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Noni Inhibits Neuronal Damage Caused by the Immune Reaction of Microglial Cells Activated by Doxorubicin

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Doxorubicin에 의해 활성화된 미세 아교세포의 면역반응으로 인한 신경손상에 Noni가 미치는 영향

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ABSTRACT

Microglial cells function as major immune cells in the brain, playing an important role in the protection and damage of neurons. BV2 microglia, activated by drug stimulation, secrete inflammatory cytokines by activating the nuclear factor kappa-light-chain-enhancer of the activated B cells pathway and are involved in neuroinflammatory and immune responses. The overactivation of microglia by stimuli can cause neuronal damage, leading to brain disease. Noni, a natural product, reduces the activity of microglia to prevent neuronal damage and is a potential natural medicine because it exerts excellent regeneration and anti-inflammatory effects on damaged cells. In this study, when noni was used to treat BV2 cells stimulated by the anti-cancer drug doxorubicin, it reduced the release of pro-inflammatory cytokines from BV2. On the other hand, neuronal damage is a side effect of doxorubicin. Therefore, the cytokines released from doxorubicin-stimulated BV2 cells treated with noni had a positive effect on the neuronal viability compared to those released from doxorubicin-stimulated BV2 cells not treated with Noni. Thus, Noni increases neuronal viability. These results suggest that noni inhibits the release of cytokines by regulating the nuclear factor kappa-light-chain-enhancer of the activated B cells pathway of BV2, thereby inhibiting neuronal damage.

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INTRODUCTION

Microglia are neurons that regulate the immune function of the brain. They phagocytose denatured neurons or foreign bodies in the tissues and play an important role in neuroprotection and neurotoxicity. In addition, microglia are beneficial to neurons under

normal conditions; however, their overactivation lead to inflammation-mediated nerve damage and degeneration [1]. Microglia are directly associated with the brain diseases such as dementia and Parkinson's disease. An abnormally misfolded protein, "amyloid beta," accumulates between the brain cells, while "tau" protein accumulates within the brain cells, causing dementia. When microglia detect misfolded protein plaques, they become active and release protein particles that aggregate and remove misfolded proteins [2]. However, protein particles such as pro-inflam-

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matory cytokines and neurotoxic substances released by microglia during this process cause brain inflammation associated with dementia. Therefore, therapeutic drugs are used to minimize or suppress the induction of immune response caused by microglia. However, these drugs have side effects; hence, many studies have been conducted to compensate for them [3, 4].

Doxorubicin is an antibiotic isolated from *Streptomyces peucetius* and is a chemotherapy drug aimed at cancer therapy. It is an anti-tumor agent widely used for various carcinomas such as malignant lymphoma, leukemia, and gastrointestinal cancer. It is included in the anthracycline and anti-tumor antibiotic classes and interferes with and inhibits cell division by participating in DNA synthesis. However, like other chemicals, it has common side effects. As the amount of doxorubicin accumulated in our body increases, toxicity to the heart occurs, impairing heart function, and vomiting and skin disease. In addition, anemia and immunity decrease due to the decrease of various components in the blood, and various inflammatory reactions and hypersensitivity reactions are accompanied [5-8].

In addition to internal substances, external substances can also activate microglia. Several studies have reported that anticancer drugs used for clinical treatment activate microglia. This is one of the side effects of artificially occurring anticancer drugs rather than normal reactions. Thus, a new strategy is needed to prevent microglia activation errors that may occur internally or externally [9-11].

Therefore, we focused on the natural substance noni to suppress the immune response of microglia. *Morinda citrifolia* L. or Noni is a tropical fruit containing a variety of ingredients; therefore, it is widely used as food and medicine after processing [12]. In particular, Noni contains more than 200 physiologically active substances, and it relieves inflammation by killing cells that cause inflammation by quickly regenerating damaged cells with an anti-inflammatory component

called arinoid. In addition, a component called scopoletin helps expel harmful substances from the body to relieve inflammation, and phytochemicals suppress inflammation in blood vessels [13, 14]. Further, noni has no known side effects or tolerance as it is a natural medicine and is currently attracting attention as a potential natural medicine [15, 16].

It is unknown what mechanisms noni influences including NF- κ B, related to immune inducing signals. However many studies have reported that potential anti-inflammatory substances of noni affect the proteins involved in inflammatory responses and regulatory pathways of inflammatory cytokines. In some *in vivo* study, when the anticancer drugs treat to mice, the inflammation-mediated substances such as inducible oxidation is induced. However, nitrogen (iNOS) and cyclooxygenase-2 (COX2) production were decreased when noni was administered orally to mice for 60 days. Moreover, it affects the expression of cytokines such as interleukin (IL)-12, IL-6, IL-17, IL-23, tumor necrosis factor- α (TNF- α), and interferon (IFN)- γ [1, 13, 14, 17].

Therefore, we evaluated how the microglia-mediated inflammation overactivated by anticancer drugs affected noni (Figure 1).

MATERIALS AND METHODS

Figure 1. Co-culture system scheme to assess activated microglia-mediated inflammation in SH-SY5Y neuron cells.

1. Cell culture

The BV2 microglial cell line and SH-SY5Y neuron cell line were obtained from Professor Seung-Ju Yang from the Department of Biomedical Laboratory Science, Konyang University. All cell lines were cultured as adherent cells using Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Utah, USA) with 10% fetal bovine serum (FBS; Hyclone, USA) and 1% penicillin G-streptomycin solution (PS; Hyclone, Thermo Fisher Scientific, Waltham, MA). All cell cultures were incubated in a humidified atmosphere containing 5% carbon dioxide (CO₂) at 37°C.

2. Coculture of BV2 microglial cells and SH-SY5Y neuron cells

SH-SY5Y neuron cells were cocultured with BV2 microglial cells to assess the effect of the neuron cell viability when activated microglia were treated with noni. BV2 microglial cells were seeded at 2×10^4 in a transwell insert (pore size, 0.4 μ m; Corning Life Sciences, Sigma-Aldrich, Germany), and SH-SY5Y cells were seeded at 1×10^5 in a 24-well plate one day before the assay. The cells were treated with noni 5% for 24 h and doxorubicin 100 nM for 6 h. After treatment, all media was removed and replaced with new media, and the transwell insert was transferred onto SH-SY5Y cells, and the secretion substances released from BV2 microglia for 24 h passed through the membrane pore without direct contact between the cells.

3. Treatment with chemicals and reagents

BV2 microglia cells were seeded in 24-well plates at 1×10^5 cells/well and cultured overnight at 37°C. Doxorubicin hydrochloride (DOX; Sigma-Aldrich, Merck, Germany) and noni juice containing arinoid and scopoletin components (SARL TAHITI NATUREL, FRANCE) were used, and optimal treatment conditions were set according to each time and concentration (from 20 to 400 nM for DOX and from 0.5 to 10% for Noni). In a combined treatment experiment to examine

the immune reaction of microglia activated by DOX, microglia were treated with noni 50 μ L/mL for 24 h and then with DOX 100 nM for 6 hours. After treatment with noni and DOX, the cells were assessed.

4. Cell viability assay

The cell viability kit Ez-CyTox (DoGen Bio, Seoul, South Korea) was used to confirm the effects of noni on microglia activated by DOX and assess cell death in the neuronal cells. After treatment with noni and DOX at various concentrations, the medium was removed and a new medium and Ez-CyTox were diluted 10:1 and treated in a well. After incubation at 37°C for at least 1 h under dark conditions, the mixture solution was transferred to a 96-well plate and the absorbance was measured using a microreader (Versamax microplate reader; Associates of cape cod incorporated, MA). The relative viability of the cells was measured by measuring the absorbance at 450 nm. Cell viability was expressed as a percentage using the absorbance value for the control.

5. Enzyme-linked immunosorbent assay

BV2 microglial cells were seeded on a six-well plate (5×10^5 cells/well). The cells were treated with noni (50 μ L/mL) for 24 h and DOX (100 nM) for 6 h, and pro-inflammatory cytokine IL-6 expression was measured using enzyme-linked immunosorbent assay (ELISA) from the condition soup. Cell-free supernatants of the drug-treated samples were collected and IL-6 levels were measured with a mouse IL-6 DuoSet ELISA kit (R&D, NJ, USA), following the manufacturer's instructions. The absorbance of the microplate reader was measured at 450 nm, and the data represent the mean of triplicate experiments. IL-6 values were calculated using the standard value obtained from the linear regression equation.

6. Western blot assay

To confirm the suppression of immune function by noni in the cell signal system, samples were prepared

after treatment with noni (50 $\mu\text{L/mL}$) and DOX (100 nM/mL) in BV2 for western blotting. Cell pellets of BV2 lysed using RIPA buffer, and samples (30 $\mu\text{g/lane}$) and SDS-PAGE loading buffer (5 $\times$) were mixed and boiled at 100°C for 5 min. Then, it was separated from 10%~12% SDS-polyacrylamide gel and transferred to a nitro-cellulose membrane. To detect the proteins related to immune reaction, NF- κB , p-NF- κB , and β -actin primary antibodies (Abcam, Milton, Cambridge, UK) were reacted overnight at 4°C and then anti-rabbit IgG HRP and anti-mouse IgG HRP secondary antibodies (Abcam, Milton, Cambridge, UK) were reacted at room temperature for 1 h. Immuno-positive bands were visualized by chemiluminescence (ECL; Bio Rad, CA, USA) and imaged using a chemiluminescence detection system (VilberLourmat, Eberhardzell, Germany).

7. Statistical analyses

All data are presented as mean $\pm$ standard error of the mean (SEM). The values were obtained from at least three repetitive experiments and graphed based on the results. Significance between each group was tested using an independent expression t-test and one-way variance analysis (ANOVA). Statistical significance was set at $*P<0.05$, $**P<0.02$, and $***P<0.01$, and SPSS Version 18 for Windows (SPSS Inc., IL, USA) was used for all statistical analyses.

RESULTS

1. Optimal concentration of doxorubicin for the induction of pro-inflammatory cytokine expression in BV2 microglia

Microglia, which exist in the brain, function as macrophages in the immune system in the presence of stimuli occurs, such as amyloid beta peptide (Ab) and tau. These stimuli are activated by treatment with chemotherapeutic agents, such as DOX. Therefore, we conducted an experiment to select the optimal concentration of DOX to activate the microglia without inducing cell death. Activation of microglia was

confirmed by the expression of the pro-inflammatory cytokine IL-6. As shown in Figure 2A, DOX cytotoxicity was induced depending on its concentration; however, the degree of cell death was not severe. However, the cell viability began to decline noticeably at concentrations of 200 nM and 400 nM.

After confirming that some cytotoxicity was induced by DOX, we assessed the expression of IL-6 via ELISA to determine whether the activity of microglia could be induced at each DOX concentration. The expression of IL-6 increased proportionally with the increase in DOX concentration compared with the expression of IL-6 in the non-treated NC group. The expression of IL-6 increased from a concentration of 200 nM. This result shows that microglia can be activated by DOX and that DOX is a simulant that can induce the activity of microglia (Figure 2B).

Figure 2. Optimal concentration of doxorubicin for the activation of BV2 microglia. (A) Cell viability of BV2 microglia according to DOX concentration. (B) The induction of IL-6 expression depending on DOX concentration from activated microglia. $***P<0.01$ for NC versus 100 nM DOX concentration.

Based on these results, we used a concentration of 100 nm or 200 nm as the optimal concentration for DOX treatment in all subsequent experiments; however, some cytotoxicity was induced at a concentration of 200 nm.

2. Inhibition of immune function by noni in BV2 microglia activated by doxorubicin

Before evaluating the effects of noni on microglia activated by DOX, we performed an experiment to select the optimum concentration of noni for the treatment of BV2 microglia. As shown in Figure 3, the cell viability of microglia after noni treatment did not show a significant difference according to the concentration. However, some cytotoxicity was observed with 10% noni. Therefore, we decided to use a noni concentration of less than 10% to examine the effect of noni. After the assessment of cytotoxicity induced by noni, we evaluated whether it can inhibit the microglia-mediated inflammation activated by DOX. BV2 microglia were first treated with noni for 24 h and then with DOX for 6 h; BV2 microglia treated with noni and DOX showed significant cytotoxicity in the group treated with 100 nM or 200 nM DOX and 10% noni (Figure 4A). This result indicates that noni 5% does not induce severe cell death in microglia activated by DOX.

Figure 4. The effects of Noni in microglia activated by DOX. BV2 microglial cells were incubated with noni for 24 hours and then DOX was treated for 6 h. After the treatment time of DOX, the cells were assessed to measure cell viability (A) $**P < 0.02$ for NC versus Noni 10% groups in the presence of 100 or 200 nM DOX concentration, respectively and (B) IL-6 expression level (IL-6 /cell viability). $*P < 0.05$ or $**P < 0.02$ for DOX only versus Noni 5% groups in the presence of 100 or 200 nM DOX concentration, respectively.

Figure 3. Cell viability of BV2 microglia treated with optimal Noni concentration. (A) Cell image and (B) cell viability of BV2 microglial cells treated with various Noni concentration.

Next, the expression results of IL-6 to evaluate the immuno-response inhibition of activated microglia actually showed the reduced effect when treated with Noni (Figure 4B). In particular, lower IL-6 expression was observed in cells treated with 5% noni and 200 nM DOX. However, the decreased degree and the best effects were observed in cells treated with 5% noni and 100 nM DOX compared with that of cells treated with DOX only, indicating that noni can inhibit or deactivate the microglia-mediated inflammation activated by DOX.

3. Increased cell survival of neuron via NF-κB inactivation in noni-treated microglia

Microglia activated by DOX has the function as macrophage of immune response. In general, macrophages activated during the initiation of the immune response activate the NF-κB signaling system to express cytokines or interferons. To address this, a western blot assay was performed to confirm that noni inducing the expression of decreased cytokines reduced the activity of NF-κB at the cytoplasmic level. In the group treated with DOX only, the expression of phosphorylated NF-κB was significantly higher, consistent with the results of IL-6 expression in previous experiments. However,

Figure 5. A decreased induction of NF-κB intracellular signal pathway by Noni in activated microglia and improved neuron cell survival. (A) The reduced expression of phosphorylated NF-κB by Noni treatment in activated microglia. (B) Increased cell viability of SH-SY5Y neuron in co-culture system with Noni-treated microglia. * $P < 0.05$ for DOX only versus 5% noni and 100 nM DOX concentration.

noni treatment in cells activated by DOX induced the expression of the reduced NF- κ B (Figure 5A). This result suggests that noni reduces the immune response associated with NF- κ B signaling in BV2 microglia, leading to decreased cytokine expression.

Based on the above results, when noni was treated with DOX, the expression of NF- κ B and cytokine secretion decreased in BV2 microglia. Accordingly, we tested whether noni can alleviate neuronal damage. Neuronal cell viability was assessed by coculture systems with BV2 microglia and SH-SY5Y cells. BV2 microglial cells were seeded into transwell inserts, treated with noni and doxorubicin according to prescribed methods, and then transferred onto SH-SY5Y cells. As shown in Figure 5B, the group treated with DOX showed lower cell viability than the non-treated group. However, cell viability in the noni-treated group noticeably increased compared with that in the DOX-treated group. In conclusion, the results indicate that noni alleviates neuronal damage by downregulating the NF- κ B pathway in BV2 microglia activated by DOX.

DISCUSSION

Our immune system protects our body from the invasion of external substances. Cells such as microglia in the brain play an important role in the immune reactions. Microglia function like the macrophages in the immune response in the brain in the presence of internal or external stimuli. If microglia are activated by stimulation, they release various cytokines for cell protection and cytotoxic factors to defend against nerve inflammation and oxidative stress and affect nerve function and cell viability [1, 18, 19]. Microglia are also activated when neurons are damaged or affected by a disease; however, when activated chronically, microglia causes neurotoxicity and becomes a major cause of damage. Microglia activated by stimulation release pro-inflammatory cytokines, reactive oxygen intermediates, and nitric oxide (NO),

which activate TLR4/MyD88/NF- κ B signaling pathways to induce neuroinflammatory response and innate immunity, leading to neuronal damage and decreased cell viability [9–11, 20]. Some studies have shown that the potential anti-inflammatory substances of Noni affect protein inflammatory mediators and regulatory pathways of inflammatory cytokines, and when Noni is orally administered to mice for 60 days, inducible oxidation is expressed by stimulation such as inflammation. The inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) was reduced. It has also been reported that it affects the expression of cytokines such as interleukin-6 (IL-6), IL-12, tumor necrosis factor- α (TNF- α), interferon-gamma (IFN- γ), IL-17, and IL-23. In addition, as a study related to the toxicity of doxorubicin to organs, kidney function and catecholamine content were measured when doxorubicin and noni were administered to mice. As a result, administration of doxorubicin and noni did not significantly affect renal function, but the content of catecholamine was significantly reduced, suggesting that noni affects the neurotransmitter pathway [13]. And Several studies have shown that Noni shows cytotoxic and apoptosis inducing affect by itself. Also enhances the efficacy of anticancer drugs like doxorubicin and Cisplatin [8, 21].

Thus, we tried to assess its effectiveness by treatment with a natural product, noni, to prevent and reduce overactivation of microglia. First, we determined the optimal concentration of DOX to artificially activate microglia, and then determined the optimum concentration of noni (Figure 2 and 3) [3]. Furthermore, Noni did not cause serious cytotoxicity (Figure 3).

DOX is an antitumor drug that induce cytokines and generates NO to induce neuronal damage and inflammatory reactions. The degree of activation of microglia by DOX was assessed by the induction level of cytokine expression. DOX treatment successfully activated microglia by inducing IL-6 expression [22, 23], indicating that DOX is a good inducer of microglial activation. Among the foods currently known, noni

contains the most powerful antioxidants, and many reports have reported that it prevents cell damage and aging. In addition, according to clinical studies, noni is rich in antioxidants and has a positive effect on suppressing inflammatory reactions, helping to improve immune function in the body. Further, the study results showed that the microglia-mediated inflammation was inhibited by noni (Figure 4). This result demonstrates that noni affects the microglia-mediated inflammation, leading to a decrease in the immune induction ability of microglia.

Macrophages in the immune system secrete various cytokines in response to antigen stimulation. These cytokines are induced by the expression of a specific protein called NF- κ B in intracellular signaling pathways. It is involved in the inflammatory response and immune system regulation [24, 25]. In most cells, NF- κ B exists in the cytoplasm in an inactive state, and when cells are stimulated by receiving signals from the outside, various cytokines are secreted by the NF- κ B activation pathway. Therefore, we confirmed the expression of NF- κ B at the cytoplasmic level according to the decreased IL-6 level due to noni and DOX treatment (Figure 5). The expression of NF- κ B significantly increased in the DOX-treated group and decreased in the noni-treated group (Figure 5A). This result suggests that noni treatment induces down-regulates the expression of NF- κ B in microglia activated by DOX, leading to decreased cytokine secretion.

Next, we evaluated the effect of decreased the microglia-mediated inflammation on the neurons following the activity of the reduced intracellular signaling pathway of NF- κ B by noni. For this, after BV2 microglial cells were treated with DOX and noni, they were cocultured with SH-SY5Y cells. The neuronal viability of the group treated with DOX only significantly decreased, but the cell viability of the group treated with noni improved. The study results indicate that noni can prevent brain cell damage that can be induced by overactivated microglia.

In this study, we assessed the role of noni, a natural product, in the prevention neuronal cell damage that can be induced by hyperactive microglia. Microglia treated with DOX and noni reduced the microglia-mediated inflammation, such as a decrease in the expression of cytokines, compared with microglia treated with DOX only. Noni also downregulated the NF- κ B activity associated with cytokine expression in this function of microglia. Hence, Noni can be used as the prevention agent against brain cell damage that could result from incorrect activity of microglia. Therefore, we attempted to verify the effect of this function of noni on cytokine secretion in activated BV2 microglial cells and whether it can alleviate the damage of neuron cells damaged by doxorubicin treatment.

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