A THESIS FOR THE DEGREE OF MASTER

Neuroprotective and immunomodulatory effects of superoxide dismutase in SH-SY5Y neuroblastoma cells and RAW 264.7 macrophage cells

SH-SY5Y 신경모세포종 세포와 RAW 264.7 대식세포에서 초과산화물불균등화효소의 신경보호 및 면역조절 효과

2023년 8월

서울대학교 대학원
수의학과 임상수의학(수의내과학) 전공
김 문 범

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지도교수 윤 화 영

이 논문을 수의학 석사 학위논문으로 제출함

2023년 4월

서울대학교 대학원

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김문범의 석사 학위논문을 인준함

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Abstract

Neuroprotective and immunomodulatory effects of superoxide dismutase in SH-SY5Y neuroblastoma cells and RAW 264.7 macrophage cells

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Superoxide dismutase (SOD) is an antioxidant enzyme that protects the body from free radicals. It has been reported to have both antioxidant and immunomodulatory properties. Macrophages are key mediators of the innate immune response and are divided into the pro-inflammatory M1 and anti-inflammatory M2 subtypes. And SOD is known to have immunomodulatory effects that cause macrophages to polarize from M1 to M2. The purpose of this study was to assess the antioxidant and neuroprotective effects of SOD in nerve cells, as well as its immunomodulatory ability in macrophages.

In order to assess cytotoxicity against SOD, nerve cells (SH-SY5Y) and macrophages (RAW 264.7) were treated with varying concentrations of SOD and

then analyzed using the cell counting kit (CCK)-8 assay. When the concentration of SOD exceeded 10 μM in nerve cells, cell viability decreased (1.13-fold, $P<0.001$), whereas the viability of macrophages was not affected by SOD concentrations. Therefore, the study was conducted at a SOD concentration of 1 μM, at which it is known to be non-cytotoxic to both cell lines. To determine the antioxidant effect of SOD, H_2O_2 was treated to induce oxidative damage in cultured nerve cells. Subsequently, alterations in reactive oxygen species (ROS) accumulation and cell viability were determined using H_2DCFDA staining and cell CCK-8 assay, respectively. When nerve cells were treated with H_2O_2, SOD inhibited the accumulation of ROS (5.5-fold, $P<0.001$) and enhanced cell viability (1.15-fold, $P<0.05$). In addition, the degree of necrosis was assessed using annexin V staining when nerve cells were treated with conditioned medium obtained after treating macrophages with lipopolysaccharide (LPS) to induce inflammation. The degree of necrosis was reduced when SOD was treated with conditioned medium (1.56-fold, $P<0.05$). In addition, quantitative real-time PCR was performed to assess the immunomodulating ability of SOD, and it was confirmed that cytokines related to M1 polarization decreased when SOD was applied to inflammatory macrophages treated by LPS (IL-1β, threefold, $P<0.001$; IL-6, 8.57-fold, $P<0.001$; TNF-α, 20.15-fold, $P<0.001$). It was confirmed by these result that SOD induces M1 to M2 transition in macrophages.

This study confirmed not only the antioxidant effect of SOD, but also its ability to protect nerve cells in inflammatory situations and to induce the transition of inflammatory M1 to anti-inflammatory M2 when applied to macrophages. In veterinary clinical medicine, SOD has the potential to be used as a treatment option

for a variety of neurological diseases, including noninfectious meningoencephalitis of unknown etiology, based on the findings of this study. However, since this study was conducted at the cellular level, additional research seems to be needed.

Keywords: superoxide dismutase; antioxidant; immunomodulation; macrophage; neuroprotection

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1. Introduction

Superoxide dismutase (SOD) is an enzyme present in all aerobic living cells (Che et al., 2016). The enzyme is generated in many cellular processes, as a product of normal respiration and as oxidative bursts from immune cells (Altobelli et al., 2020). It regarded as the most essential line of enzymatic antioxidant defense against reactive oxygen species (ROS), and there have been identified three different isoforms of SOD to date (Nguyen et al., 2020; Corpas et al., 2006). SOD1, or Cu/Zn-SOD, was the first enzyme to be characterized and is found in the intracellular cytoplasmic spaces. SOD2, or Mn-SOD, is found in the mitochondrial matrix. SOD3, or extracellular (EC)-SOD, exists as a tetramer containing copper and zinc and is synthesized with a signal peptide directing the enzyme to the extracellular area (Kim et al., 2015; Nojima et al., 2015). It converts superoxide (O_2^-) radicals into hydrogen peroxide (H_2O_2) and ordinary molecular oxygen (O_2) (Fujii et al., 2022). Superoxide is the most important ROS in the progression of a number of inflammatory diseases (Mittal et al., 2014). The intracellular concentration of ROS is controlled by antioxidant systems and the main antioxidant enzymes in mammalian cells are SOD, catalase, and glutathione peroxidase (He et al., 2017). A previous study had reported that the concentration of ROS increased in a SOD knock-out mouse model, leading to dilated cardiomyopathy, severe anemia, and neurodegeneration, resulting in a shorter lifespan than that in wild type (Rosa et al., 2021).

Recent research has revealed that, in addition to being an antioxidant enzyme, SOD possesses immunomodulatory properties that polarize macrophages into the

M2 phase (Sah et al., 2020). Macrophages are a key component of the innate immune system and have an important function in the interaction between innate and adaptive immunity (He and Carter, 2015). They are distinctly subdivided into the classical M1 and M2 categories (Yao et al., 2019). Typically, IFN- γ or lipopolysaccharide (LPS) activate M1 macrophages (or classically activated macrophages, CAMs) to produce proinflammatory cytokines, phagocytose microorganisms, and initiate an immune reaction. M2 macrophages (or alternatively activated macrophages, AAMs) are activated by cytokines including IL-4, IL-10, or IL-13. Macrophages are associated with tissue repair and wound healing (Orecchioni et al., 2019; Wang et al., 2014). Studies have demonstrated that SOD affects macrophages, and SOD knockout (KO) mice have diminished phagocytosis and bacterial killing abilities, indicating that SOD increases bacterial clearance and reduces inflammation (Manni et al., 2011). Another study found that SOD could polarize macrophages from M1 to M2 phenotype and that SOD-mediated H_2O_2 levels could regulate M2 gene expression at the transcriptional level by redox modulation of a key cysteine in STAT6 (He et al., 2013).

Several studies have investigated the relationship between neurodegenerative and neuroinflammatory diseases and macrophages (Devanney et al., 2020; Munawara et al., 2021). Immune cells, particularly macrophages, are intimately involved in the initiation, progression, and resolution of neuroinflammatory processes. The cell populations have been recognized as key participants in the pathology of the central nervous system (CNS), including autoimmune disorders, such as multiple sclerosis, and degenerative diseases, such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) (Mammana et al., 2018). Therefore,

the therapeutic potential of SOD has attracted attention and demands further investigation.

The present study aimed to investigate the antioxidant and neuroprotective effects of SOD in a human neuroblastoma cell line SH-SY5Y, and evaluate the immunomodulatory potency of SOD in LPS-treated macrophage cell line RAW 264.7.

2. Materials and Methods

2.1. Cell cultures

The human neuroblastoma cell line SH-SY5Y (Korea Cell Line Bank, Seoul, Korea) and murine macrophage cell line RAW 264.7 (Korea Cell Line Bank, Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium-high glucose (Welgene, Gyeongsan, Korea) supplemented with 100 units/mL penicillin G (Sigma-Aldrich, St. Louis, MO), 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO), and 10% fetal bovine serum (FBS, Gibco, Waltham, MA), and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Culture medium was replaced every 2~3 days, and subculture was performed when the cells reached 70~80% confluency.

2.2. Cell viability test

Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay (D-Plus™ CCK Cell Viability Assay Kit, Dong-in Biotech, Seoul, Korea) to determine whether SOD had any influence on the growth of SH-SY5Y neurons and RAW 264.7 cells. SH-SY5Y were seeded at a density of 2.0×10^4 cells/well and RAW-264.7 were seeded at a density of 1.0×10^4 cells/well in 96-well plates. SOD was added next, at different final concentrations (in SH-SY5Y: 0, 0.01, 0.1, 1, 10, 100, 200, 500, and 1,000 μM; in RAW-264.7: 0, 10, 100, 200, 500, and 1,000 μM), to the wells. The

cytotoxic effects were calculated using the CCK-8 assay.

2.3. Reactive oxygen species (ROS) detection test

A concentration of 1 μM SOD was applied to SH-SY5Y cells for 24 h, and H_2O_2 was added at 200 μM. Intracellular ROS levels were measured based on the detection of the fluorescent product 2′,7′-dichlorofluorescein (DCF) obtained from the oxidation of H_2DCFDA. Cell cultures were incubated in a medium containing 10 μM H_2DCFDA for 1 h. After incubation, the medium was removed, and cells were washed with D-PBS. Stained cultures were analyzed for green fluorescence using an inverted fluorescence microscope (EVOS FL microscope, Life Technologies, Darmstadt, Germany) and a fluorescence microplate reader (Infinite M200 Pro, TECAN, Zurich, Switzerland).

2.4. Immunofluorescence analysis

For immunofluorescence (IF) staining, RAW 264.7 macrophage cells were fixed with 4% paraformaldehyde and blocked with a buffer containing 5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 30 min. The cells were then incubated at 4 °C for 1 h with antibodies against allophycocyanin (APC)-conjugated CD11c (1:100, Santa Cruz Biotechnology, Dallas, TX) and fluorescein isothiocyanate (FITC)-conjugated CD206 (1:100, Santa Cruz Biotechnology, Dallas, TX). After three washes, the cells were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-

phenylindole (DAPI, Vector Laboratories, Burlingame, CA), and observed under an EVOS FL microscope (Life Technologies, Darmstadt, Germany).

2.5. RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from RAW 264.7 macrophage cells using the Easy-Blue RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea), according to the manufacturer's instructions. Cell Script All-in-One 5X First Strand cDNA Synthesis Master Mix (Cell Safe, Yongin, Korea) was used to synthesize cDNA, and RNA expression was analyzed using 400 nM forward and reverse primers (Bionics, Seoul, Korea) and AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY) in an Applied Biosystems™ QuantStudio 5 qPCR System (Thermo Fisher Scientific, Waltham, MA). Expression of each gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used are listed in Table 1.

2.6. Conditioned medium from RAW 264.7 treated by LPS

To induce M1 macrophage polarization, 200 ng/mL LPS was added to the RAW 264.7 cell culture. After 6 h, the cells were washed with PBS to ensure that the cytokines used in the culture were completely removed and the medium was replaced with complete culture medium. After culturing for another 24 h, the medium supernatants were collected, centrifuged at 1,200 rpm for 5 min, and then filtered through 0.22 μm-pore filters to remove cells and debris.

2.7. Treating SH-SY5Y cells with conditioned medium

In 6-well cell-culture plates, SH-SY5Y cells were seeded at a density of 1.0×10^6 cells/well. After cell adhesion, the medium was replaced by fresh medium consisting of 30% conditioned medium and 70% original medium. To compare the neuroprotective effects of SOD, some groups were treated with 1 U/mL of SOD. All the plates were incubated at 37 °C for 24 h.

2.8. Annexin V staining

According to the manufacturer's instructions (BD Biosciences, San Jose, CA), SH-S5Y5 cells, treated with conditioned medium and SOD, were stained with annexin V-fluorescein isothiocyanate/propidium iodide (PI), and subsequently analyzed using a fluorescence-activated cell sorting instrument (FACS Aria II, BD Biosciences, San Jose, CA). The stained cells were categorized as follows: PI- and annexin V-negative (lower left quadrant), normal; PI-negative and annexin V-positive (lower right quadrant), early apoptotic; PI- and annexin V-positive (upper right quadrant), late apoptotic; and PI-positive and annexin V-negative (upper left quadrant), necrotic.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6.01) software (GraphPad Software, La Jolla, CA). Data were analyzed using Student's t-test and one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test. Data are presented as mean ± standard deviation (SD). Differences were considered statistically significant at a P-value of 0.05.

3. Results

3.1. Cell viability test using SOD in SH-SY5Y and RAW 264.7 cells

Cytotoxicity of SOD was evaluated using the CCK-8 assay, and cell viability was confirmed after treating SH-SY5Y and RAW 264.7 cells with various concentrations of SOD. In SH-SY5Y, cell viability was stable at concentrations 0, 0.01, 0.1 and 1 μM, and was reduced at concentrations above 10 μM. (P<0.01, P<0.001, Fig. 1A). In contrast, the viability of RAW 264.7 was not affected by SOD up to 1,000 μM (Fig. 1B).

3.2. Neuroprotective effect of SOD in SH-SY5Y cells

H_2O_2 was applied to SH-SY5Y neurons to cause oxidative damage, and then the 1 μM SOD-pretreated and untreated SH-SY5Y groups were compared. Cell viability was evaluated using the CCK-8 assay, and was confirmed to increase in the SOD-treated group (P<0.05, Fig. 2). In addition, the degree of ROS relaxation was compared using H_2DCFDA via fluorescent dyeing method. ROS was expressed in SH-SY5Y cells treated with H_2O_2, and the expression level decreased when SH-SY5Y cells were pretreated with SOD (P<0.001, Fig. 3).

3.3. Immunomodulatory effect of SOD in RAW 264.7 cells

We evaluated the changes in macrophage polarization after treatment of RAW 264.7 cells with SOD, based on immunofluorescence staining of CD 11c (red stain) and CD 206 (green stain) (markers of M1 and M2 phases, respectively). CD 11c expression, associated with M1 polarization, increased in LPS-treated macrophages, and CD 206 expression, associated with M2 polarization, increased in SOD-treated macrophages (Fig. 4A).

In addition, cytokines associated with the macrophage M1 phase, such as IL-1β, IL-6, and TNF-α, were measured by qPCR. Expression of IL-1β, IL-6, and TNF-α was increased when RAW 264.7 cells were treated with LPS, whereas it decreased significantly when RAW 264.7 cells were treated with SOD (P<0.001, Fig. 4B).

3.4. SOD protected SH-SY5Y cells by the conditioned medium of RAW 264.7 cells

Conditioned medium of RAW 264.7 cells was applied to SH-SY5Y cells. To confirm the neuroprotective ability of SOD, SH-SY5Y cells were treated with 1 μM SOD. Annexin V-FITC staining was performed to evaluate the necrosis of SH-SY5Y cells. Necrosis was found to increase in the nerve cells treated with conditioned medium, and was clearly reduced when SOD was added (P<0.05, Fig. 5), thereby indicating the neuroprotective properties of the latter.

4. Discussion

By regulating ROS levels, SOD is an enzyme found in living cells that prevents oxidative damage to the body. SOD is recognized not only for its antioxidant properties but also for its neuroprotective ability (Huang et al., 2012). Several studies have confirmed that SOD acts on immune cells, such as lymphocytes and macrophages, to induce macrophage M2 polarization (Agrahari et al., 2021; Tan et al., 2016). In this study, human neuroblastoma cells, namely SH-SY5Y, were used to evaluate the antioxidant and neuroprotective effects of SOD, and mouse macrophages, RAW 264.7, were used to evaluate the immunomodulatory ability of SOD.

To evaluate the non-cytotoxic dose of SOD, CCK-8 assay was performed; results indicated that SOD is not toxic at all concentrations in macrophages, although cell viability decreased above a certain SOD concentration in nerve cells. Therefore, the study was conducted on nerve cells at a SOD concentration of 1 μM, at which it is known to be non-cytotoxic to both cell lines. To investigate the antioxidant effect of SOD, nerve cells were treated with H_2O_2 to induce oxidative damage first, and ROS build-up was measured using a fluorescent product. Accumulation of ROS was confirmed to increase in the H_2O_2-treated group; however, it decreased significantly in the SOD-treated group than in the H_2O_2-treated group. Thus, SOD was confirmed to have antioxidant potential in SH-SY5Y cells. Concurrently, cell viability was examined. The decreased cell viability in presence of H_2O_2 was reversed when SOD was administered concurrently. As expected, SOD was proven to not only exert an

antioxidant effect that reduces ROS, but also exert a protective effect on nerve cells, as established in previous studies (Huang et al., 2012; Dohare et al., 2014).

To assess SOD's immunomodulating capacity, it was applied to mouse macrophages RAW 264.7. Macrophages were treated with LPS to induce inflammation, and immunofluorescence staining and qPCR were used to detect the inflammatory cytokines released. Immunofluorescence staining indicated that LPS-treated macrophages had abundant red staining of CD11c (linked to M1 polarization), whereas SOD-treated cells displayed green staining of CD206 (associated with M2 polarization). Quantitative real-time PCR indicated that macrophages treated with LPS generated cytokines, such as IL-1β, IL-6, and TNF-α, which are associated with M1 polarization; however, when SOD was added, levels of the cytokines were significantly reduced (P<0.001). Considering the results, SOD was concluded to have immunomodulatory potential from M1 to M2 phase in macrophages.

According to a previous study, LPS treatment of macrophages stimulates the shift of macrophages to M1 phase as well as the release of cytokines associated with this phase. Thus, conditioned medium derived from LPS treatment of macrophages was produced and applied to nerve cells. When neural cells were treated with conditioned medium, necrosis of the cells increased relative to that in the control group; however, necrosis decreased when SOD was treated along with the conditioned medium (P<0.05). Considering that conditioned medium from LPS-stimulated macrophages is enriched with inflammatory cytokines, SOD may inhibit the function of inflammatory cytokines in neural cells. Previous studies had shown that SOD inhibits the production of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, from neutrophils (Yasui and Baba, 2006). However, in this study, SOD not only

inhibited the production of macrophages but also had the potential to protect cells from inflammatory cytokines.

In this study, we confirmed the direct antioxidant effect of SOD as well as its neuroprotective effect. Through an experiment with conditioned medium, the protective effect on nerve cells, against inflammatory substances, was confirmed. Immunomodulation by SOD was demonstrated, and SOD was found to induce the polarization of macrophages from M1 to M2.

Based on this ability, SOD was proposed as a potential treatment tool for several disorders (Younus, 2018); in fact, active research is being conducted on the use of SOD in neurodegenerative diseases. Neurodegenerative disorders, such as AD, Parkinson's disease (PD), ALS, and multiple sclerosis, frequently exhibit microglia-mediated neuroinflammation (Appel et al., 2010). Neuroinflammation requires microglial activation, and in response to different microenvironmental disturbances, microglia can polarize either the M1 pro-inflammatory phenotype or the M2 anti-inflammatory phenotype (Guo et al., 2022). The balance between microglial M1/M2 polarization offers therapeutic potential against neurodegenerative disorders (Song and Suk, 2017), and the promotion of microglial polarization shift from the M1 to M2 phenotype is a promising treatment target. As confirmed in our experiment, SOD can simulate macrophage polarization into the M2 phase. SOD supplementation in mice reduces lipid peroxidation and maintains neurogenesis in the hippocampus to prevent against cognitive decline in stress-induced cells (Balendra and Singh, 2021).

However, in this study, SOD was found to be toxic to nerve cells when its concentration exceeded a certain level. Previous studies had shown that high doses of SOD in post-ischemic heart models decrease myocardial contractility and trigger

toxic reactions (Nelson et al., 1994). Consequently, dosage is of the highest concern, when using SOD as a treatment, and care must be taken against an overdose.

Experiments confirmed the antioxidant and neuroprotective effects of SOD on nerve cells, as well as its ability to regulate the immune response of macrophages. It appeared that SOD could be used as a treatment for a variety of diseases, particularly neurological diseases, based on these characteristics. However, since its effectiveness has been demonstrated at the level of nerve cells, future research is required to determine whether the same effect will be demonstrated in actual animal models. Furthermore, if the efficacy of SOD is demonstrated in animal models, the toxicity of SOD at high concentrations should be thoroughly assessed.

5. Conclusion

In this study, we demonstrated the antioxidant and neuroprotective effects of SOD in human nerve cells, besides its ability to control immunity in mouse macrophages. The characteristics suggested that SOD has potential as a treatment option for various diseases. Although SOD has been demonstrated to be effective at the level of nerve cells, additional research in animals would be required before it can be applied in a therapeutic setting. Further research is recommended to elucidate the mechanism of protection from inflammatory cytokines.

6. References

1. Agrahari G, Sah SK, Bang CH, Kim YH, Kim TY: Superoxide dismutase 3 controls the activation and differentiation of $CD4^{+}$T cells. *Frontiers in Immunology* 2021, 12, Article 628117.

2. Altobelli GG, Noorden SV, Belato A, Cimini V: Copper/zinc superoxide dismutase in human skin: Current knowledge. *Frontiers in Medicine* 2020, 7, Article 00183.

3. Appel SH, Beers DR, Henkel JS: The T cell-microglial dialogue in Parkinson's disease and amyotrophic lateral sclerosis: are we listening?. *Trends Immunology* 2010, 31, 7–17.

4. Balendra V, Singh SK: Therapeutic potential of astaxanthin and superoxide dismutase in Alzheimer's disease, *Open Biology* 2021. 11, Article 210013.

5. Che M, Wang R, Wang HY, Zheng XF: Expanding roles of superoxide dismutases in cell regulation and cancer. *Drug Discov. Today* 2016, 21, 143–149.

6. Corpas FJ, Ocana AF, Carreras A, Valderrama R, Luque F, Esteban FJ, Serrano MR, Chaki M, Pedrajas JR, Sandalio LM, Rio LA, Barroso JB: The expression of different superoxide dismutase forms is cell-type dependent in olive (Olea europaea L.) leaves. *Plant Cell Physiology* 2006, 47, 984–994.

7. Devanney NA, Stewart AN, Gensel JC: Microglia and macrophage metabolism in CNS injury and disease: The role of immunometabolism in neurodegeneration and neurotrauma. *Experimental Neurology* 2020, 329, Article 113310.

8. Dohare P, Garcia MC, Vipani A, Bowens NH, Nalwalk JW, Feustel PJ, Keller Jr. RW: The neuroprotective properties of the superoxide dismutase mimetic tempol correlate with its ability to reduce pathological glutamate release in a rodent model of stroke. *Free Radical Biology Medicine* 2015, 77, 168–182.

9. Fujii J, Homma T, Osaki T: Superoxide radicals in the execution of cell death. *Antioxidants* 2022, 11, 501, Article 501

10. Guo S, Wang H, Yin Y: Microglia polarization from M1 to M2 in neurodegenerative diseases. *Frontiers in Aging Neuroscience* 2022, 14, Article 815347.

11. He C, Carter AB: The metabolic prospective and redox regulation of macrophage polarization. *Journal of Clinical and Cellular Immunology* 2015, 6(6), Article 371.

12. He C, Ryan AJ, Murthy S, Carter AB: Accelerated development of pulmonary fibrosis via Cu,Zn-superoxide dismutase-induced alternative activation of macrophages. *The Journal of Biological Chemistry* 2013, 288, 20745–20757.

13. He L, He T, Farrar S, Ji L, Liu T, Ma X: Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cellular Physiology and Biochemistry* 2017, 44, 532–553.

14. Huang HF, Guo F, Cao YZ, Shi W, Xia Q: Neuroprotection by manganese superoxide dismutase (MnSOD) mimics: Antioxidant effect and oxidative stress regulation in acute experimental stroke. *CNS Neurosciences & Therapeutics* 2012, 18, 811–818.

15. Kim SH, Kim SH, Lee JH, Lee BH, Yoon HJ, Shin DH, Park SS, Jang SB, Park JS, Jee YK: Superoxide dismutase gene (SOD1, SOD2, and SOD3) polymorphisms and antituberculosis drug-induced Hepatitis. *Allergy Asthma Immunology Research* 2015, 7(1), 88-91.

16. Mammana S, Fagone P, Cavalli E, Basile MS, Petralia MC, Nicoletti F, Bramanti P, Mazzon E: The role of macrophages in neuroinflammatory and neurodegenerative pathways of alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis: Pathogenetic cellular effectors and potential therapeutic targets. *International Journal of Molecular Science* 2018, 19, Article 831.

17. Manni ML, Tomai LP, Norris CA, Thomas LM, Kelley EE, Salter RD, Crapo JD, Chang LL, Watkins SC, Piganelli JD, Oury TD: Extracellular superoxide dismutase in macrophages augments bacterial killing by promoting phagocytosis. *American Journal of Pathology* 2011, 178(6), 2752-2759.

18. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB: Reactive oxygen species in inflammation and tissue injury. *Antioxidant and Redox Signaling* 2014, 20, 1126–1167.

19. Munawara U, Catanzaro M, Xu W, Tan C, Hirokawa K, Bosco N, Dumoulin D, Khali A, Larbi A, Levesque S, Ramassamy C, Barron AE, Cunnane S, Beauregard PB, Bellenger JP, Rodrigues S, Desroches M, Witkowski JM, Laurent B, Frost EH, Fulop T: Hyperactivation of monocytes and macrophages in MCI patients contributes to the progression of Alzheimer's disease. *Immunity and Ageing* 2021, 18, Article 29.

20. Nelson SK, Bose SK, McCord JM: The toxicity of high-dose superoxide dismutase suggests that superoxide can both initiate and terminate lipid peroxidation in the reperfused heart. *Free Radical Biology and Medicine* 1994, 16, 195–200.

21. Nguyen NH, Tran GB, Nguyen CT: Anti-oxidative effects of superoxide dismutase 3 on inflammatory diseases. *Journal of Molecular Medicine* 2020, 98, 59–69.

22. Nojima Y, Ito K, Ono H, Nakazato T, Bono H, Yokoyama T, Sato R, Suetsugu Y, Nakamura Y, Yamamoto K, Satoh JI, Tabunoki H, Fugo H: Superoxide dismutases, SOD1 and SOD2, play a distinct role in the fat body during pupation in silkworm Bombyx mori. *Plos One* 2015, 10(2), Article 0116007.

23. Orecchioni M, Ghosheh Y, Pramod AB, Ley K: Macrophage polarization: Different gene signatures in M1(LPS+) *vs.* classically and M2(LPS-) *vs.* alternatively activated macrophages. *Frontiers in Immunology* 2019, 10, Article 1084.

24. Rosa AC, Corsi D, Cavi N, Bruni N, Dosio F: Superoxide dismutase administration: A review of proposed human uses. *Molecules* 2021, 26, Article 1844

25. Sah SK, Agrahari G, Kim TY: Insights into superoxide dismutase 3 in regulating biological and functional properties of mesenchymal stem cells. *Cell and Bioscience* 2020, 10, Article 22.

26. Song GJ, Suk K: Pharmacological modulation of functional phenotypes of microglia in neurodegenerative diseases. *Frontiers in Aging Neuroscience* 2017, 9, Article 139.

27. Tan HY, Wang N, Li S, Hong M, Wang X, Feng Y: The reactive oxygen species in macrophage polarization: Reflecting its dual role in progression and treatment of human diseases. *Oxidative Medicine and Cellular Longevity* 2016, 2016, Article 2795090.

28. Wang N, Liang H, Zen K: Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Frontiers in Immunology* 2014, 5, Article 614.

29. Yao Y, Xu XH, Jin L: Macrophage polarization in physiological and pathological pregnancy. *Frontiers in Immunology* 2019, 10, Article 792.

30. Yasui K, Baba A: Therapeutic potential of superoxide dismutase (SOD) for resolution of inflammation. *Inflammation Research* 2006, 55, 359–363.

31. Younus H: Therapeutic potentials of superoxide dismutase. *International Journal of Health Sciences* 2018, 12, 88–93.

32. Song WJ, Li Q, Ryu MO, Ahn JO, Bhang DH, Jung YC, Youn HW: TSG-6 released from intraperitoneally injected canine adipose tissue-derived mesenchymal stem cells ameliorate inflammatory bowel disease by inducing M2 macrophage switch in mice. *Stem cell research & therapy* 2018, 9(1), Article 91.

Table 1. Sequences of PCR primers for detecting gene expression of immune related factors and macrophage target genes

Species	Genes	Nucleotide sequence (5' to 3')	References
Mouse	IL-6	F: AGT TGC CTT CTT GGG ACT GA	*
		R: TCC ACG ATT TCC CAG AGA AC	
	IL-1β	F: TGG ACC TTC CAG GAT GAG GAC A	*
		R: GTT CAT CTC GGA GCC TGT AGT G	
	TNF-α	F: CCC TCA CAC TCA GAT CAT CTT CT	*
		R: GCT ACG ACG TGG GCT ACA G	
	GAPDH	F: AGT ATG TCG TGG AGT CTA CTG GTG T	Song et al., 2018
		R: AGT GAG TTG TCA TAT TTC TCG TGG T	

*Primers with asterisk in reference section were designed by own.

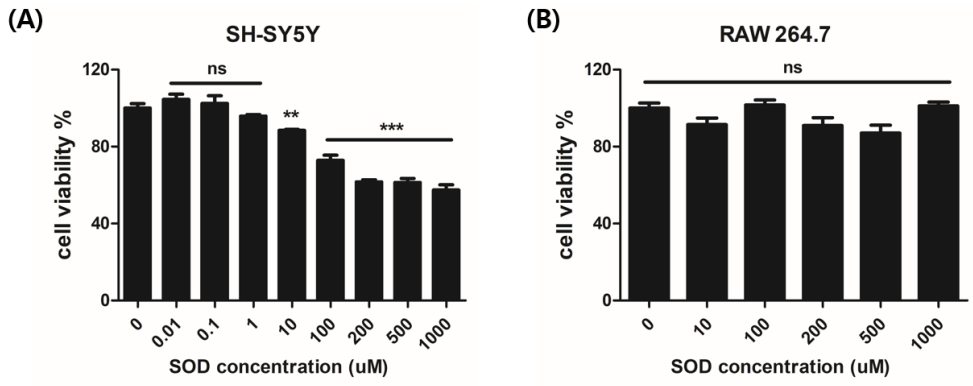

Figure 1. Cell viability assessment using the CCK-8 assay in SH-SY5Y neuronal cells and RAW 264.7 macrophage cells. (A) In SH-SY5Y neuronal cells, viability reduction was observed at SOD concentrations above 10 μM. (B) In RAW 264.7 macrophage cells, viability was not affected by up to 1,000 μM SOD. $^{**}P<0.01$, $^{***}P<0.001$. ns, not significant.

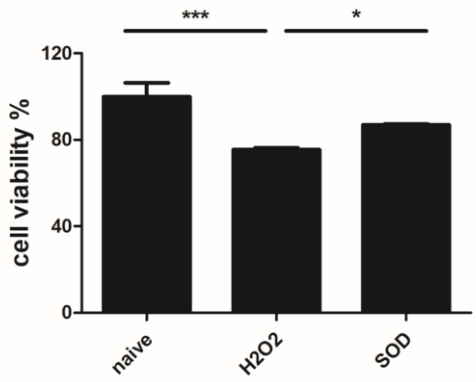

Figure 2. Effects of SOD on the viability of H_2O_2-treated SH-SY5Y. When SH-SY5Y cells were treated with H_2O_2, cell viability was reduced; however, when pre-treated with SOD, cell viability increased. $^{*}P<0.05$, $^{***}P<0.001$.

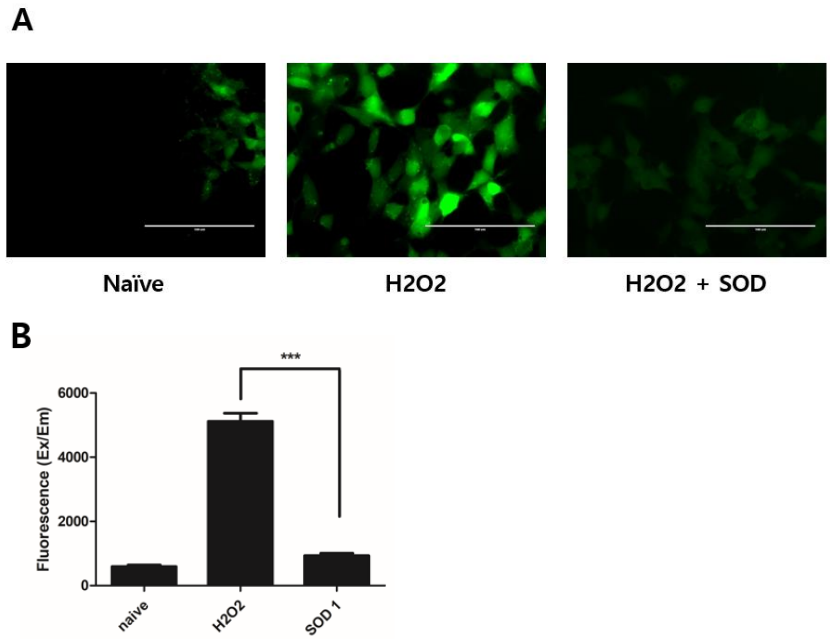

Figure 3. Effects of SOD on reducing ROS accumulation. (A) H_2O_2 treatment of SH-SY5Y enhanced fluorescence intensity, although the increase diminished upon administration of SOD. (B) H_2O_2 treatment of SH-SY5Y enhanced ROS generation, but SOD treatment decreased it. $^{***}P<0.001$.

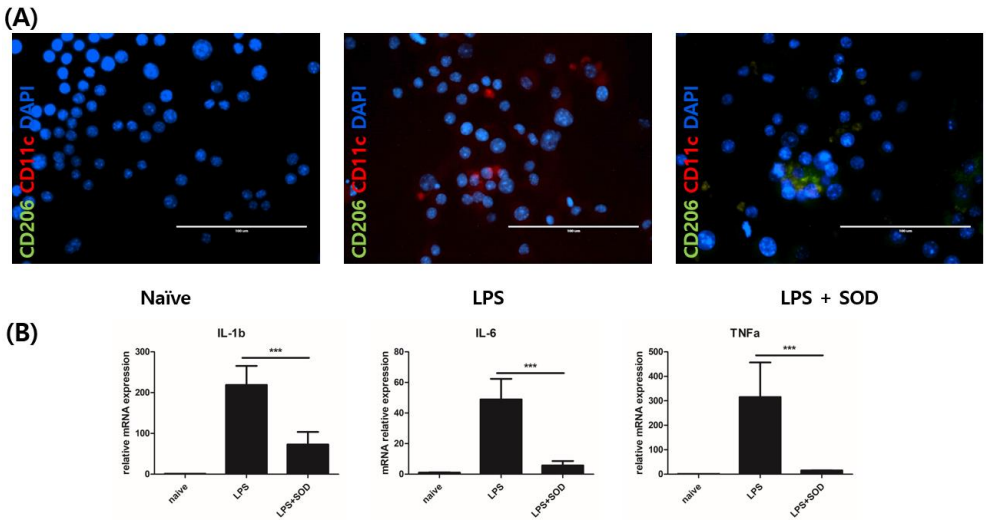

Figure 4. Changes in macrophage polarization from M1 to M2 phase upon SOD treatment. (A) Detection of CD11c (M1 marker, red) cells and CD 206 (M2 marker, green) cells using an immunofluorescence assay. The proportion of CD11c cells increased in the LPS-treated group while that of CD206 cells increased when SOD was treated with LPS. (B) mRNA expression levels of cytokines related to M1 macrophage marker. The M1 phase was induced by LPS stimulation of macrophages and reversed by SOD treatment. After stimulation with LPS, expression of IL-1β, IL-6, and TNF-α increased significantly. When LPS-stimulated macrophages were treated with SOD, cytokine expression was decreased. $^{***}P<0.001$.

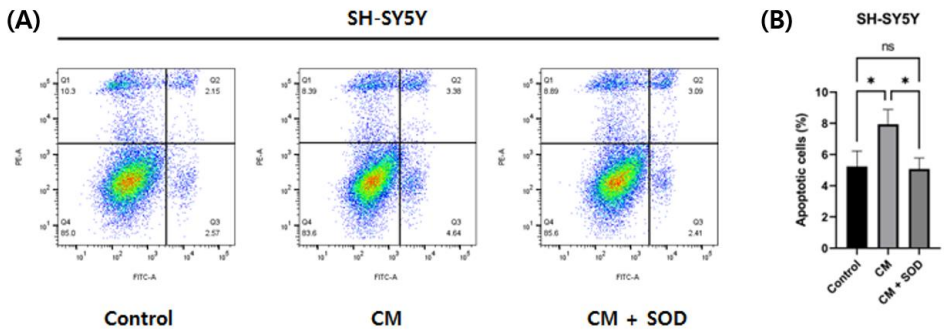

Figure 5. Reduction of neuronal necrosis in SH-SY5Y cells treated with conditioned medium upon SOD treatment. (A) The apoptotic ratio was determined using flow cytometry. (B) Quantification of data shown in (A). Neuronal necrosis increased in the group treated with conditioned medium compared to that in the control group. In contrast, neuronal necrosis decreased in the group treated with SOD compared to that in the SOD-untreated group. $^{*}P<0.05$. ns, not significant.

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국문초록

SH-SY5Y 신경모세포종 세포와 RAW 264.7 대식세포에서 초과산화물불균등화효소의 신경보호 및 면역조절 효과

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초과산화물불균등화효소(superoxide dismutase, SOD)는 활성산소(reactive oxygen species, ROS)로부터 몸을 보호하는 항산화 효소이다. 이는 항산화 효과뿐만 아니라 대식세포에서 면역조절 특성을 가지고 있다고 보고되었다. 대식세포는 선천성 면역반응의 주요 매개자이며, 염증을 유발하는 M1 대식세포와 염증을 억제하는 M2 대식세포의 형태로 나뉜다. 그리고 SOD는 대식세포의 M1에서 M2로의 분극을 유도하는

면역조절 특성을 가지고 있다고 알려졌다. 이 연구의 목적은 신경세포에서 SOD의 항산화 및 신경 보호 효과를 확인하는 것뿐만 아니라 대식세포에서 SOD의 면역 조절 능력을 평가하는 것이었다.

신경세포(SH-SY5Y)와 대식세포(RAW 264.7)에서 SOD의 세포 독성을 평가하기 위해 다양한 농도의 SOD를 배양된 세포에 처리한 후 세포 계수 키트(CCK)-8을 통해 분석을 진행하였다. 실험결과 신경세포에서는 SOD의 농도가 10μM을 넘어설 때 세포생존율이 1.13배 감소하였으며(P<0.001), 대식세포에서는 SOD의 농도에 따른 세포생존율이 차이가 확인되지 않았다. 따라서 실험은 SOD가 신경세포, 대식세포의 세포생존율에 영향을 주지 않았던 1μM의 농도에서 진행되었다. SOD의 항산화 효과를 평가하기 위해 우선 배양한 신경세포에 H_2O_2를 처리하여 산화적 손상을 유발한 후 SOD 처리 유무에 따른 활성산소종의 축적 정도 및 세포생존율의 변화를 각각 H_2DCFDA 염색법과 세포 계수 키트-8을 통해 평가하였다. SOD를 신경세포에 같이 처리한 경우 처리하지 않은 경우에 비하여 활성산소종의 축적을 5.5배 억제하였고(P<0.001) 세포 생존율을 1.15배 향상시켰다(P<0.05). 또한 대식세포에 lipopolysaccharide(LPS)를 처리하여 염증을 유발한 뒤 얻은 조건화 배양액을 신경세포에 처리하였을 때 괴사되는 정도를 annexin V 염색을 통해 평가하였고, SOD를 조건화 배양액과 같이 처리한 경우 괴사되는 정도가 1.56배 감소하였다(P<0.05). 그리고 SOD의 면역조절 능력을 평가하기 위해 실시간 중합효소 연쇄반응(RT-qPCR) 실험을 진행하였

고, LPS를 처리하여 염증을 유발한 대식세포에 SOD를 처리하는 경우 M1 분극과 관련된 IL-1β, IL-6, TNF-α와 같은 사이토카인들이 감소하는 것을 확인하였다(IL-1β, 3배, P<0.001; IL-6, 8.57배, P<0.001; TNF-α, 20.15배, P <0.001). 이 결과를 통해 대식세포에서 SOD가 M1에서 M2로의 전이를 유도하는 것을 확인하였다.

이 연구를 통하여 SOD의 항산화효과 뿐만 아니라 염증상황에서 신경세포를 보호하는 효과를 확인할 수 있었고, 대식세포에 적용했을 때 염증성 M1에서 항염증성 M2로의 전이를 유도하는 면역조절 능력도 확인할 수 있었다. 비록 세포 수준에서 확인한 효과로 더 많은 후속 연구가 필요할 것으로 보이지만, 본 연구의 결과는 수의임상에서 원인불명의 비감염성 뇌수막염을 비롯한 다양한 염증성 신경계 질환의 치료제로서 SOD가 적용될 수 있는 가능성을 보여주었다.

주요어: 초과산화물불균등화효소, 항산화제, 면역조절, 신경보호, 대식세포

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