Effects of Curcumin and Melatonin on Lipopolysaccharide/Manganese-Induced Microglial Activation

미세교세포 활성을 유도하는 LPS와 Manganese에 대한
Curcumin 과 Melatonin의 효과

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ABSTRACT

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Microglia activation induces inflammation mediators and causes neuroinflammation response which is directly related with various neurodegenerative diseases. In this study, the beneficial effects of curcumin and melatonin against LPS/$MnCl_2$-induced microglial activation were investigated. Curcumin (1 μM) and melatonin (10 μM) effectively reduced lipid peroxidation induced by LPS (500 ng/ml)/$MnCl_2$ (250 μM) exposure. Furthermore, both curcumin and melatonin exerted the antioxidant effects by elevating and maintaining GSH activity which was significantly reduced by LPS/$MnCl_2$. Moreover, curcumin and melatonin markedly attenuated LPS/$MnCl_2$-stimulated inducible nitric oxide synthase (iNOS) mRNA

and protein expression, as well as NO production and expression of proinflammatory cytokines including TNF-α and IL-1β in BV-2 miroglial cells. Both curcumin and melatonin also showed inhibition of LPS/$MnCl_2$-induced IκB-α phosphorylation and subsequent NF-κB activation in BV-2 cells. In addition, pretreatment of BV-2 cells with curcumin and melatonin dramatically inhibited LPS- and/or $MnCl_2$-induced phagocytosis. These results suggested that both curcumin and melatonin are bioactive microglia regulators having anti-inflammatory functions.

Ⅰ. Introduction

Many studies have been reported regarding the possible factors such as environmental toxins and genetic factors induce mitochondrial dysfunction as well as free radical-mediated neuronal cell death behind the neuronal degeneration. Recently, several studies have demonstrated that the neuroinflammation is the major key event in various neurodegenerative disease including Alzheimer's disease, Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS) [1-2]. Inflammation is associated with glial cells such as microglia and astrocyte activation. Microglial cells are a resident immunocompetent and phagocytic cells and play a role as the contributor of proinflammatory cytokines including Tumor necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6 [3]. Microglial activation can be easily induced by lipopolysaccharide (LPS) and manganese ($MnCl_2$) in experimental condition [4].

A gram-negative bacterial endotoxin (LPS) is very well known miroglial activating agent. LPS binds to membrane receptor complex on microglial cells and/or interact with specific receptors on the surface of a various host cells and induces the generation of Reactive Oxygen Species (ROS), proinflammatory cytokines and nitric oxide (NO) [2, 5]. It has been shown that the activated microglial cells by LPS treatment are 4-8 times more in the substantia nigra (SN) than in other brain regions and upregulate the level of cyclooxygenase-2 (COX-2), an enzyme for the synthesis of inflammation-related prostaglandins [6].

Manganese is a common environmental contaminant as an industrial metal contained in alloys, steel and iron, fertilizers,

gungicides and dry-cell batteries [4]. When exposed to Mn, it is leads to progressive and irreversible brain damage, neuronal degeneration and similar symptoms with PD characterized by rigidity and bradykinesia [7]. Manganese induced significant increase of nitric oxide production in microglial cells. Moreover, cotreatment of Mn and LPS increases the inducible nitric oxide synthase (iNOS), TNF-α and IL-6 expression and nuclear factor-kappa B (NF-κB) activation. [4, 8-9]

Curcumin (fig. 1A) an active dietary ingredient of dietary spice tumeric (Curcuma longa) is a yellow phenolic pigment that has anti-inflammatory, anti-oxidative, anti-carcinogenic, anti-infectious, chemopreventive and chemotherapeutic activities [10]. Curcumin has inhibitory effects against nitric oxide production in LPS-stimulated microglial cells and the expression of proinflamatory genes, such as cyclooxygenase-2, cytokines and iNOS through suppression on NF-κB activation [11] and AP-1 binding to DNA in BV-2 microglial cells [12].

Melatonin (N-acetyl-5-methoxytryptamine) (fig. 1B), an indole hormone from the mammalian pineal gland, has beneficial effects preventing neuronal cell death in a number of degenerative conditions [13]. Recent studies have suggested that melatonin has neuroprotective effects against 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mediated PD models [14]. It has been known that melatonin stimulates defensive system and effectively elevates the superoxide dismutase activation, glutathione peroxidation activation and glutathione level. Furthermore, melatonin also prevents inflammation and immune responses in various cell types [15]. In recent years, it has become apparent that melatonin has effectiveness for the reduction of lipid peroxidation, mitogen-

activated protein kinase (MAPK) activation, NF-κB activation, iNOS expression and nitric oxide production which are induced by LPS [16]. Indeed, numerous recent studies suggested evidence that various inflammatory mediators and inflammation processes act as a direct and/or indirect neuronal damage in neurodegenerative disorders [17]. Curcumin is known to prevent nitric oxide, production by LPS in primary microglial cells [18]. Melatonin has been found to suppress COX expression and NOS activation by LPS in glioma cells [14] through the down-regulation of NF-κB active subunits-p50 and p65 expression [16].

This study demonstrated whether curcumin and melatonin can modulate LPS and/or $MnCl_2$-induced neuroinflammation responses in BV-2 microglial cells. The present results confirmed that the nitric oxide production, iNOS, and proinflammatory cytokines expression were induced through the NF-κB activation. In addition, p-IκB-α expression were measured by Western blotting and NF-κB p65 expression was revealed by immunocytochemistry. With a focus on phagocytotic activation of microglial cells, this study clearly demonstrated that curcumin and melatonin effectively inhibit the LPS and/or $MnCl_2$-induced phagocytotic activation of BV-2 cells.

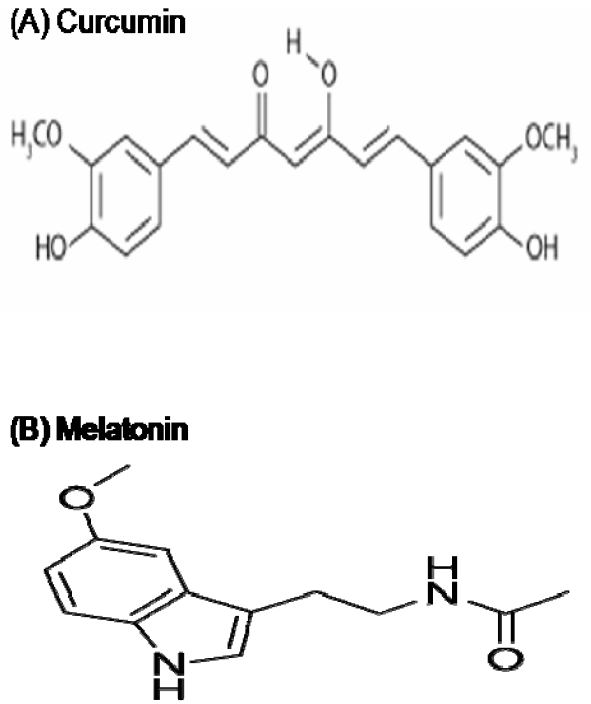


Figure 1. Structures of curcumin (A) and melatonin (B)

II. Materials and Methods

II-1. Materials

Curcumin from curcuma longa (tumeric), LPS from *Escherichia coli* O111:B4 and melatonin were obtained from SIGMA (St Louis, Missouri, USA). Antibodies against NFκB p65, actin, iNOS, IκB-α and p-IκB-α were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). All other chemicals were obtained from SIGMA (St Louis, Missouri, USA).

II-2. Methods

II-2-1. Cell Culture

The murine microglial cell line, BV-2 cells cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, CA, USA) and 1% penicillin (100 unit/ml)/streptomycine (100 μg/ml). Culture were maintained at 37 ℃ under a 95% humidified condition with 5% CO_2. Usually, the culture medium were changed to medium with 0.5% FBS before any treatment to reduce the serum effect.

II-2-2. Cell viability assay

The cell viability was determined by the 3'-(4,5-dimethylthiazol-2-ly)-2,5-diphenylthetrazolium bromide (MTT)

assay. The BV-2 cells were plated at a density of 1.0×10^4 cells/well in a 96well plate for 24 hr. When indicated, 1 μM curcumin and 10 μM melatonin were added 1 hr before the treatment with 500 ng/ml LPS and/or 250 μM $MnCl_2$ and maintained for 24 hr. After cells were treated with chemicals, 10 μl MTT (dissolved in phosphate buffered saline, final concentration of 500 μg/ml) was added to each well of 96well plate and incubated for further 4 hr. At the end of incubation, media were removed and formazan products were extracted for 15 min by 200 μl dimethylsulfoxide (DMSO). The absorbance was read at 540 nm with ELISA plate reader (VersaMax, Molecular Devices, Sunnyvale, USA). The viability of cells was expressed as the percentage compared with non-treated control cells.

II-2-3. Nitric Oxide (NO) assay

The production of NO was measured by the accumulation of nitrite in culture media. The cell supernatants were collected after chemical treatments for 24 hr. The collected supernatants were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthyl-ethylenediamine dihydrochloride in water) in the dark at room temperature for 15 min. The absorbance was read at 540 nm using a plate reader.

II-2-4. Isolation of RNA and Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated with Easy-blue™ (Intron, Korea) according to the manufacturer's instruction. The cultured cells

were harvested and homogenized with 1 ml of Easy-blue. The lysed cells were added 200 μl chloroform and then vortex for 5 sec. Cells were centrifuged at 13,000 rpm at 4 ℃ for 10 min. The aqueous phase was transferred to new centrifuge tube containing same volume of isopropanol (Sigma, CA, USA), and mixed. After incubation for 10 min, centrifuged at 13,000 rpm at 4 ℃ for 5 min. The supernatant was removed and the pellet was washed with 1 ml 70% ethanol and centrifuged 10,000 rpm 4 ℃ for 5 min. Watching steps were repeated three times. Total RNA (2 μg) was reverse transcribed using M-MLV reverse transcriptase (Intron, Korea). PCR was performed with Taq polymerase (Intron, Korea) using appropriate primer sets.

II-2-5. Isolation of Protein and Western blot analysis

The BV-2 cells were seeded 1.0 x 10^6 into 100 mm cell culture plates (Corning, NY, USA) and the BV-2 cells were treated with various chemicals such as curcumin, melatonin, LPS and/or $MnCl_2$ for 24 hr. Cells were lysed with RIPA buffer (1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1mg/ml phenylmethylsulfonyl fluoride, 1.4U/mg aprotinin, 1mM Na_3VO_4). Lysates were centrifuged at 13,000 rpm for 20 min at 4 ℃ after incubated on ice for 30 min. The supernatants were transferred to new tubes and protein concentration was determined with protein assay kit (Bio-Rad, CA, USA). The samples were boiled and electrophoresized in sodium dodecyl sulfate (SDS)-polyacrylamide gel and then electrotransferred to PVDF membrane. The membranes were blocked with TBS buffer containing 5% non-fat milk plus 0.1% Tween20 for 4hr. The membrane were incubated with primary antibodies (Actin, iNOS, IκB-α or p-IκB-α) for overnight at

4 ℃ and washed with TBS-T for 15 min three times and incubated with horseradish conjugated secondary antibody for 2 hr at room temperature. Detection of proteins was carried out by ECL chemiluminescence kit (Amersham, Arlington Heights, Illinois, USA).

Table 1. Primer sequences

Genes	Primer sequences	PCR product	Annealing temperature
GAPDH (MOUSE)	R:5'-GGT GCT GAG TAT GTC GTG GA	451 bp	52℃
	F:5'-GAC ACA TTG GGG GTA GGA AC		
iNOS	R:5'-GTG TTC CAC CAG GAG ATGTTG	356 bp	53℃
	F:5'-CTC CTG CCC ACT GAG TTC GTC		
IL-1β	R:5'-ATG GCA ACT GTT CCT GAA	811 bp	52℃
	F:5'-TTT AGG AAG ACA CGG ATT		
IL-6	R:5'-GCC TTC CCT ACT TCA CAA	564 bp	52℃
	F:5'-TAG GTT TGC CGA GTA GAT		
TNF-α	R:5'- TTC ACA GAG CAA TGA CTC C	711 bp	50.5℃
	F:5'- CCA TGA GCA CAG AAA GCA T		

II-2-6. Determination of glutathione (GSH)

Total glutathione levels were determined as previously described [19]. The BV-2 cells were plated at a density of $0.8x10^6$ cells into 6 mm cell culture plates for 24 hr. Cells were treated with chemicals for 24 hr at 37 ℃. The treated cells were washed two times with PBS and lysed with 600 μl ice-cold Tris-buffered saline (TBS, 25mM Tris-HCl. pH 7.4, 130mM NaCl, 5mM KCl) on ice. The lysed cells were collected by rubber policeman and transferred to centrifuged tube and centrifuged at 2,000 g for 10 min at 4 ℃. The cell pellets were resuspended using 50 μl tris-buffered saline (25mM, pH 7.4) and homogenized with 50 μl 10% ice-cold trichloroacetic acid (TCA) solution. And then, cells were vigorously shaked for 10 min and centrifuged at 21,000 g for 10 min at 4 ℃. The supernatants were transfered to new tube and mixed with 10 μl sample and 177.5 μl assay buffer (0.01M phosphate buffer (pH 7.0) 140 μl + 4mM NADPH 12.5 μl + 6U/ml glutathione reductase 25 μl) and then incubated at 37 ℃ for 5 min. After incubation, add 10mM DTNB (5,5' -dithiobis (2-nitrobenzoic acid) for 6 min and the absorbance was measured at 412 nm.

II-2-7. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured using the method of previously describe by [20]. The BV-2 cells were plated at a density of $0.8x10^6$ cells into 6 mm cell culture plates for 24 hr. After curcumin, melatonin, LPS and/or $MnCl_2$ treatments, cells were maintained at 37 ℃ in a 5% CO_2 for 24 hr. The treated cells

were washed twice with PBS and treated with 600 μl ice-cold tris-buffered saline (TBS, 25mM Tris-HCl. pH 7.4, 130mM NaCl, 5mM KCl) on ice. Cells were collected by rubber policeman and transferred to new centrifuged tube and centrifuged at 2,000 g for 10min at 4 ℃. The cell pellets were homogenized in potassium phosphate buffer (pH 7.8) and centrifuged at 12,000 g for 15 min at 4 ℃. The supernatants were used to determine the quantity of protein. Each 25 μl of supernatants of samples were mixed with 200 μl reaction buffer (50mM potassium phosphate buffer pH7.8, 1.33mM diethylenetriaminepenta acetic acid : DTPA, 1.0U/ml catalase, 70μM nitroblue tetrazolium : NBT, 0.2 mM xanthine, 50 μM bathocuproinedisulfonic acid, 0.13mg/ml bovine serum albumin) at 37 ℃. The final reaction was incubated with 25 μl xanthine oxidase (0.1-0.2U/ml) at 37 ℃ for 5 min and measured in absorbance of 560 nm.

II-2-8. Estimation of lipid peroxidation

Lipid peroxidation was estimated as previously describe [21]. The BV-2 cells were plated at a density of $0.8x10^6$ cells into 6mm cell culture plates for 24 hr. After chemical treatments, cells were maintained at 37 ℃ in a 5% CO_2 for 24 hr. The treated cells were washed two times with PBS and treated with 600 μl ice-cold tris-buffered saline (TBS, 25mM Tris-HCl. pH 7.4, 130mM NaCl, 5mM KCl) on ice. Cells were collected and added 6 μl BHT (the ration of 10 μl/ml cell suspension) and homogenized by sonication on ice (sonicator was setted at 40V). The homogenated cells were centrifuged at 3,000 g for 10 min at 4 ℃ and transferred the supernatant to new tube clear supernatant. The 100 μl supernatant was mixed with 900 μl reaction buffer (150mM Tris-

HCl buffer, pH7.1, 1mM FeSO4, 1.5mM ascorbic acid) and incubated at 37 ℃ for 15 min. 1 ml 10% trichloroacetic acid (TCA) solution was added to stop the reaction and 2 ml 0.375%(w/v) thiobarbituric acid added and then boiled at 100 ℃ for 15 min. The boiled cells were centrifuged at 4,500 g for 10 min and obtained supernatant and measured by absorbance at 532 nm.

II-2-9. Phagocytosis experiment

The BV-2 microglial cells were seeded at $1.5x10^4$ cells/well into 24well plates and treated with curcumin, melatonin, LPS and/or $MnCl_2$. The incubated cells were treated with 2.5 μl latex beads into each well. After 24 hr LPS and/or $MnCl_2$ treatment, the media were removed and washed four times with PBS at room temperature. Cells were fixed with methanol for 5 min, removed and dried. The fixed cells were then stained with giemsa solution (Giemsa:PBS:EtoH=1:3.5:0.5) for 10min at room temperature. After remove giemsa solution, the cells were rinsed with PBS and evaluated on a microscope.

II-2-10. Immunocytochemistry

The BV-2 microglial cells were plated $0.8x10^4$ cells/well into chamber slide (Nunc, Naperville, USA) and treated with curcumin, melatonin, LPS and/or $MnCl_2$ for 2 hr. The BV-2 cells were fixed with 3.5% paraformaldehyde in PBS for 30 min and washed three times with PBS. The fixed cells were then permeabilized with 0.1% triton X-100 for 15 min at room temperature and washed with PBS three times. The permeabilized cells were then blocked with 1% bovine serum albumin (BSA) for 1 hr at room temperature. The

blocked cells were incubated with 1 μg/ml of monoclonal mouse anti-NF-κB P65 antibody for 2 hr at room temperature and washed with PBS three times. Cells were then incubated with Fluorescein (FITC)-conjugated secondary antibody (1:500 diluted, Jackson Immunoresearch, USA) for 2 hr at room temperature and washed with PBS twice.

III. Results

III-1. Curcumin and Melatonin had an effects against LPS and/or MnCl2-induced cell death in BV-2 microglial cells.

The effects of curcumin and melatonin on the cell viability were measured by MTT assay. Firstly, the cytotoxicity of BV-2 microglial cells was determined with various concentrations of curcumin (0.1 μM ~ 8 μM), melatonin (0.1 μM ~ 10 μM), LPS (100 ng/ml ~ 1000 ng/ml) $MnCl_2$ (10 μM ~ 100 μM) for 24 hr. As shown in figure 2, the percentage of cell viability of BV-2 cells with curcumin (1μM) (Fig, 2A) and melatonin (10μM) (Fig, 2B) was almost at the same level as that of untreated control cells. When treated with 2 μM or more concentration of curcumin the cell viability was slightly decreased to 90% (2 μM treatment), 84% (4 μM treatment) and 77% (8 μM treatment) respectively. According to this result, 1 μM curcumin and 10 μM melatonin were used in subsequent experiments. As shown in figure 2(C), $MnCl_2$ treatment induced BV-2 cell death in dose-dependent manner. After $MnCl_2$ treatment with 250, 500, 1000 μM for 24 hr, the percentages of viable cells were 53%, 36% and 14% respectively. The LPS concentration was determined as shown in figure 2(D). The percentages of cell viabilities were not significant decrease in the various concentrations of LPS treatments.

In the next step, BV-2 microglial cells were pretreated with 1 μM curcumin or 10 μM melatonin for 1 hr and then treated with

LPS and/or $MnCl_2$ for 24hr to investigate whether curcumin and melatonin could protect against neuroinflammation-related toxicants-induced activation of microglia. As shown in figure 3, the treatment of $MnCl_2$ alone and LPS/$MnCl_2$ cotreatment on BV-2 cells reduced the viabilities were to 53% and 50%, respectively. However, when the curcumin and/or melatonin were applied LPS/$MnCl_2$-induced cytotoxic effect decreased remarkably in BV-2 cells.

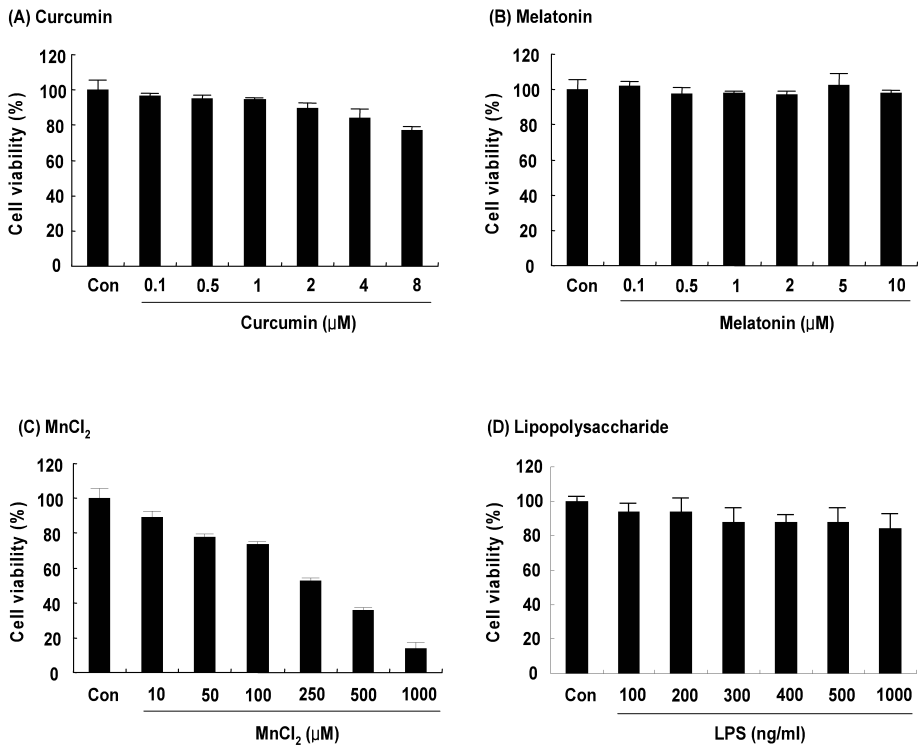


Figure 2. Effects of curcumin (A), melatonin (B), $MnCl_2$ (C) and LPS (D) on microglial cell viabilities. The cell viability of BV-2 cells was determined by MTT assay. Values are means ±SEM of triplicate determinations of two sets of experiments. Con: untreated control

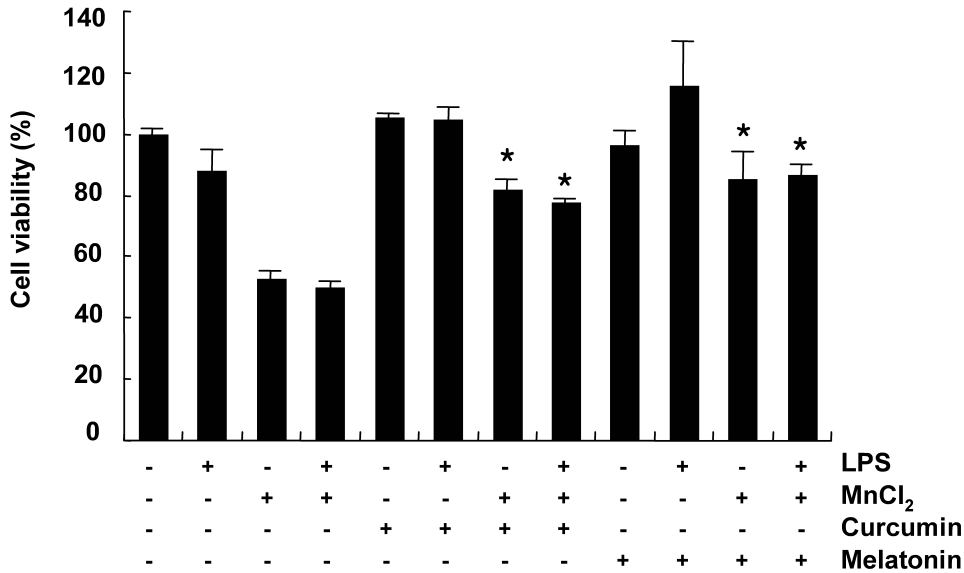


Figure 3. Effects of cucumin (1 μM) and melatonin (10 μM) on the LPS (500 ng/ml) and/or $MnCl_2$ (250 μM)-induced decrease of cell viability in BV-2 cells. The cell viability of BV-2 cells were determined by MTT assay. Data are the mean ±SEM from three independent experiments. *, $p<0.05$ as compared with the corresponding group.

III-2. Effects of Curcumin and Melatonin on the activities of antioxidant and lipid peroxidation in BV-2 microglial cells

The activities of glutathione (GSH) and superoxide dismutase (SOD), and the level of lipid peroxidation were determined in BV-2 microglial cells (Table 2). The GSH activity was significantly down-regulated by LPS (54.4% of control), $MnCl_2$ (67.8% of control), or LPS/$MnCl_2$ (43.4% of control) treatment. In contrast, curcumin and melatonin single treatment notably up-regulated (4 folds by curcumin and 2 folds by melatonin) the GSH activity in BV-2 cells. Although, curcumin or melatonin pretreatment before LPS and/or $MnCl_2$ treatment showed less effect than curcumin or melatonin alone treatment, both curcumin and melatonin pretreatment exhibited up-regulated GSH activities to 1.6~2.6 folds of control (Table 2). Next, the lipid peroxidation change was measured in the same conditions. The level of lipid peroxidation was notably increased by LPS (14.8% increase), $MnCl_2$ (19.3% increase), and LPS/$MnCl_2$ (51.2% increase) when compared with control. However, curcumin and melatonin effectively suppressed the lipid peroxidation by LPS and/or $MnCl_2$. In contrast the GSH activity changes, the antioxidant superoxide dismutase (SOD) activity was no significant changes in BV-2 microglial cell when pretreated with curcumin or melatonin.

Table 2. The measurement of GSH, SOD and lipid peroxidation expression level in BV-2 microglial cells.

	GSH (μM/μg)	Lipid peroxidation (μM/μg)	SOD (unit/μg)
Con	6.55±1.00	4.92±0.12	9.05±0.12
LPS	3.56±0.97	5.65±0.31	9.41±0.23
MnCl2	4.44±0.03	5.87±0.48	9.32±0.14
LPS/MnCl2	2.84±0.55	7.44±0.26	8.05±0.86
Curcumin	26.45±1.41	4.75±0.15	9.71±0.12
Cur+L	17.05±1.34	5.09±0.07	8.68±0.23
Cur+Mn	13.75±1.01	5.23±0.18	8.68±0.23
Cur+L/Mn	11.64±1.36	5.38±0.12	8.65±0.27
Melatonin	13.60±2.02	5.09±0.13	6.04±0.81
Mel+L	11.55±0.93	5.29±0.03	8.16±0.18
Mel+Mn	10.80±0.78	5.20±0.11	8.16±0.18
Mel+L/Mn	10.54±1.49	5.76±0.22	8.16±0.18

The cells were pretreated with curcumin (1 μM) and melatonin (10 μM) for 1 hr and then treated with LPS (500 ng/nl) and/or $MnCl_2$ (250 μM). After 24 hr of chemical treatments, the antioxidants and lipid peroxidation expression levels were measured. Con; untreated control. Cur; curcumin, L; LPS, Mel; melatonin, Mn; $MnCl_2$

III-3. Curcumin and melatonin inhibited $MnCl_2$ and/or lipopolysaccharide-induced nitric oxide production in BV-2 microglial cells

When BV-2 microglial cells were exposed to LPS or $MnCl_2$ for 24 hr, the nitrite production was increased about 2.9 folds, and 1.5 folds, respectively. To determine the effects of curcumin and melatonin on nitrite production, BV-2 cells were pretreated with curcumin or melatonin for 1 hr before LPS and/or $MnCl_2$ treatment and then nitrite concentration was measured in culture medium after 24 hr (Fig. 4). The levels of nitrite production were significantly reduced by curcumin or melatonin single treatment. Also, when the curcumin or melatonin pretreatment attenuated LPS or $MnCl_2$-induced NO productions, compared to control level (Fig 4).

III-4. Curcumin and Melatonin attenuated LPS and/or $MnCl_2$-induced iNOS and inflammatory cytokines expression levels

Previous studies suggested that NO is generated due to the increase of an iNOS and cytokines in microglial cells. To determine the effect of curcumin and melatonin on inflammatory cytokines and iNOS expression in LPS and/or $MnCl_2$-activated BV2 cells, RT-PCR and Western blot analysis were performed.

III-4-1. iNOS mRNA and protein expression

First, RT-PCR was performed to determine the iNOS mRNA expression level (Fig. 5A). As show in figure 5B the iNOS mRNA expression levels were significantly increased up to 115% by LPS

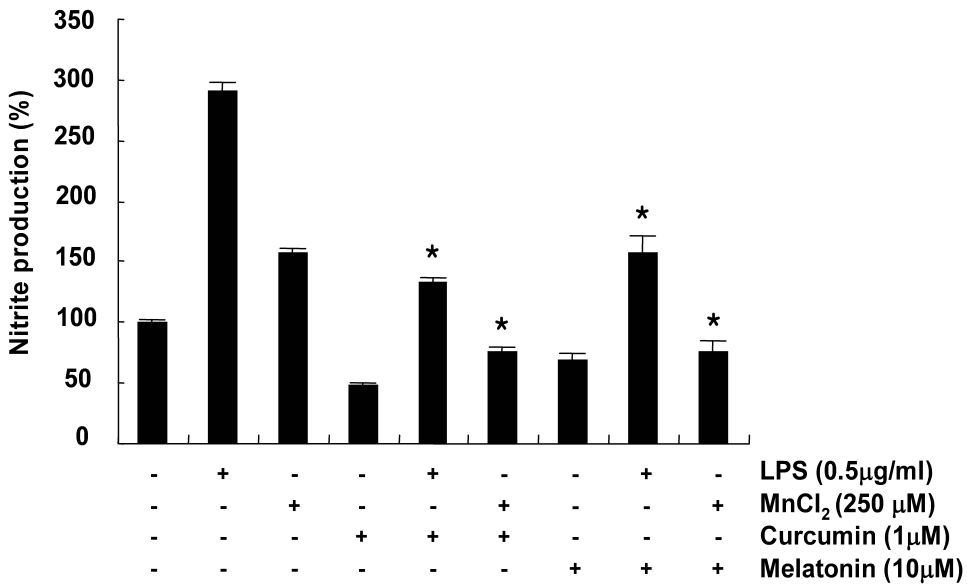


Figure 4. Effects of curcumin and melatonin on nitric oxide production in lipopolysaccharide (LPS) or MnCl2-activated BV-2 microglial cells. The BV-2 cells were pretreated with curcumin or melatonin at indicated concentrations for 1 hr before LPS or MnCl2 treatment. After 24 hr of chemicals treatment, nitrite levels in the culture medium were determined by Griess reaction. *, $p<0.05$

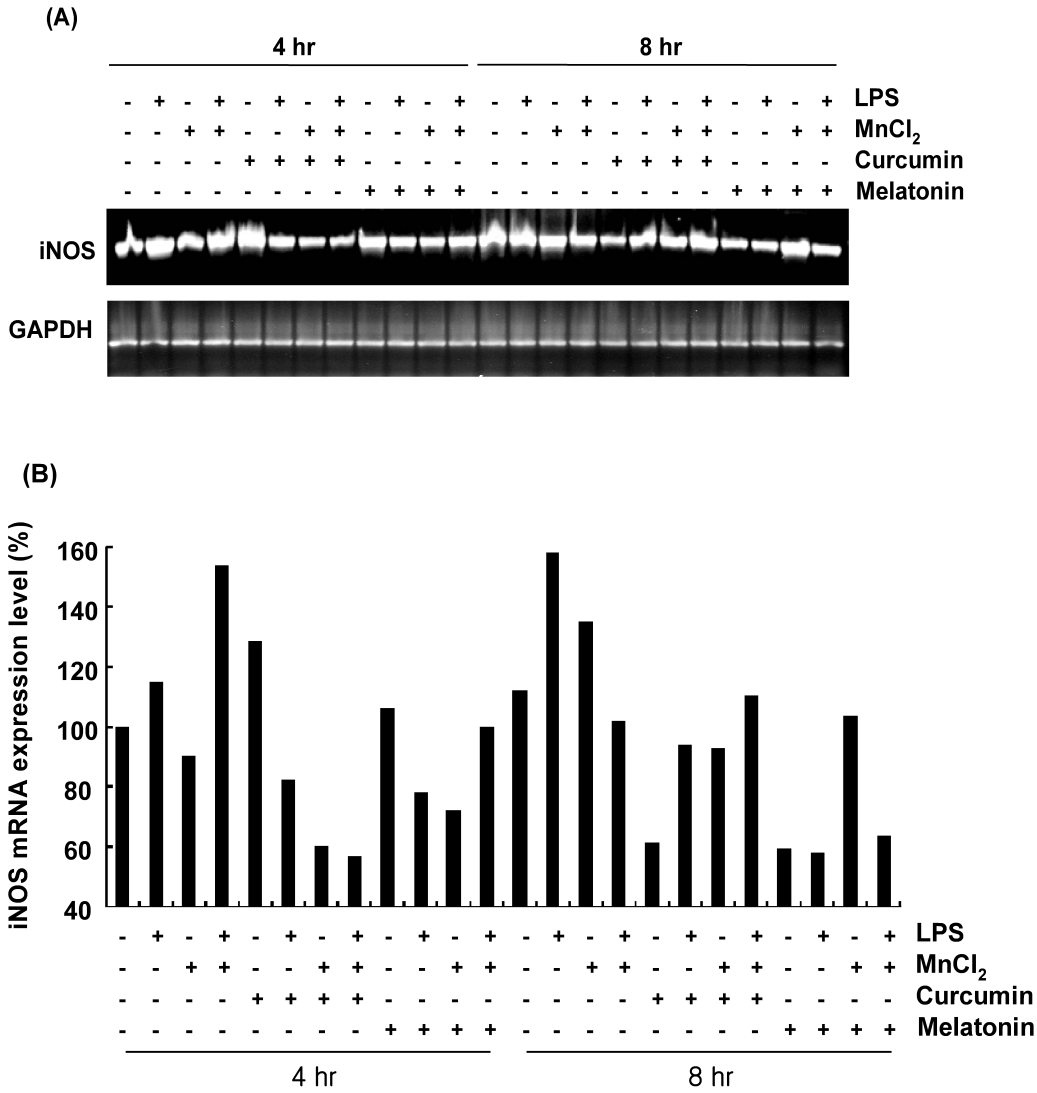

(A)
4 hr
8 hr
LPS
MnCl2
Curcumin
Melatonin
iNOS
GAPDH
(B)
iNOS mRNA expression level (%)
160
140
120
100
80
60
40
LPS
MnCl2
Curcumin
Melatonin
4 hr
8 hr

Figure 5. Inhibitory effects of curcumine (1 μM) and melatonin (10 μM) on iNOS mRNA expression levels in BV-2 microglial cells. (A) The cells were pretreated with curcumine (1 μM) and melatonin (10 μM) for 1 hr before the addition of LPS (500 ng/ml) and/or $MnCl_2$ (250 μM) for 4 hr and 8 hr. The iNOS mRNA expression levels (4 hr, 8 hr) were determined by RT-PCR. (B) Band intensities were measured by densitometric analysis and normalized to GAPDH 4 hr quantified as the ratio against the rate of untreated control.

alone and 154% by LPS/$MnCl_2$ treatment for 4 hr. However, $MnCl_2$ single treatment induced slight reduction of the iNOS mRNA expression (90% of control). When curcumin and melatonin was treated for 4 hr, the iNOS mRNA expression levels were significantly rise up to 128% and 106% of control, respectively. As expected, curcumin and melatonin pretreatment effectively attenuated LPS-and/or LPS/$MnCl_2$-induced iNOS mRNA levels (Fig 5). The iNOS mRNA expression by LPS and $MnCl_2$ treatment for 8 hr was more increased than for 4 hr treatment conditions. On the other hand, the LPS/$MnCl_2$ cotreatment for 8 hr decreased iNOS mRNA expression level from 154% (4hr) to 102% (8hr) of control. As same as 4 hr treatment conditions, 8 hr treatment conditions revealed that the inhibitory effects of curcumin and melatonin on the LPS and/or $MnCl_2$-induced production of iNOS mRNA expression were significant.

Next, the levels of iNOS protein expression were measured after 12 hr LPS and/or $MnCl_2$ treatment with or without curcumin and melatonin pretreatment for 1 hr by western blotting (Fig. 6A). As shown in figure 6B, BV-2 microglial cells stimulated by LPS and $MnCl_2$ for 12 hr increased the iNOS protein expression levels to 148% (500 μg/ml LPS), 129% (250 μM $MnCl_2$) and 143% (LPS/$MnCl_2$) of control, respectively. However, curcumin and melatonin pretreatment induced significant reduction of iNOS protein expression in LPS and/or $MnCl_2$-stimulated BV-2 cells level.

III-4-2. Cytokines mRNA expression

In the same conditions that we measured the iNOS mRNA expression levels, the proinflammatory cytokines expression level was determined by RT-PCR (Fig 7). There was no significant change

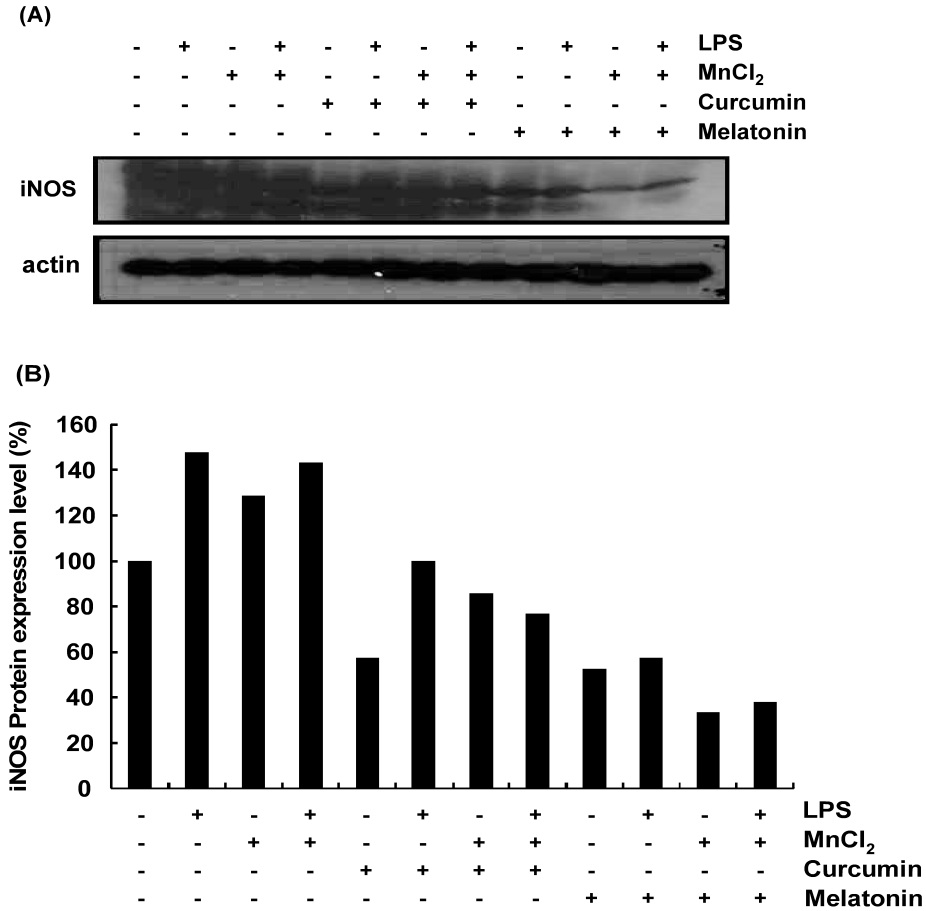


Figure 6. Inhibitory effects of curcumine (1 μM) and melatonin (10 μM) on iNOS protein expression levels in BV-2 microglial cells. (A) The cells were pretreated with curcumine (1 μM) and melatonin (10 μM) for 1 hr before the addition of LPS (500 ng/ml) and/or $MnCl_2$ (250 μM). The expression of iNOS protein was determined by western blot analysis after 12 hr LPS and/or MnCl2 stimulation. (B) Band intensities were measured by densitometric analyis and normalized to anti-actin and then quantified as the ratio against the value of untreated control.

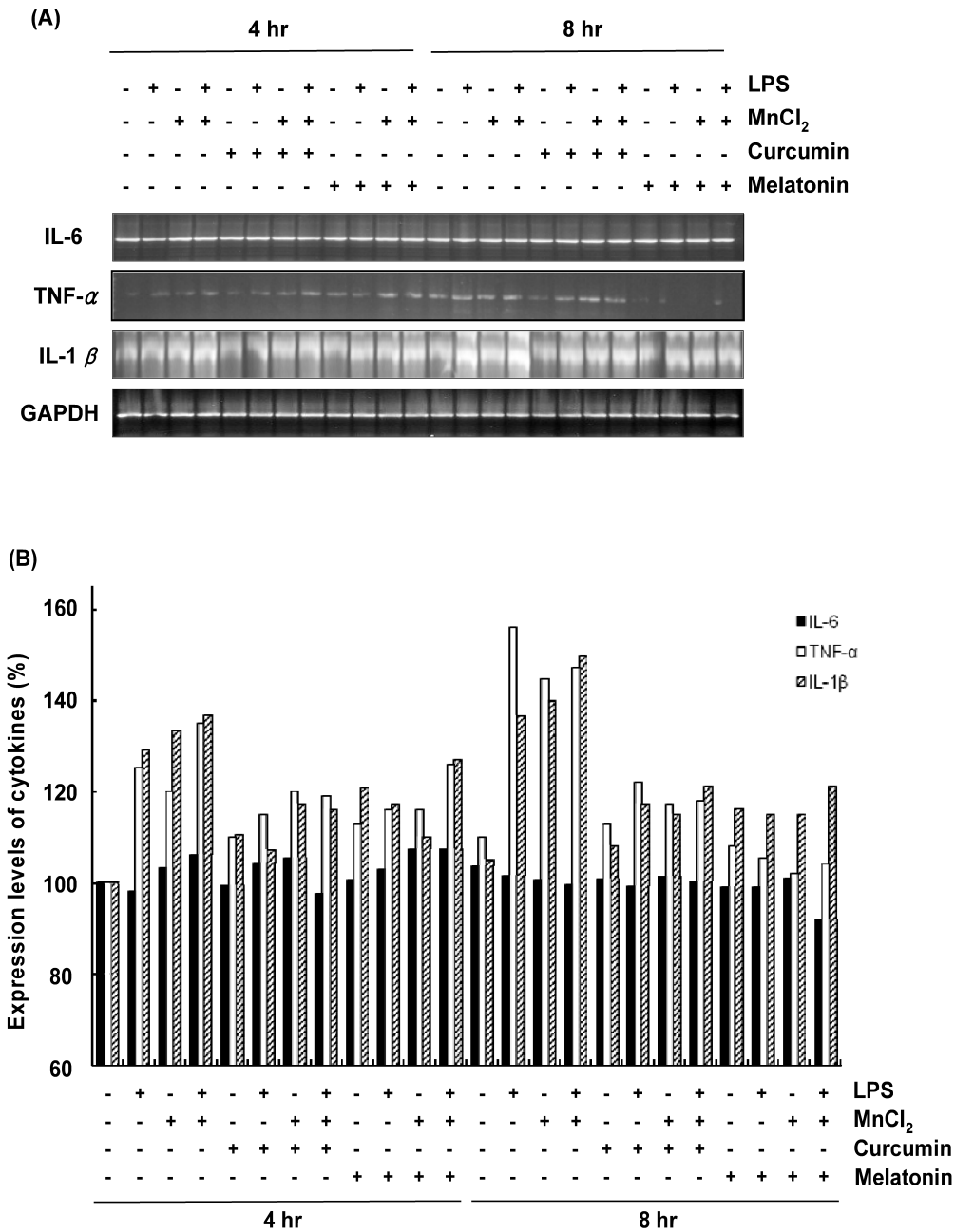

(A)
4 hr
8 hr
LPS
MnCl2
Curcumin
Melatonin
IL-6
TNF-α
IL-1 β
GAPDH
(B)
Expression levels of cytokines (%)
160
140
120
100
80
60
IL-6
TNF-α
IL-1β
LPS
MnCl2
Curcumin
Melatonin
4 hr
8 hr

Figure 7. Effects of curcumin or melatonin on LPS and/or $MnCl_2$-induced cytokines mRNA expression in BV-2 microglia. (A) BV-2 microglial cells were pretreated with curcumine (1 μM) or melatonin (10 μM) for 1 hr prior to the addition of LPS (500 ng/ml) and/or manganese (250 μM) for 4 and 8 hr, and then the mRNA expression levels of IL-6, IL-1β, TNF-α and GAPDH were determined by RT-PCR. (B) Band intensities were measured by densitometric analysis and normalized quantification values against GAPDH expression level were assessed as the ratio against the value of untreated control.

of IL-6 expression level in BV-2 microglial cells when preincubated with curcumin and melatonin for 1 hr and then added LPS and/or $MnCl_2$ for 4 hr or 8 hr. However, TNF-α and IL-1β mRNA expression levels were significantly elevated by LPS (TNF-α; 125% of control, IL-1β; 130% of control), $MnCl_2$ (TNF-α; 120% of control, IL-1β; 133% of control), and LPS/$MnCl_2$ (TNF-α; 135% of control, IL-1β; 137% of control) after 4 hr treatment (Fig 7). And, after 8 hr treatment, TNF-α and IL-1β mRNA expression levels were significantly elevated by LPS (TNF-α; 156% of control, IL-1β; 136% of control), $MnCl_2$ (TNF-α; 145% of control, IL-1β; 140% of control), and LPS/$MnCl_2$ (TNF-α; 147% of control, IL-1β; 150% of control). The expression levels of cytokines in 8 hr higher than in 4 hr treatment.

Both curcumin and melatonin pretreatment effectively suppressed LPS and/or $MnCl_2$-induced TNF-α and IL-1β mRNA levels at both time points (4 hr and 8 hr).

These results suggest that both curcumin and melatonin did not modulate the IL-6 mRNA expression level, but those antioxidants could suppress LPS and/or-induced TNF-α and IL-1β mRNA expression in BV-2 microglial cells.

III-5. Curcumin and Melatonin modulate p-iκB-α protein expression levels

To observe the NF-κB signaling associated with proinflammatory cytokines and iNOS gene expression, western blot analysis was performed and determine the IκB-α and phosphorylated IκB-α protein expression in BV-2 microglial cells (Fig 8).

As shown in figure 8B, p-IκB-α expression was increase by LPS

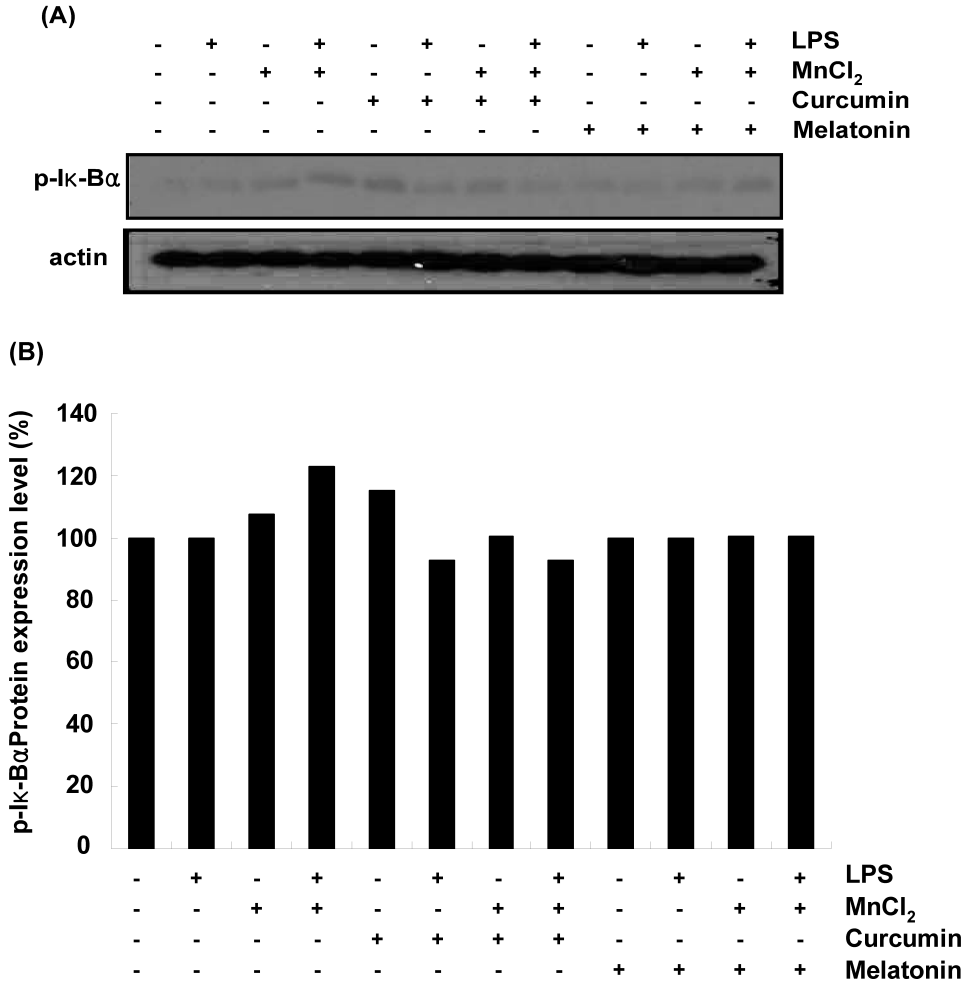


Figure 8. Effects of curcumine and melatonin on the expression of p-IκB-α in microglial cells. (A) BV-2 microglial cells were activated with LPS (500 ng/ml) and/or $MnCl_2$ (250 μM) for 2hr with or without curcumine (1 μM) or melatonin (10 μM). The expression of p-IκB-α and actin was determined by western blot using specific antibodies. (B) Intensity of each band was estimated by densitometric analysis.

and/or $MnCl_2$ treatment in BV-2 microglial cells. However curcumin and melatonin pretreatment 1 hr prior to LPS and/or $MnCl_2$ reduced the p-IκB-α expression level to untreated control level. Previous studies suggested that NF-κB is sequestered in the cytoplasm by IκBs and various stimulates including LPS induce phosphorylation of IκBs, which release active NF-κB. Therefore, these results suggest that curcumin and melatonin inhibits the IκB-α phosphorylation and subsequent NF-κB activation in BV2 cells.

III-6. Curcumin and Melatonin attenuate the trlanslocation of the p65 subunit of NF-κB into nucleus

Next, we determined the effects of curcumin and melatonin on LPS and/or $MnCl_2$ stimulation activities NF-κB and translocates the p65 subunit of NF-κ B into nucleus by immunocytochemistry (Fig 9). Our results found that melatonin attenuated the translocation of NF-κB p65 into nucleus in BV-2 cells stimulated with $MnCl_2$. In contrast LPS- and/or $MnCl_2$ treatments in BV-2 cells, curcumin or melatonin had not shown significant effects.

III-7. Phagocytosis

To assess whether curcumin and melatonin prevent LPS and/or MnCl2-induced phagocytotic activity in the BV-2 cells, latex beads ingestion was counted.

As shown in figure 10, phagocytotic activity was significantly increased in cells treated with LPS (2.4 folds), $MnCl_2$ (2.6 folds),

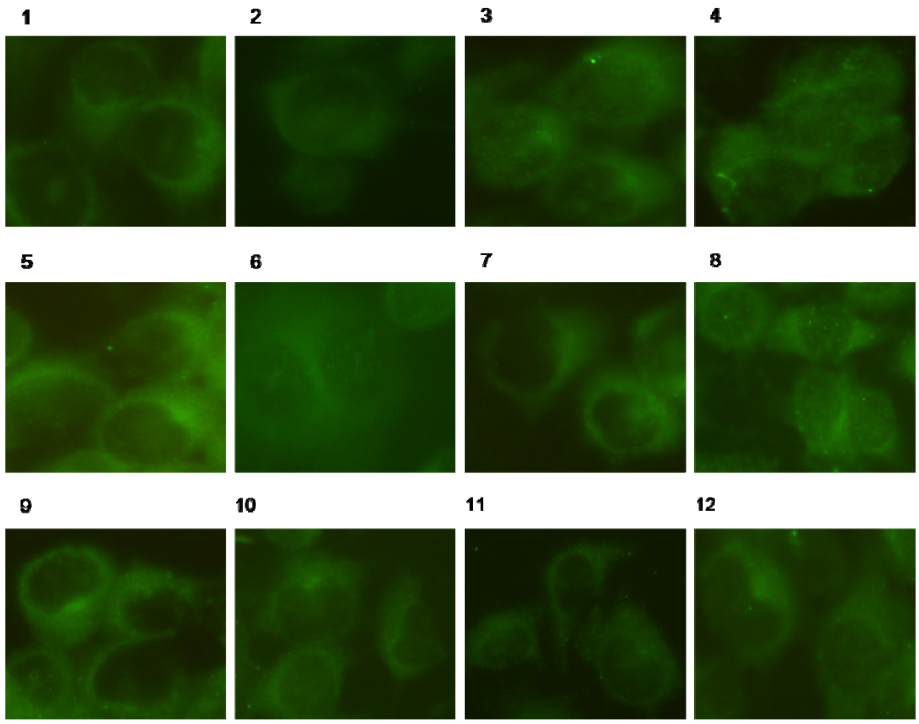
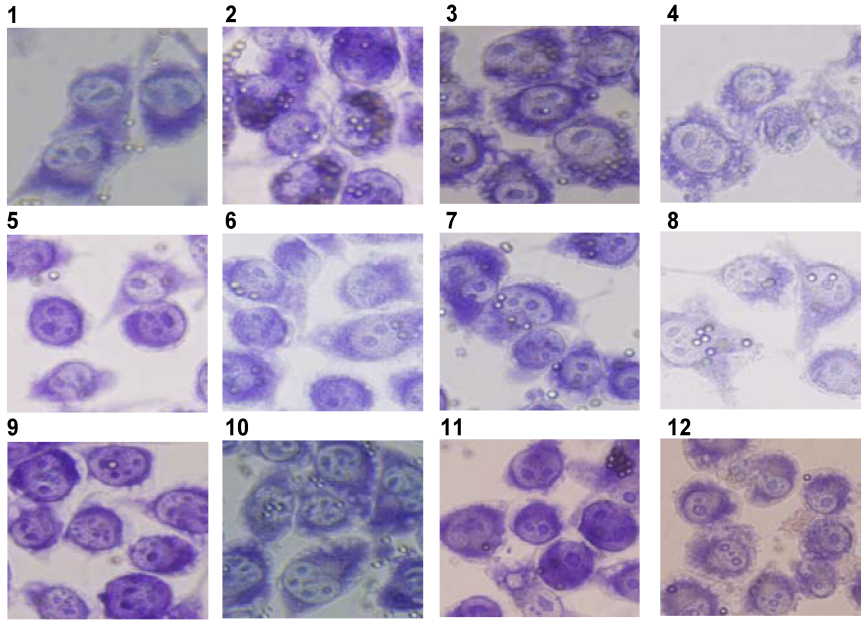


Figure 9. Effects of curcumine and melatonin on the translocation of p65 subunit of NF-κB of BV-2 microglial cells. BV-2 microgllial cells were activated with LPS (500 ng/ml) and/or $MnCl_2$ (250 μM) for 3 hr with or without curcumine (1 μM) or melatonin (10 μM). The translocation of p 65 subunit of NF-κB was determined by immunocytochemistry. 1; Control, 2; LPS 500ng/ml, 3; $MnCl_2$ 250 μM, 4; LPS+MnCl2, 5; Curcumin 1 μM, 6; Curcumin+LPS, 7; Curcumin+$MnCl_2$, 8; Curcumin+LPS/$MnCl_2$, 9; Melatonin 10 μM, 10; Melatonin+LPS, 11; Melatonin+ $MnCl_2$, 12; Melatonin+LPS/$MnCl_2$

(A)


1
2
3
4
5
6
7
8
9
10
11
12

(B)

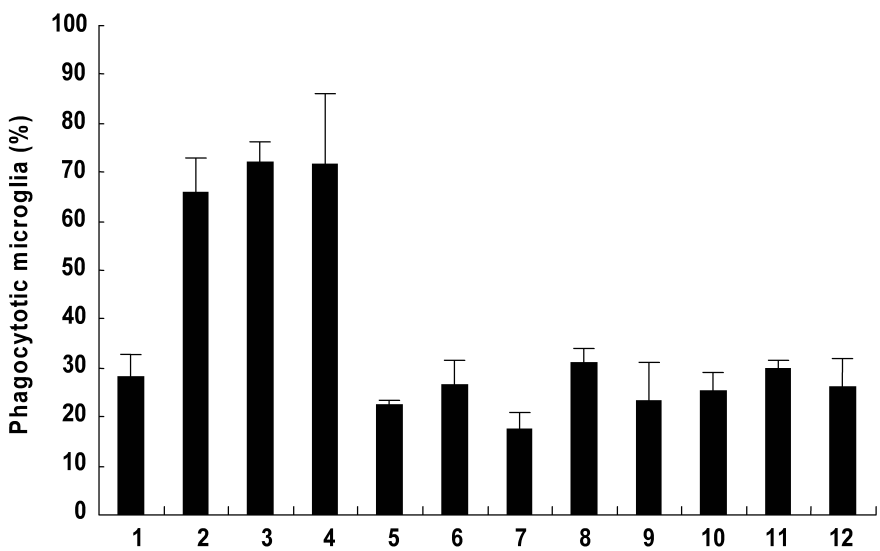

Phagocytotic microglia (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12

Figure 10. Effects of curcumin and melatonin on phagocytotic activity of BV-2 microglia cells. (A) cells were pretreated with curcumin and melatonin for 1 hr before LPS and/or MnCl2 treatment. After 24 hr treatment, phagocytosed beads were observed by microscope. Representiativa micrographs are shown for each condition. (B) the potical bead density was counted and quantified as percentage. 1; Control, 2; LPS 500ng/ml, 3; $MnCl_2$ 250 μM, 4; LPS+MnCl2, 5; Curcumin 1 μM, 6; Curcumin+LPS, 7; Curcumin+$MnCl_2$, 8; Curcumin+LPS/$MnCl_2$, 9; Melatonin 10 μM, 10; Melatonin+LPS, 11; Melatonin+ $MnCl_2$, 12; Melatonin+LPS/$MnCl_2$.

and LPS/$MnCl_2$ (2.6 folds), respectively. However, the curcumin or melatonin pretreatment dramatically reduced the LPS- and/or $MnCl_2$-induced phgocytosis. These results clearly indicate a prominent bioactive effect of curcumin and melatonin on the phagocytotic function of microglia.

IV. Discussion

Several previous studies suggested that neuroinflammation is a major determinant of the pathogenesis in various neurodegenerative diseases including Alzheimer disease, Parkinson's disease, Amyotrophic lateral sclerosis (ALS), and Huntington's disease [22-23]. Those neurodegenerative diseases are directly related to the production of various inflammatory mediators by the microglia activation. The activated microglia induces neuronal loss through the inflammation in which secretes inflammatory mediators such as TNF-α, IL-1β, IL-6, prostaglandin E2, NO, iNOS and ROS [24-25]. Therefore, inhibiting neuroinflammatory mediators could be an effective method to cure the neurodegenerative diseases. In this study, I confirmed the anti-inflammatory effects of curcumin and melatonin that modulated neuroinflammatory mediators generated from BV-2 microglial cells.

BV-2 microglial cells damaged by LPS induce oxidative stress accompanied by inflammation. Although the oxidative stress generates the reactive oxygen species (ROS) free radicals and NO increase in BV-2 microglia, antioxidants including glutathione (GSH), and antioxidant enzymes such as superoxide dismutase (SOD) and catalase are decreased in the BV-2 cells when treated with LPS [26]. In addition, lipid peroxidation is increased in the LPS treatment [27]. Those antioxidants and antioxidant enzymes are known as the free radical scavengers, which are increased by curcumin [27] or melatonin [26] treatment. In this study, both curcumin and melatonin made the lipid peroxidation, GSH and SOD expression levels changed. Both curcumin and melatonin

effectively suppressed the lipid peroxidation which was elevated by LPS and/or $MnCl_2$. Although GSH activity was up-regulated by both curcumin and melatonin, SOD activity was not affected by curcumin and melatonin in BV-2 cells (Table 2). Those results suggest that curcumin and melatonin have some specific regulatory mechanisms suppressing lipid peroxidation and elevating GSH activity in BV-2 microglial cells.

Exposure of microglial cells to LPS and neurotoxins [28] elicited the excessive inflammatory mediators such as nitric oxide (NO) and cytokines, which induced neuroinflammatory responses and subsequent neuronal cell damage [29]. Transcribed and expressed inducible nitric oxide synthase (iNOS) is a key molecule to produce nitric oxide (NO) through the NO synthetic pathway in macrophage activated by LPS [30]. Inhibition of the iNOS production could reduce the LPS-induced NO production in macrophages and microglia [31-32]. In this study, I found that curcumin and melatonin completely inhibited the LPS-induced iNOS mRNA (Fig. 5) and protein (Fig. 6) expression and subsequent NO production (Fig. 4) in BV-2 cells. NO production was additionally increased by manganese and LPS treatment in microglial cells (Fig 4). Previous study reported that, either $MnCl_2$ or $MnSO_4$ increased the NO level but other transition metals, such as $NiCl_2$, $CoCl_2$, $FeCl_2$, $CuCl_2$ and $AnCl_2$ did not significantly change the LPS-induced NO production [8]. Moreover, $MnCl_2$ clearly induced iNOS mRNA and protein expression in BV-2 cells when treated with LPS together [33]. In accordance with those previous studies, present study confirmed that LPS and/or $MnCl_2$ treatment significantly elevated iNOS expression and NO production in BV-2 cells (Figs 4 and 5). In addition present study indicates that both curcumin and melatonin could control iNOS expression from transcription

phase and further those antioxidants suppress iNOS protein expression and effectively decrease NO production caused by LPS and/or $MnCl_2$ in BV-2 cells. Although previous studies have focused on the direct antioxidant actions of melatonin and curcumin in neuronal cells, this study suggested the considerated evidence of suppression of NO release by both melatonin and curcumin in microglial cell. Even though it has been suggested that curcumin attenuates LPS-induced iNOS and NO production [34]. There are no studies on the inhibitory effects of curcumin or melatonin against LPS/$MnCl_2$ cotreatment-induced iNOS and NO expression in BV-2 microglial cells. Taken together, present study demonstrated the first evidence of inhibitory effects of curcumin and melatonin on microglia activation induced by LPS and/or $MnCl_2$. In contrast to other antioxidants, both curcumin and melatonin were found to cross the blood-brain barrier, and reach every neuronal compartments [35]. Several studies have shown that melatonin is a multifunctional molecule acting as an antioxidant, free radical scavenger, and regulator of immune responses [35-36]. A variety of oxygen and nitrogen species could be scavenged but several antioxidative enzymes, such as glutathione peroxidase and superoxide dismutase were increased by melatonin [37]. It has been known that NO-donor inhibits dopamine release while NOS inhibitor increases dopamine release from the retina. Melatonin inhibited retinal NOS activity and regulated NO production caused by SNP in retina [38-39]. Several studies have indicated that melatonin protected against neuronal damage induced by 3-nitropropionic acid [40] and lipopolysaccharide (LPS)-induced gastrointestinal motility disturbances [16]. LPS-activated microglia clearly indicated that inflammatory cytokines such as TNF-α, IL-1β and IL-6, induce blood-brain barrier breakdown

[41-42] and elevated the immune reactions in the central nervous system which play a major role for the inflammation in the neurodegenerative diseases [43]. $MnCl_2$ markedly elevates proinflammatory cytokines expression in the presence of inflammogen LPS [4]. This study showed that curcumin or melatonin pretreatment significantly attenuated neuroinflammatory cytokines, such as TNF-α, IL-1β and IL-6 genes expression caused by LPS and/or $MnCl_2$ stimulated BV-2 cells (Fig. 7). Considering anti-inflammatory effects of curcumin and melatonin it seems likely that both curcumin and melatonin could be used as a potential therapeutics in neurodegenerative diseases associated with neuroinflammatory reactions. NF-κB, a transcription factor, plays a central role in modulating inflammatory responses in activated immune cells and is induced by various inflammatory stimuli such as LPS and/or $MnCl_2$ treatment [9]. NF-κB exists in a cytoplasma an inactive state bound to its inhibitor IκB, but activated NF-κB by external stimulus can be translocated into the nucleus. Activation of NF-κB depends on phosphorylation and subsequent degradation of IκB. When NF-κB is into the nucleus, it binds to specific promoter regions of target genes such as TNF-α, IL-1β and IL-6 [44-45]. Therefore, prevention of NF-κB activation can be a useful strategy to prevent inflammatory damage in neurodegenerative diseases. It has been reported that curcumin can inhibit LPS-induced NO production and proinflammatory cytokines gene expression through the regulation of AP-1 and NF-κB signaling pathways[12, 34]. Present study suggested that both curcumin and melatonin inhibited the IκB-α phosphorylation and subsequent NF-κB activation in BV-2 cells (Fig. 8). The ability of melatonin to inhibit the translocation of p65 subunit of NF-κB was higher than that of curcumin (Fig.

9).

In brain immune system, the microglial cells are rapidly activated by inflammogens including LPS and inflammatory show phagocytotic activity [46]. Phagocytosis is the major mechanism that is used in removing a pathogene and cell debris and leads to NF-κB activation which regulate genes responsible for innate immune response [9]. Present results showed the protective effects of curcumin and melatonin against LPS and/or $MnCl_2$ induced phagocytotic activity in BV-2 cells (Fig. 10). It means that both curcumin and melatonin can be used as critical compounds for regulating microglial activation.

In conclusion the present study demonstrated that both melatonin and curcumin have the anti-inflammation effects to block the production of inflammatory mediators, such as NO and cytokines through the inhibition of NF-κB signaling pathway in BV-2 cells. The use of both curcumin and melatonin could be a promising treatment option for microglia activation-associated neurodegenerative diseases.

Ⅴ. 적요

미세교세포(microglia) 활성은 염증 유발 관련 사이토카인들 (TNF-α, IL-6, IL-1β)과 nitric oxide (NO)와 같은 여러가지 염증매개물질들을 분비하여 신경염증 반응을 유발 하고, 신경세포의 사멸을 유도함으로써 다양한 신경퇴행성 질환의 한 가지 원인으로 여겨지고 있다.

Lipopolysaccharide (LPS)/Manganese ($MnCl_2$)에 의해 활성화 된 미세교세포는 NF-κB signaling mechanism을 통해 neuroinflammogens를 분비하고, 미세교세포의 phagocytotic 활성을 갖게 한다. 따라서, 미세교세포의 활성을 억제함으로써 신경퇴행성 질환의 발생을 억제 시킬 수 있다고 생각 되어 진다. 본 연구에서 미세교세포 활성을 유도하는 LPS와 manganese에 대한 curcumin과 melatonin의 효과를 확인하였다.

먼저, MTT assay를 통해 미세교세포에 대한 curcumin과 melatonin, LPS 그리고 manganese의 적정농도를 찾았다. LPS/ $MnCl_2$ 처리에 의해 활성화를 갖는 미세교세포는 염증반응과 함께 산화적 스트레스를 동반한다. 이는 reactive oxygen species (ROS) free radicals과 NO를 과하게 생성하고, antioxidant인 GSH와 antioxidant enzyme인 SOD를 감소 시키며 lipid peroxidation을 증가시켰다. 미세교세포에 curcumin과 melatonin을 전 처리하였을 때에는 GSH 발현 수준은 오히려 증가하였고 lipid peroxidation은 효과적으로 억제되었지만, SOD 활성의 변화에는 영향을 미치지 않았다.

다음으로, curcumin과 melatonin은 LPS/ $MnCl_2$에 의해 활성화된 미세교세포로부터 증가하는 NO 생성수준을 효과적으로 감소시켰고, 염증사이토카인들의 mRNA 발현과, NO 합성효소인 iNOS mRNA및 단백질 발현을 억제하였다.

LPS/ $MnCl_2$가 미세교세포를 자극하면서 NF-κB 억제제인 IκB를 인산화 시키고 NF-κB로부터 분리 되어 세포질에 존재하던 NF-κB 전사조

절 인자가 활성화 되면서 핵 안으로 들어가 염증사이토카인들의 유전자를 발현시킨다. Curcumin과 Melatonin은 IκB-α 인산화를 억제하였고, 또한 NF-κB p65가 핵 내로 이동하는 것을 억제하였다.

뇌의 면역 계에서 LPS/$MnCl_2$에 의해 빠르게 활성화 된 미세교세포는 식세포 작용을 갖는데, curcumin과 melatonin은 식세포 활성을 유도하는 LPS/ $MnCl_2$에 대해 억제 효과를 보였다.

이 실험 결과들을 통해 curcumin과 melatonin은 미세교세포 활성을 조절하는 중요한 물질들로써 사용 되어 질 수 있음을 증명하였다.

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감사의 글

석사 졸업을 앞두고 제가 가장 먼저 감사 드리고 싶으신 분은 제가 꿈을 갖고 포기하지 않도록 지도해 주신 전홍성 교수님 이십니다. 그리고 응원과 지지, 꾸짖음 까지 잊지 않고 해주신 생명공학과 김성준 교수님, 양영기 교수님, 정현숙 교수님, 이정섭 교수님, 박윤경 교수님 감사 드립니다. 제 논문 심사를 맡아 주신 김춘성 교수님께도 감사 드립니다.

가장 긴 시간을 보내는 실험실에서 2 년 반 동안 참 많은 일이 있었습니다. 공부와 실험을 하는 곳임과 동시에 사람과 사람이 살아가는 곳 이였습니다. 존경 받는 사람이 되기 위해선 다른 사람을 위한 배려와 위로 그리고 친절이 필요하다는 것을 배우게 되었습니다. 실험실에 처음 들어왔을 때 편안하게 잘 적응할 수 있게 해주신 실험실 선배님들께 도 감사 드립니다.

같이 대학원에 입학에 함께 힘들어했던 대학원 동기분들 수고하셨습니다. 가끔 내 일탈을 도와준 친구분들 많이 고맙습니다. 내 기분 변화를 함께 느끼면서 많이 힘들었을 my first 유수연 양과 간디 에게 진심을 다해 고마운 제 마음을 전합니다.

마지막으로 든든한 후원자 순호씨, 복자씨, 도리, 4 가지, 달용이, 삼촌들, 이모, 고모 우리 가족들 전부 다 고맙고 고맙고 또 고맙습니다. 절 위해 기도해 주시고 흔들리 않도록 버팀목이 되어 주시고 끝까지 지지해 주시고 그래서 제가 힘든 시간들 잘 이겨낼 수 있었던 것 같습니다.

정말 모든 분들께 다시 한번 감사 드립니다. 앞으로도 꿈을 잃지 않고 건강하게 살아가는 박정애 되겠습니다.

저작물 이용 허락서

학 과	생명공학과	학 번	20087063	과 정	석사
성 명	한글: 박 정 애	한문: 朴 正 愛		영문: Park Jeong Ae	
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논문제목	한글: 미세교세포 활성을 유도하는 LPS 와 Manganese 에 대한 Curcumin 과 Melatonin 의 효과				
	영문: Effects of Curcumin and Melatonin on Lipopolysaccharide/ Manganese-Induced Microglial Activation				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2010 년 2 월

저작자: 박 정 애 (인)

조선대학교 총장 귀하