organ transplantations and

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other diseases. However, the exact apoptotic mechanisms in hypoxic / ischemic condition have not been settled yet.

In general, apoptosis is driven by the activation of a family of cysteine protease called caspases, which cleave a critical set of cellular proteins to initiate apoptotic cell death[9,10]. Two main pathways for activating caspases are the death receptor-mediated mechanism and the mitochondria-mediated mechanism. The mitochondrial pathway is stimulated by hypoxic / ischemic condition, cytotoxic reagents, radiation, and growth factor deprivation[11,12]. The death receptor pathway is stimulated by the death receptor of cell surface such as Fas and tumor necrosis factor (TNF) receptor[13]. The receptors, activated by ligands, lead to activation of caspase-8, with subsequent activation of caspase-3. There have been no investigations regarding the mechanism of the death receptor-mediated apoptosis in hypoxic / ischemic condition.

In addition to the caspases, members of the Bcl-2 family are also critical for the regulation of apoptosis[14]. The Bcl-2 family control the release of mitochondrial cytochrome-c by modulating the permeability of the outer mitochondrial membrane. Bcl-2 family members are functionally divided into anti-apoptotic molecules (Bcl-2, Bcl-XL, Bcl-w, Mcl-1) and pro-apoptotic molecules (Bax, Bcl-Xs, Bak, Bid, Bad, Bim, Bik)[14-16]. All members possess at least one or four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Most anti-apoptotic members, which can inhibit apoptosis in the face of a wide variety of cytotoxic insults, contain at least BH1 and BH2, and those most similar to Bcl-2 have all four BH domains. The pro-apoptotic molecules differ in their correlation with Bcl-2 markedly. Bax and Bak, which contain BH1, BH2, and BH3, resemble Bcl-2 quite closely. The Bcl-2 family are regulated by cytokines and other death-survival signals at different levels. Several anti-apoptotic genes are induced transcriptionally by certain cytokines, and Bax is induced in some cells as part of the p53-mediated damage response[17]. Bcl-2 protects against diverse cytotoxic insults - for example, γ- and ultraviolet-irradiation, cytokine withdrawal, dexamethasone, staurospotine, and cytotoxic drugs[18]. Recent studies have shown that pro-apoptotic family members may act as tumor suppressors. Bax is mutated in human gastrointestinal cancer and some leukemias[19]. Moreover, its expression is activated in some cell types by the p53 tumor suppressor, which can provoke apoptosis[19]. However, the roles of the Bcl-2 family and caspases in hypoxic / ischemic - induced neuronal apoptosis have not been well elucidated.

Cobalt chloride ($CoCl_2$) can mimic hypoxic / ischemic conditions, including generation of reactive oxygen species and transcriptional change of some genes such as hypoxia inducible factor (HIF-1α), and p53, p21, and PCNA in promoting the cell death[20,21]. PC12 is a cell line derived from rat pheochromocytoma widely used for investigating neuronal apoptosis[22]. $CoCl_2$-induced apoptosis may serve as a simple and convenient in vitro model to elucidate molecular mechanism in hypoxia-linked neuronal cell death and to research its treatment methods.

In the present study, we investigated whether $CoCl_2$-induced apoptosis is accompanied by the Bcl-2 family and associated with the mitochondria-mediated pathway and the death receptor-mediated pathway in neuronal hypoxic / ischemic conditions, and whether LED irradiation can provide protection against apoptosis induced by $CoCl_2$.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma Chemical Co. (USA) unless otherwise specified. Colorimatric immunoassay kit (Roche Molecular Biochemicals, Germany), antibodies (Santo Cruz, USA), and Multi-probe RNase protection assay system (BD biosciences, USA) were used for this project. For analytical apparatuses of gel electrophoresis, Bio-Rad (USA) was used.

Cell Culture

Human SH-SY5Y neuroblastoma cells were maintained with Medium A [DMEM(Gibco BRL, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA] and Antibiotic Antimycotic Solution (100 units / ㎖ penicillin, 100 ㎍ / ㎖ streptomycin, 0.25 ㎍ / ㎖ amphotericin B) at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2. Cells were allowed to subculture for 3 days, and the medium was replaced with a fresh medium. Then cells were trypsinized using trypsin-EDTA (0.5%, 5.3 mM) solution (Gibco BRL, UK) and plated again. Starvation of SH-SY5Y was induced with Medium B (FBS free medium A containing 0.05% BSA and 2 mM sodium pyruvate) for 24 hours, and all the experiments on SH-SY5Y were con-

ducted with Medium C (Medium B plus 0.5 μmol/L of insulin, 5 μg/ml of transferrin, 5 μg/ml of sodium selenite and 0.2 mmol/L of ascorbic acid).

Cell treatment condition

For individual experiments, cells were seeded at 1×10^4 cells/well in 96-well plates or 1×10^5 cells/well in 6-well plates for Diff-Quik staining or 5×10^5 cells/10 cm^2 culture dish for the DNA fragmentation assay. These cells were cultured for 1 day and quiescent cells were obtained by cultivation for 24 hours with medium B before being treated with $CoCl_2$ with medium C. Concentrations of $CoCl_2$ being used were 0, 50, 100, 150, 200, 250, 300, and 400 μM. Incubation time for $CoCl_2$ were 0, 4, 8, 12, 24, 36, and 48 hours. There were three groups in this experiment, the first group was the non-$CoCl_2$ treated and non-LED irradiated group, the second group was $CoCl_2$-treated and non-LED irradiated group, and the third group was $CoCl_2$-treated and LED-irradiated group.

Light source and irradiation

The source of light for irradiation was a continuous-wave LED (U-JIN LED Co., LTD., Korea) emitting at a wavelength of 590 nm or 730 nm, and manufactured so that the energy density was 5 mW/cm^2 on the sample surface (Biophoton Co., Gwangju, Korea). To determine optimum irradiation time to proliferate the SH-SY5Y cells, cells were irradiated once for 40 minutes, 60 minutes, 80 minutes, or 100 minutes after treating with $CoCl_2$ in a 5% CO_2 humidified chamber. Cells were detached after irradiation.

Cell proliferation assay by BrdU incorporation

For determination of cell proliferation, DNA synthesis was evaluated by the BrdU incorporation method, according to the manufacturer's description, by using an enzyme-linked immunosolvent assay (ELISA) kit (Roche Molecular Biochemicals, Germany). Briefly, after treatment with $CoCl_2$ as described above, BrdU was added to cells and cells were incubated for another 2 hours in a 5% humidified chamber. Cells were washed with 200 μl of 1 ×PBS twice, fixed with 200 μl of Fix/Denat. solution at room temperature for 15 minutes, and 100 μl of Anti-BrdU-POD was added to the cells at room temperature and left for 90 minutes. Thereafter, cells were washed with 300 μl of 1× washing buffer for 3 times and another wash was done with distilled water. 100 μl of substrate solution was added and incubated at room temperature in a dark place for 5 minutes. Finally, 25 μl of stop solution was added and cell proliferation was measured with an ELISA reader (excitation at 450 nm and emission at 630 nm). In the assay of proliferation of cells by LED irradiation, BrdU and Medium C were added to the cells after they had been irradiated at 590 nm or 730 nm for various times described above and cell proliferation was assayed using the BrdU incorporation method.

Diff-Quik staining

Quiescent SH-SY5Y cells in 6 wells were stimulated with medium C in the absence or presence of $CoCl_2$ (400 μM) for 12 hours. After 12 hours incubation, the cells were washed with PBS and pre-fixed with 95% ethanol at room temperature for 10 minutes. Next, the pre-fixed cells on coverglass were fixed with Hemacolor 1 solution (Merck, Germany) for 1 minute, the cytosol was stained with Hemacolor 2 solution for 1 minute, and the nucleus was stained with Hemacolor 3 solution for 1-3 seconds. The stained cells on coverglass were washed in the inverted way with Double Distilled Water (DDW), air dried, and mounted onto slide for light microscopy.

DNA fragmentation assay

The SH-SY5Y cells were seeded at 5×10^5 cells/10 cm^2 culture dish. After treatment with $CoCl_2$ (400 μM), cells were scraped using a cell scraper, transfered to a eppendorf tube, and washed once with 200 μl of PBS. DNA was extracted using the Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI, USA) according to manufacturers instructions. Briefly, 600 μl nuclei lysis solution and 3 μl RNase solution were added to the cells, and the mixture was incubated for 30 minutes at 37 ℃. After cooling the tube at room temperature, 200 μl of protein precipitation solution was added, the tube was vortexed and chilled on ice for 5 minutes. Next, the mixture was centrifuged at 16,000 g for 4 minutes, the supernatant was transferred to a eppendorf tube containing 600 μl 100% isopropanol, mixed by inversion, centrifuged again for 1 minute. The pellet was resuspended in 70% ethanol and centrifuged at 16,000 g for 1 minute, and the supernatant was discarded. The precipitated pel-

let was air dried and then hydrated with hydration solution and incubated at 65 ℃ for 1 hour. Finally, the extracted DNA was electrophoresed on 1.5% agarose gel containing ethidium bromide (EtBr), and the remaining DNA was stored at -20 ℃.

Western blot for HIF-1α

To determine whether the cells had entered an acute hypoxic state, the hypoxia marker HIF-1α was measured at protein level. Incubation time with $CoCl_2$ was varied from 0 hour to 24 hours. The SH-SY5Y cells grown on 10 cm^2 culture dish were harvested with a cell scraper and lysed with 130 ㎕ of NP-40 lysis buffer containing 50 mM Tris-cl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin, 2 ㎍/ml aprotinin, and 1 ㎍/ml pepstatin[23]. The mixture was incubated on ice for 20 minutes and then subjected to 4 ℃ centrifugation at 12,000 rpm for 20 minutes. Protein concentrations were determined using the BCA protein assay kit (Pierce, USA) following manufacturer`s instructions. Aliquots of the proteins were fractionated by 8-10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Protran, pore size 0.45 ㎛, Schleicher & Schuell bioscience, Germany)[24]. The membranes were blocked for 1 hour at room temperature with 0.1% Tween 20 in PBS (PBST) containing 5% powdered skim milk, and then incubated for 1 hour with 5% skim milk in PBST containing antibodies diluted according to manufacturer's recommendations against the target proteins. Membranes were then washed with PBST, incubated for 1 hour at room temperature in 5% skim milk in PBST containing alkaline phosphatase conjugated IgG against primary antibodies, and then rinsed in PBST. Antibody binding was visualized by colorimetric detection using NBT/BCIP solution (Calbiochem, Germany).

RNase Protection Assay (RPA)

To detect mRNA levels of apoptosis-related genes, the RNase protection assay was performed using the hAPO Multi-probe Template sets (RiboQuant; BD PharMingen, San Diego, CA, USA). The first Multi-probe Template set contained DNA templates for human Bcl-x, p53, GADD45, c-fos, p21, Bax, Mcl-1, two housekeeping genes, L32 and GAPDH as internal controls. Another Multi-probe Template set contained DNA templates for human Bcl-w, Bcl-x, Bid, Bax, Bak, Mcl-1 and the same housekeeping genes. Each reaction (20 ㎕) contained 10 mM biotin-labeled UTP, 500 μM each GTP, CTP, and ATP, 100 mM DTT, 1× transcription buffer, and enzyme mixture. After 2 hours of incubation at 37 °C, the reaction was terminated by addition of 2 U of a RNase free DNase for 30 minutes at 37 °C. The RNA was extracted with phenol and chloroform and precipitated with ethanol and resuspended in 50 ㎕ of hybridization buffer. For the hybridization reaction, the total RNA 20㎍ was mixed with riboprobes and heated to 90 °C for 5 minutes. The reaction was cooled down slowly to 56 °C and incubated for 16 hours at this temperature. Then RNA was digested with RNase A and T1 and proteinase-K treated. Using another phenol:chloroform extraction RNA was precipitated with ethanol again and resuspended in 1× loading buffer. After gel electrophoresis on a vertical 5% acrylamide gel, hybridized RNA were transferred to nylon membranes and UV crosslinked. The protected RNA bands were detected with an X-ray film and quantified by a phosphorimager (Canberra Packard, Dreieich, Germany). Quantification was performed by measuring digital light units (DLU) using the applied software. In analogy to a densitometric measurement, signals (i.e. bands on the electrophoresed gel) could be quantified as peaks in the densitometric electropherogram, normalization was performed with the DLU measured for GAPDH.

RESULTS

Decreased cell proliferation after exposure to $CoCl_2$

After SH-SY5Y cells were exposed to $CoCl_2$, DNA synthesis was markedly decreased in a dose- and time-dependent manner (Fig. 1). Within the 100 μM $CoCl_2$ treatment for 12 hours, the relative cell proliferation was similar to the control (Fig. 1A). The 400 μM of $CoCl_2$ induced cell death triggered in over 4 hours, suggesting the apoptosis occurrence (Fig. 1B). The cell proliferation was reduced to about 50% when the cells were treated with 400 μM $CoCl_2$ for 12 hours.

Morphological changes of SH-SY5Y cells after $CoCl_2$ treatment

Apoptosis was assessed in terms of both morphological changes and DNA ladder formation. When Diff-Quik

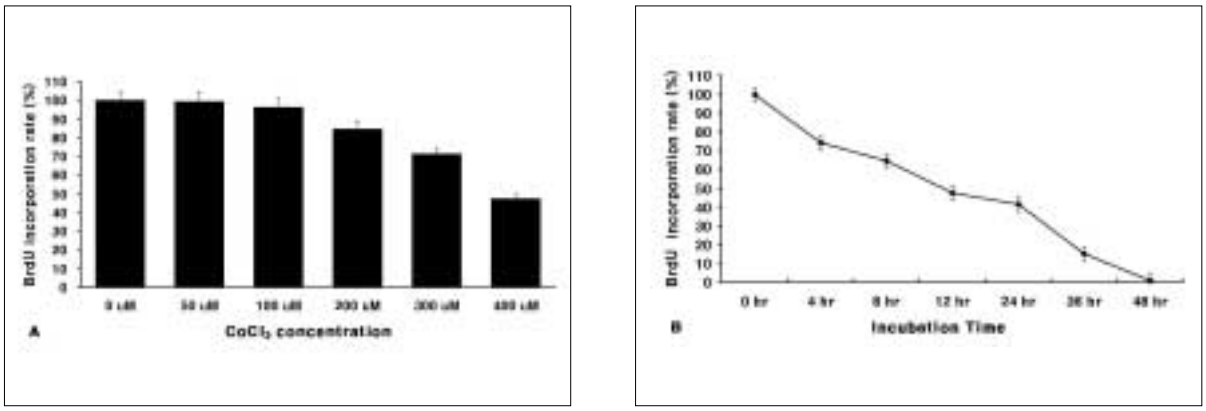

Fig. 1. $CoCl_2$ decreased cell proliferation in SH-SY5Y cells. (A) Cells were incubated with different concentrations of $CoCl_2$ for 12 hours. (B) Cells were incubated with 400 μM $CoCl_2$ for various time periods. Cell proliferation was measured by BrdU assay. Data are mean ± S.D. from three independent experiments.

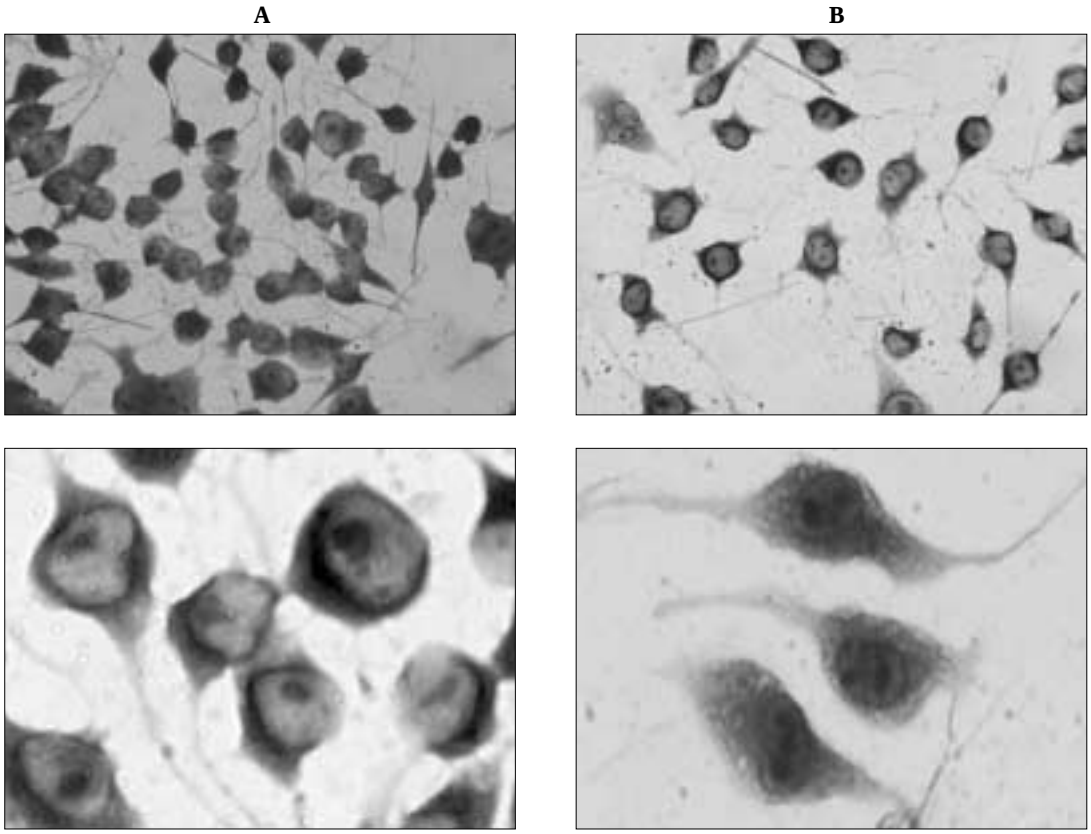

Fig. 2. $CoCl_2$ increased the number of cells with apoptotic morphology in SH-SY5Y cells. Cells were incubated in the absence $CoCl_2$ (A) or in the presence of 400 μM $CoCl_2$ (B) for 12 hours and stained using the Diff-Quik method.

staining was done, no condensed nuclei were seen in non-$CoCl_2$-treated cells (Fig. 2A). But highly condensed and fragmented nuclei can be clearly seen in the $CoCl_2$-treated cells (Fig. 2B).

Analysis of internucleosomal DNA fragmentation

The biochemical hallmark of apoptosis is the fragmentation of DNA in multiples of ~ 200 bp. Non-$CoCl_2$

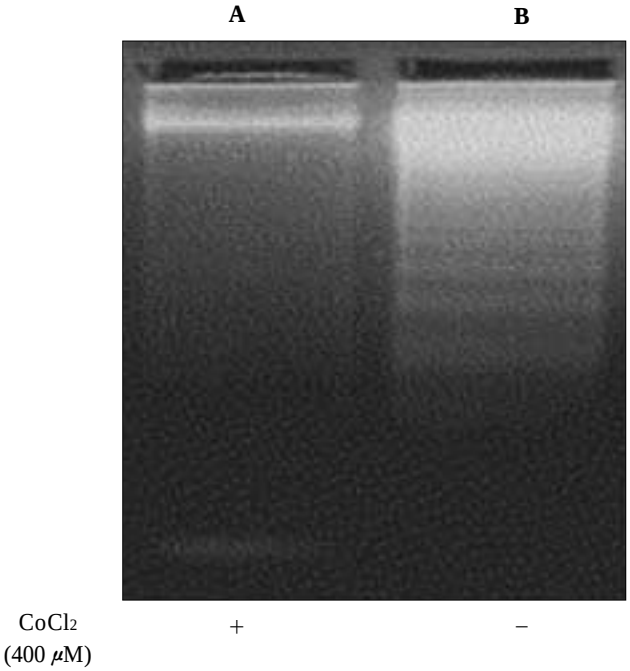

Fig. 3. $CoCl_2$ induced fragmented DNA in SH-SY5Y cells. Cells were incubated without $CoCl_2$ (A), and with 400 μM $CoCl_2$ for 16 hours (B). Fragmentations of genomic DNA were detected on a 1.8% agarose gel.

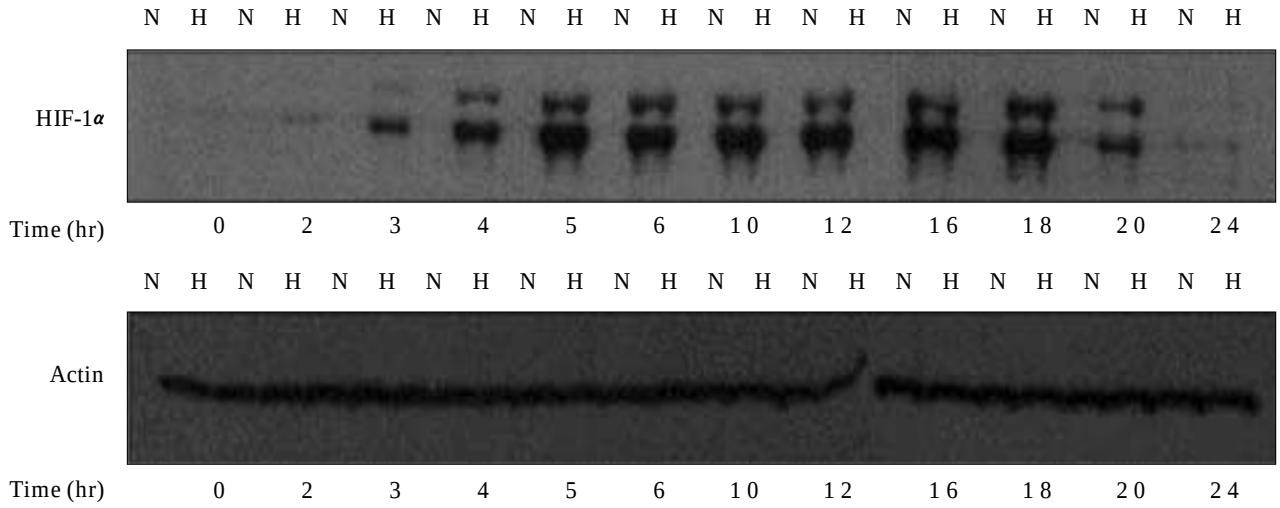

Fig. 4. HIF-1α expression in Human SH-SY5Y cells indicated acute hypoxia. Actin was used as an internal control. Data are representative blots of three separate experiments. (N, Normoxic; H, Hypoxic ($CoCl_2$)).

treated cells showed high molecular DNA which meant no apoptosis occurred (Fig. 3A). However, $CoCl_2$-treated cells for 16 hours showed DNA fragmentation (Fig. 3B).

HIF-1α expression in SH-SY5Y cells after $CoCl_2$ treatment

To verify that exposure to $CoCl_2$ induces a hypoxia-mimicking response in SH-SY5Y cells, expression of hypoxia-inducible factor-1α (HIF-1α), a marker of hypoxia, was examined. In $CoCl_2$-treated cells, HIF-1α levels were greatly elevated (Fig. 4). HIF-1α protein was induced in a time-dependent manner. HIF-1α appeared at 3 hours and peaked at 16 hours and disappeared at 24 hours.

Proliferative effects of LED irradiation on SH-SY5Y cells

To examine the proliferative effects of LED irradiation on SH-SY5Y cells, the cells were LED-irradiated for 40 minutes, 60 minutes, 80 minutes, and 100 minutes each. The effects of LED irradiation at 590 nm and 730 nm on DNA synthesis of cells was examined by BrdU incorporation. The BrdU incorporation reached its maximal point at 590 nm for 80 minutes irradiation, showing an slightly more increase over the control group (Fig. 5). But at 730 nm, DNA synthesis of SH-SY5Y cells has no differences between that of the control group. When the cells were irradiated for 100 minutes at 590 nm, the DNA synthesis was lower than the group irradiated for 80 minutes and the control group, suggesting that a high dose of irradiation can damage the cells in other ways.

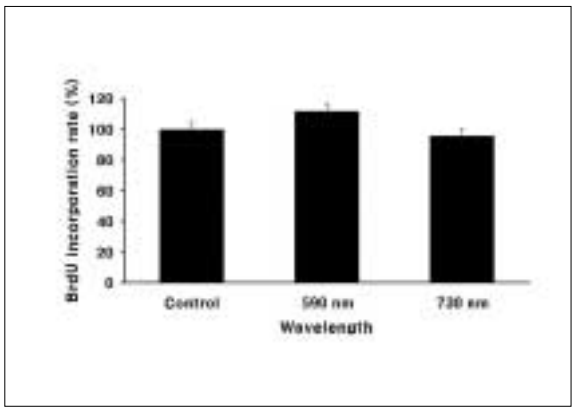

Fig. 5. Proliferative effects of LED irradiation on DNA synthesis in Human SH-SY5Y cells (all groups were not treated with $CoCl_2$). All samples were irradiated for 80 minutes except for the control.

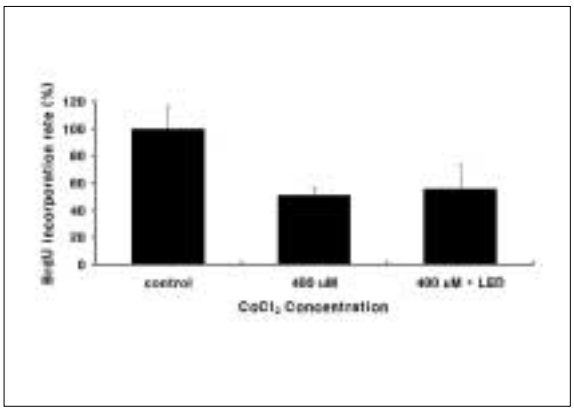

Fig. 6. Effects of LED irradiation (590 nm, 80 minutes) on human SH-SY5Y cells treated with or without $CoCl_2$ (400 μM). Results are expressed as mean of percentage over that control.

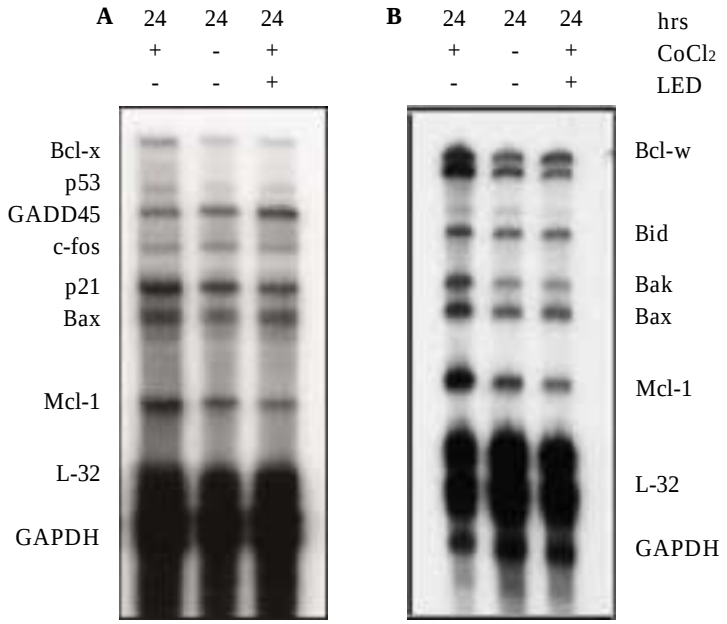

Fig. 7. After LED irradiation, $CoCl_2$ treated Human SH-SY5Y cells showed decreased mRNA level for Bid, Bak, and Bax at 24 hours. Following $CoCl_2$ treatment, there were elevated mRNA levels for Bcl-x, p53, p21, Bax, Mcl-1, Bak, and Bid compared to non-$CoCl_2$ tretated cells (A, B). Results portrayed are representative of two independent cultures.

Inhibitory or protective effects of LED-irradiation against $CoCl_2$

To verify the inhibitory or protective effects of LED-irradiation against $CoCl_2$, LED were used to irradiate SH-SY5Y cells which had been already treated with $CoCl_2$ (Fig. 6). In the group which were treated with $CoCl_2$ (400 μM) but without LED-irradiation (non-LED-irradiated), DNA synthesis was greatly reduced to approximately comparing with the control group (non-$CoCl_2$, non-LED). But when cells were LED-irradiated (590 nm, 80 minutes) after exposure to 400 μM of $CoCl_2$, the DNA synthesis was elevated to a level above that of the group treated with only $CoCl_2$.

Relative mRNA levels in SH-SY5Y cells after exposure to LED

Generally, expression of Bcl-2 family has proven to be significant for apoptosis determination, since a high ratio of anti-apoptotic versus pro-apoptotic molecules denotes a lower apoptotic threshold, while a low ratio indicates a higher apoptotic threshold. The genes shown in this figure are the pro-apoptotic gene family (Bid, Bad, Bak, Bax) and the anti-apoptotic Bcl-2 family (Bcl-2, Bcl-x, Bcl-w, Mcl-1). p53, p21, and GADD45 are used as cell damage markers. L32 and GAPDH are used as internal controls. Using the RNase protection assay (RPA), $CoCl_2$-treated SH-SY5Y cells had high constitutive mRNA levels for p53, p21, Bax, Bid, Bak, Bcl-x, and Mcl-1 (Fig. 7). This means that the cells have undergone an apoptotic state. At 24 hours after LED irradiation, the mRNA levels of those genes were lower than those of $CoCl_2$-treated cells, while the GADD45 mRNA level was elevated. This means that the cells were prevented from undergoing apoptosis and damaged DNA was repaired by LED irradiation.

DISCUSSION

Hypoxia is a common occurrence in everyday life during events such as breath holding, climbing to altitude and childbirth as well as in pathological states like sleep apnea, some types of congenital heart disease, various types of pulmonary disease, myocardial infarction and stroke. There are many reports that $CoCl_2$ could mimic the hypoxic responses in cultured cells, including transcriptional changes of some genes, such as hypoxia-induced factor 1α (HIF-1α), p53, p21, and PCNA[24]. To elucidate the mechanism by which neuronal cells respond to hypoxia and the effects of LED irradiation, the present study used $CoCl_2$-treated SH-SY5Y cells. This study demonstrated that $CoCl_2$ triggered apoptosis in SH-SY5Y cells and LED irradiation could reduce apoptosis in the cells.

The most characteristic traits of apoptosis are the fragmentation of the nucleus with condensed chromatin and extensive membrane blebbing[25,26]. All of these morphologic features were observed in SH-SY5Y cells treated with $CoCl_2$. Another reliable biochemical marker for apoptosis is the double-strand cleavage of nuclear DNA at the linker regions between nucleosomes. Gross nuclear changes and DNA fragmentation patterns are critical events to differentiate apoptosis from necrosis[27]. In the present study, $CoCl_2$-induced cells demonstrated DNA fragmentation showing the ladder pattern, and dose-dependent internucleosomal DNA fragmentation in $CoCl_2$-treated SH-SY5Y cells confirmed apoptosis occurring.

This study examined whether $CoCl_2$ caused the SH-SY5Y cells to enter into a hypoxic state. In this step, HIF-1α was used as an indicator of hypoxic state and confirmed that $CoCl_2$ made the SH-SY5Y cells enter a hypoxic state from 3 hours after $CoCl_2$-treatment.

Many of the central molecular regulators of apoptosis have been well characterized. Bcl-2 and Bcl-x play a specific role to inhibit apoptosis and they seem to be interchangeable[28]. A given cell type may express either or both of them. On examination of the expression of Bcl-x in SH-SY5Y cells after $CoCl_2$ treatment, it was found that its expression was up-regulated by $CoCl_2$. Therefore, a Bcl-x-dependent signal pathway might be involved in the $CoCl_2$-induced apoptosis. This result was consistent with the fact that Bcl-x predominantly inhibits apoptosis induced by hypoxia in PC12 cells[29]. Bcl-x, acting as an inhibitor of apoptosis, is abundantly expressed in both the developing and the mature nervous system. Bcl-x may function as an ion channel or an adapter of a docking protein[30,31]. Further work is needed to address this issue. It is well known that overexpression of Bax accelerates the apoptotic death of cells triggered by certain apoptotic stimuli. Other members of the Bcl-2 family such as Bcl-w, Bad, Bak, and Bid might also be involved in the regulation of apoptosis[15,20], and further investigation of these members will provide further informations on apoptosis induced by $CoCl_2$.

It was reported that when cultured skeletal muscle satellite cells were irradiated by He-Ne laser, the light irradiation increased expression of the anti-apoptotic protein Bcl-2 and reduced the pro-apoptotic protein Bax[32]. Of course, the mechanism of these intriguing phenomena is to be further investigated. As can be partly seen in these data, different wavelengths and doses of LED irradiation have different effects on the apoptosis. In the present study, irradiation time was varied from 40 minutes to 100 minutes at the same irradiation intensity. It was found that the duration of irradiation is a significant factor in the effect of LED irradiation on apoptosis. When the irradiation intensity was relatively high, the effect of light on apoptosis was the reverse of that found in other previous works[33,34]. In this study, $CoCl_2$ increased apoptosis in SH-SY5Y cells by approximately 50%. But when the $CoCl_2$-treated cells were irradiated by LED, the cells somewhat were recovered.

The intercellular signal molecules, especially those associated with apoptosis, were assayed after $CoCl_2$ treatment and LED irradiation. In this study, it was shown that the effect of LED irradiation on apoptosis induced by $CoCl_2$ was to markedly down-regulate pro-apoptotic molecules. As compared with the control group (non-$CoCl_2$, non-LED), pro-apoptotic molecules such as Bax, Bid, Bak were overexpressed in the $CoCl_2$-treated group, whereas those molecules were down-regulated in the LED irradiated group. This result suggested that LED irradiation could inhibit the cell damage or help the damaged cells to recover.

The mechanism of these intriguing effects has been discussed in another paper[35]. Because of the lack of good animal models of the disease, no one has been able to test whether apoptosis intervention will help slow the disease progression. But given the possibility of a good therapeutic payoff, Altzheimer disease (AD) researchers would continue to try to overcome the diseases such as AD. Our data suggest a potential role for LED in protection against neuro-degeneration.

CONCLUSION

To verify the inhibitory or protective effects of LED irradiation on apoptotic cell death induced by $CoCl_2$, human SH-SY5Y cells were treated with $CoCl_2$ and LED were used to irradiate the cells.

In the cell viability assay, cells were died slowly from 50 μM to 250 μM, and about 50% of cells died after 12 hours at 400 μM of $CoCl_2$. The Diff-Quik staining revealed that cells showed condensation of DNA and blebbing of the cell membrane. The DNA fragmentation assay revealed the DNA fragmentation, which is another apoptosis marker, occurred in cells treated with 400 μM $CoCl_2$ for 16 hours. In the western blot for HIF-1α, HIF-1 α was expressed after 3 hours from induction and peaked maximally at 16 hours. In the cell viability assay of the effects of LED irradiation (at 590 nm for 1 hour 20 minutes), the cells showed more proliferation (about 20%) than the control group. The RPA assay of various apoptosis-related molecules showed that pro-apoptosis molecules such as Bax, Bak, and Bid were upregulated in the $CoCl_2$ treatment group. This means that the apoptotic cell population was increased. However there was some significant changes in LED irradiated cells. In the $CoCl_2$-treated and LED irradiation group, those molecules were down-regulated more than in the only $CoCl_2$-treated group.

In conclusion, these results have shown that $CoCl_2$ may induce apoptotic cell death in human SH-SY5Y neuroblastoma cells. This was shown through the finding of DNA fragmentation, cell shrinkage, membrane blebbing, and activation of the Bid, Bak, and Bax, such as molecules are found in the death-related pathway. LED irradiation has a positive effect on apoptotic cells by down-regulation of pro-apoptotic molecules. According to the results of this experiment, LED irradiation could be proposed as an effective therapeutic tool to treat neuronal disease without side effects.

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