의학박사 학위논문

TNF-α 에 의해 유발된 청각세포 손상에서 α-Lipoic acid 의 세포 보호 효과

Protective effect of α-Lipoic acid on TNF-α induced ototoxicity

울산대학교 대학원

의학과

심명주

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이 논문을 의학박사 학위논문으로 제출함

2024 년 8 월

울산대학교 대학원

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심명주

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2024 년 8 월

영문요약

As our society becomes more industrialized and aging, the number of people experiencing hearing loss is increasing globally. This can be attributed to factors such as increased noise exposure, age-related deterioration, and the growing usage of ototoxic drugs like anti-cancer drugs. It is a matter of concern because a higher population of individuals with hearing impairments brings significant economic and social costs.

However, there has been limited availability of possible treatment and prevention for hearing loss, such as avoidance of ototoxic drugs or noise, hearing rehabilitation through hearing aids, and cochlear implantation in profound hearing loss.

Therefore, extensive research has been conducted to understand the mechanisms behind hearing loss to prevent and treat it effectively.

Reactive oxygen species (ROS) and TNF-α related apoptosis of inner ear cells is a common mechanism of sensorineural hearing loss of various causes.

α-Lipoic acid (ALA) inhibits cell membrane injury caused by reactive oxygen species (ROS) as a powerful antioxidant. Thus, previous studies reported that it has a protective effect on various types of hearing loss, including ototoxic, age-related, noise-induced, and sudden sensorineural hearing losses. Also, TNF-α, one of the major inflammatory mediators, promotes the production of ROS and causes cellular apoptosis of inner ear cells.

ALA has antioxidant action and a cytoprotective mechanism that prevents the accumulation of inflammatory cells. So, studies on the mechanism that inhibits the over-expression of ROS and its relation to TNF-α can play a positive role in future sensorineural hearing loss prevention and treatment strategies.

In this study, the generation of ROS, genes, and proteins involved in the Nrf2/KEAP1 signaling pathway, and glutathione (GSH) and glutathione disulfide (GSSG) were quantified in the TNF-α treated and ALA pre-treated House-Ear Institute-Organ of Corti 1 (HEI-OC1) cells. Using the results, we tried to measure the antioxidative action through the Nrf2/KEAP1 signaling pathway and intracellular redox signal regulation mechanism of ALA and to figure out the possibility of application to the prevention and treatment of hearing loss.

The experimental concentration of TNF-α and ALA was determined by quantification of the cell viability and ROS production in the HEI-OC1 cells treated with different concentrations of TNF-α and ALA. Next, cytoprotective effect and ROS reduction were measured and compared within the control, TNF-α alone, and the ALA preconditioned group. In addition, Western blot was performed in three fractionated cell groups in the nucleus and cytoplasm, the Nrf2 in the nucleus and KEAP1 in the cytoplasm, SOD1, GCLC, HMOX1, and NQO1 were quantified using RT-qPCR. Then, the expressed proteins SOD1, γ-GCSc, NQO1, and HO-1 were measured. Furthermore, the redox potential of ALA was also confirmed by measuring glutathione (GSH), glutathione disulfide (GSSG), and the GSH/GSSG ratio in the three groups.

As a result, cell viability was about 17% higher, and the production of ROS was about 64% lower in the ALA preconditioning group than in the TNF-α alone group. And the Nrf2/KEAP1 signaling-related genes SOD1, GCLC, NQO1, HMOX1, Proteins SOD1, γ-GCSc, NQO1, HO-1, and nuclear Nrf2 expressed all significantly higher in ALA preconditioning group. KEAP1, an Nrf2 inhibitory protein, was lower than 1/3 of the TNF-α-only treated group in the ALA preconditioning group. In addition, the intracellular glutathione (GSH) was about 4 times higher, the glutathione disulfide (GSSG) was about 30% lower, and the GSH/GSSG ratio was about 6 times higher in the ALA preconditioning group than in the TNF-α alone group.

Based on these findings, it has been suggested that ALA has a cytoprotective effect. It inhibits the generation and accumulation of ROS, which are responsible for TNF-α-induced oxidative stress. Additionally, it activates the Nrf2/KEAP 1 signaling system. The increase in intracellular redox potential could be a mechanism that contributes to hair cell preservation of ALA pre-treatment.

However, because the above results are in vitro data on the HEI-OC1, they could be primary data for in vivo experiments. Therefore, it may be helpful to prepare further research for the possibility that ALA administration can be applied as a preventive and therapeutic strategy for hearing loss.

차례

그림목차

Introduction

Increasing hearing loss is a global concern [4]. The World Health Organization recently released its World Hearing Report in 2021, which revealed that nearly 2.5 billion people around the world are projected to suffer from some degree of hearing loss. The report further noted that around 30% of these individuals will require rehabilitation to manage their condition effectively. Such a high prevalence of hearing loss is likely to result in significant financial losses due to the exclusion of individuals with hearing loss from education, employment, and other forms of communication [5, 6]. Unfortunately, especially sensorineural hearing losses caused by inner ear damage are difficult to manage because damaged inner ear structures cannot be restored or regenerated to their previous state due to neuronal characteristics and complexities[7]. Currently, avoidance of noise exposure or ototoxic drugs is the strategy for prevention. Hearing aids and cochlear implants are possible treatment options for patients with sensorineural hearing loss.

So, preventive strategies could become more critical as the limited therapeutic approaches for sensorineural hearing loss.

Sensorineural hearing loss can have various causes, including genetic, environmental, and age-related changes. However, studies have shown that reactive oxygen species (ROS) overexpression is commonly observed in sensorineural hearing loss [8-10]. ROS is a toxic cellular metabolite that can induce cellular apoptosis through intrinsic mitochondrial cell death and the extrinsic cell death receptor pathway (Figure 1)[2, 11].

ROS disturbs the balance of mitochondrial pro- and anti-apoptotic factors, such as Bcl2 family proteins, which can lead to apoptosis. It breaks the integrity of the outer and inner mitochondrial membranes (OMM and IMM), releasing mitochondrial apoptogenic factors that induce cellular apoptosis in caspase-dependent or independent manners [12, 13]. Different stimuli can activate signaling molecules via cell surface receptors in mitochondria-independent mechanisms [11].

Additionally, ROS-mediated peroxidized lipid products can lead to apoptosis of the hair cells and reduce blood flow in the cochlea [14]. Furthermore, ischemia and reperfusion promote further ROS generation by taxing mitochondria and increasing ROS leakage [15].

Increased ROS can cause various sensorineural hearing losses, such as noise-induced hearing loss[16], age-related hearing loss[17], and ototoxic hearing loss[18, 19] by activating cellular apoptosis.

The excess ROS in the cochlea can also trigger the release of pro-inflammatory cytokines, which can cause further damage [20]. Recent studies have reported that ROS is involved in damaging the cochlear ribbon synapse, leading to sensorineural hearing loss without hair cell loss [21, 22].

Another common feature observed in sensorineural hearing loss is increased expression of tumor necrosis factor-alpha (TNF-α), inflammatory mediators involved in cell proliferation, differentiation, and apoptosis[23]. TNF-α plays a role in propagating inflammation by activating and recruiting immune cells through its receptor TNF receptor 1 (TNFR1) (Figure 2). TNF-α is associated with various inflammatory diseases [24]. When TNF-α binds to TNFR1, assembles a primary signaling complex (complex I) on the cell membrane, which leads to the activation of survival genes. Subsequently, a secondary cytoplasmic complex (complex II) is formed that mediates cell death [25]. NF-κB, as the survival protein, regulates cell survival by promoting the transcription of numerous anti-apoptotic genes.

When TNFR1 signaling to NF-κB is compromised, it can be re-directed to initiate cell death. Caspase-3 is one of the apoptotic caspases. Generally, proteases of the caspase family have a pro-apoptotic role by inhibiting NF-κB activation[26]. TNF-α-induced sensorineural hearing loss is described as vibration, noise-induced, followed by bacterial meningitis, autoimmune, and cisplatin-induced hearing loss [27-31]. Also, TNF-α has been reported to stimulate ROS production by activating the c-jun NH2-terminal kinases(JNK) mitogen-activated protein kinase (MAPK) signaling pathway [25, 32, 33]. TNF-α initiates inflammation by recruiting leukocytes in infected tissue or injured site[34]. Thus, elevated TNF-α is usually measured in chronic immune-mediated diseases such as Crohn's disease[35], hematologic malignancies[36], and rheumatoid and psoriatic arthritis[36, 37].

As it does in other diseases, TNF-α causes an increase in inflammatory cell infiltration in the cochlea. Cochlear inflammation does not induce hair cell loss but elevates TNF-α production by compromising microvascular circulation [38]. In studies related to hearing loss, after noise exposure, fibroblasts in the spiral ligaments, outer hair cells, and supporting cells excreted TNF-

α, which interacts with a TNFR1, inducing ROS accumulation and activating the caspase-dependent apoptotic signaling pathway in the spiral ligaments[39].

Additionally, TNF-α, apart from being pro-inflammatory, plays a crucial role in the development of sensorineural hearing loss. It reduces blood flow in the cochlea and actively constricts the stria vascularis, leading to inadequate perfusion[40]. This disruption in perfusion causes an imbalance in the electrochemical composition of endolymph, increasing hearing thresholds [41]. In a recent study, TNF-α perfusion within the cochlea caused cochlear ischemia, leading to synaptic degeneration at the IHC and peripheral nerve junction, and reduced compound action potential[42].

Therefore, there have been studies investigating the possibilities of various antioxidants, such as All-trans retinoic acid (ATRA)[43], N-acetylcysteine[44], vitamin E[45], and glutathione[46], and an anti-TNF-α agent are used to protect inner hair cells in hearing loss of different causes.

The studies using TNF-α antagonists, blockage of the TNF-α pathway as a treatment for hearing loss, such as etanercept, a fusion protein that competitively binds the tumor necrosis factor receptors (TNFR1) have been shown to restore cochlear blood flow and preserves ABR threshold in animal models [47, 48]. In several human studies, TNF-α antagonists such as etanercept[49], infliximab[50], golimumab[51], and adalimumab[52] have been used for the treatment of SNHL, reporting hearing improvement or attenuating hearing loss in some patients[53]. Furthermore, in a study in rats with noise-induced hearing, TNF-α blockade by silencing the TNF-α gene preserves the auditory brain stem response (ABR) threshold and amplitude of waves [54].

In particular, there have been and are still in progress studies using various antioxidants as a preventive strategy against cisplatin cytotoxicity[46, 55, 56]; among these antioxidants, have been reported superior or promising cytoprotective effects of the α-Lipoic acid (ALA)[57].

α-Lipoic acid (ALA), also known as thiotic acid or 1,2-diothiolane-3-pentanoic acid, is an antioxidant that plays an essential role in mitochondrial activities [58]. α -Lipoic acid (ALA) is a crucial nutrient that has numerous health benefits. Also, it reacts with ROS and protects cell membranes. It can be synthesized in the animal's body, but the quantities of the ALA are not

enough to meet the requirements of the cells [1]. Its effects can be observed after increasing its intake through one's diet (600-1800 mg/day). Thus, most of it is obtained from diet or supplements [59]. It can be found in plants such as tomatoes and broccoli as well as in the liver, kidney, and heart of animals [60].

ALA has two chiral isomers, called S and R enantiomers(Figure 3). R isomer is a natural form, and S isomer is a chemically processed form. Most of the R enantiomers are from foods, while synthetic supplements consist of R and S enantiomers [61]. The most distinctive difference from other antioxidants is that ALA can react with fat- and water-soluble substances. Thus, it is called the universal antioxidant that can neutralize intra- and extracellular ROS [62].

A reduced form of ALA is called dihydrolipoic acid (DHLA). ALA, the oxidized form, can inactivate free radicals, and DHLA interacts with ROS[63]. It could be a scavenger of various ROS, and DHLA could regenerate other endogenous antioxidants such as vitamins C and E[64].

In addition, ALA and DHLA bind many metal ions, such as Cu 2+, Zn2+, Fe3+, Hg2+, and Pb2+[65]. Therefore, this metal chelation could modulate redox-active transitional metals without metal depletion[66].

ALA also increases intracellular antioxidants, such as vitamin C[67], and glutathione (GSH)[68] by improving the uptake and synthesis of endogenous antioxidant or antioxidant enzymes.

Indeed, ALA increases Nrf2-dependent transcription as a pro-oxidant and activates protein kinase C (PKC)[69], Erk1 and 2[70], PI3 kinase[71], Akt[72], and MAPK[71], which affects the intracellular redox state as kinases and phosphatases. In addition, ALA acts on the vascular system through endothelium-dependent nitric oxide (NO)-mediated vasodilation by activating endothelial nitric oxide synthase (eNOS)[73].

Some studies have reported that ALA can act as an anti-inflammatory agent. Because oxidative stress is essential in chronic inflammation by activating NF-κB, transcription factors induce the expression of inflammation and cell migration genes. ALA, as an antioxidant, could inhibit NF-κB. As a result, downstream proteins of NF-κB, including vascular cell adhesion molecule-1 (VCAM-1)[74], intercellular adhesion molecule-1 (ICAM-1)[74], and metalloproteinase-9[75], were reduced.

Studies on the cytoprotective effect of ALA in inner hair cells include cisplatin-induced ototoxicity; ALA reduces oxidative injury in HEI-OC1 cells and the organ of Corti by activating

the Nrf2/HO-1 signaling pathway[76]. In the aging mice model, the administration of ALA preserved outer hair cells, stria vascularis, and spiral ganglion neurons in the cochlea[77]. ALA showed hearing preservation via down-regulation of p28 and p-JNK apoptotic pathways in kanamycin-induced ototoxicity mice[78]. In a diabetic zebra fish, ALA protected hair cell loss from hyperglycemia-induced damage[79].

The Nrf2 is a crucial antioxidant element as one of the essential transcription factors [80]. Its activation is primarily regulated by KEAP1 (kelch-like ECH-associated protein 1) by ubiquitination and proteasome degradation[3] (Figure 4.). Kelch-like ECH-associated protein (KEAP1), an adaptor protein for cullin 3 (CUL 3), regulates the degradation of Nrf2 by binding to Nrf2 with CUL 3, an E3 ubiquitin ligase [81]. Nrf2/KEAP1-CUL 3 complex is a target for ubiquitination and proteasome-dependent degradation [82]. The KEAP1-NRF2 system is a major regulatory machine involved in responding to oxidative and electrophilic stresses. When KEAP1 senses ROS and toxic chemicals, its reactive cysteine residues are modified, which attenuates its ubiquitinase activity, resulting in NRF2 stabilization. Stabilized NRF2 then enters the nucleus and activates the transcription of cytoprotective genes[3]. Without oxidative stress, the Nrf2/KEAP1-CUL 3 complex exists in the cytoplasm and degrades by ubiquitination. However, when intracellular ROS was increased, Nrf2 was released from KEAP1 and entered the nucleus. Then intranuclear Nrf2 transactivates antioxidant-response element (ARE)-dependent genes by binding to an ARE in the genes[83]. Nrf2 target genes are encoding factors for the glutathione (GSH) synthesis (GCLC and GCLM), expression of antioxidant-generating enzymes (HO-1, glutathione reductase, thioredoxin reductase 1, glutathione peroxidase 2, thioredoxin, peroxiredoxin, sulfiredoxin, glutaredoxin, and ferritin light and heavy chain), xenobiotic-metabolizing enzymes (NQO1, GSTa1, GSTa3, aldehyde dehydrogenase, and carbonyl reductase), and NADPH generation (G6PDH, 6PGDH, transaldolase, transketolase, and malic enzyme) [3, 84-89].

GSH, also one of the essential redox determinants, is a critical detoxifier of xenobiotics and modulates cellular immunity, apoptosis, proliferation, and fibrogenesis [90]. GSH is primarily found in its reduced form, while its oxidized form is known as GSSG. The ratio of GSH to GSSG is a reliable indicator of the redox status of cells. GSH is part of an antioxidant network

that includes molecules like superoxide dismutase and catalase. Together, they work to neutralize the damage caused by ROS such as superoxide, hydrogen peroxide, or hydroxyl radicals [91]. The Redox cycle of GSH begins with the oxidation of GSH to GSSG by GSH peroxidase-catalyzed reactions in the peroxisome. Then GSSG reductase reduces GSSG to GSH at the expense of NADPH(Figure 5.)[92]. The first step of GSH synthesis is forming γ-glutamyl cysteine from glutamate and cysteine by catalyzing GCL. The next step is adding glycine to γ-glutamyl cysteine by GSH synthase to form GSH, γ-glutamyl cysteinyl glycine[90]. The otoprotective effect of GSH is well-defined, especially in noise-induced hearing loss [93-95]. There are also studies describing its role in ototoxic and age-related hearing loss [96-98].

The House Ear Institute-Organ of Corti (HEI-OC1) is the most widely used mouse auditory cell line in the hearing research field[99]. Until the HEI-OC1 cell was developed, experiments on outer hair cells had been complex. Because of the location of the inaccessible organ of the Corti, the small number of outer hair cells (the most commonly used research animal, the mouse, has about up to 4000 OHCs), and the structural vulnerability of the cells. In the case of non-specific systems, including Chinese Hamster Ovary (CHO), HeLa, TSA201, HEK293, and human embryonic kidney cells, which were widely used in other in vitro experiments, were challenging to have the characteristics of outer hair cells [59, 99, 100]. So, their application in hearing research has been limited. The HEI-OC1 cells were derived from an Immortomouse (H-2Kb-tsA58 transgenic mouse). In comparative studies between Mouse OHCs and Immortomouse-derived cell lines, the HEI-OC1 cells were more representative in sensitivity and expression of auditory sensory cell-specific markers than other Immortomouse-derived cochlear cell lines [101, 102]. Originally, it developed as an in vitro screening system for ototoxic drugs [96]. However, it is now widely used in research to study cytoprotection, substances-induced apoptotic pathways, genetic effects of drugs, and molecular changes during endogenous or exogenous stress, including hypoxia or oxidative damage[99].

Although the therapeutic effect of the ALA in noise-induced hearing loss and cisplatin-induced ototoxicity is relatively well verified [76, 103]. However, for application as a therapeutic and preventive strategy in general areas of sensorineural hearing loss, such as age-related hearing loss and sudden hearing loss, explanations of the applicability and mechanism of the ALA are

needed. Furthermore, the relationship between ROS and TNF-α, common mechanisms found in sensorineural hearing loss of various causes, is still not fully elucidated.

Thus, in this study, we measured intracellular ROS accumulation and cell viability of House Ear Institute-Organ of Corti (HEI-OC1) cells treated with TNF-α. We investigated the effect of ALA on these treated cells, as well as the relationship between TNF-α and ROS generation. Our study aims to determine the safe dose of ALA at the cellular level and to evaluate the cytoprotective effects of ALA against oxidative stress. We also aim to investigate the activation of the Nrf2/KEAP1 signaling pathway and the enhancement of intracellular redox potential in the HEI-OC1 cell line.

The study compared the expression of SOD1, GCLC, NQO1, and HMOX-1 genes between a group treated with only TNF-α and a group preconditioned with ALA. The SOD1 gene encodes superoxide dismutase 1 (SOD1), an enzyme that converts superoxide to hydrogen peroxide. As an antioxidant, SOD scavenges superoxide anions [104]. The GCLC gene encodes the catalytic subunit of glutamate cysteine ligase (GCL). GCL is a rate-limiting enzyme of GSH biosynthesis, composed of catalytic (GCLC) and modifier (GCLM) subunits[90]. Quinone oxidoreductase 1 (NQO1) is a cytosolic flavoprotein that reduces quinones into hydroquinones as an essential detoxification mechanism. Hydroquinone generated by NQO1 has potential roles in redox processes[105]. HMOX1 is a hemeoxygenase 1 (HO-1) coding gene. HO-1 degrades heme into antioxidant, anti-inflammatory, and pro-angiogenic products, including iron ions, carbon monoxide, and biliverdin, the precursor of bilirubin, by cooperating with NADPH cytochrome P450[106].

Glutathione (GSH), glutathione disulfide (GSSG), and the ratio of GSH/GSSG were also measured and compared to quantify the intracellular redox potential in the HEI-OC1 cell.

Material & Methods

Cell culture and treatment

Immortomouse™-derived HEI-OC1 cells were provided by Dr. Federico Kalinec (Dept. of Cell and Molecular Biology, House Ear Institute, Los Angeles, CA, USA)[20]. HEI-OC1 cells were cultured under permissive conditions (33°C, 10% CO2) in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA). HEI-OC1 cells were treated with different concentrations of Tumor necrosis factor-α (TNF-α) for 24 hours to evaluate dose-dependent TNF-α cytotoxicity. In α-Lipoic acid (ALA) administration experiments, HEI-OC1 cells were incubated with varying concentrations of ALA for 1 and 24 hours. For measuring the ALA's preconditioning effect, HEI-OC1 cells were exposed to 1mM ALA and incubated for 1 hour. Then ALA containing culture medium was discarded and set for 24 hours with 10ng/ml of TNF-α-containing culture media.

Recombinant TNF-α was purchased from BioLegend (San Diego, CA, USA), and α-Lipoic acid was from Sigma-Aldrich (St. Louis, MO, USA)

Cell viability

To examine cell viability using a D-Plus™ CCK cell viability assay kit from Dongin LS (Seoul, Korea) following the manufacturer's protocols. In brief, HEI-OC1 cells were seeded at a density of 5 x 103 cells/well and incubated for 24 hours in 96-well plates. After serum starvation for 3 hours, cells were treated with TNF-α or ALA as described above. The optical density (OD) values were measured at 450 nm using a microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA).

ROS assay

The intracellular Reactive Oxygen Species (ROS) level was detected using a fluorescent dye from Invitrogen (CM-H2DCFDA, Waltham, MA, USA). After treatment with TNF- α only or ALA preconditioning, cells were washed twice with Hank's balanced salt solution (HBSS) and

incubated with 5μM CM-H2DCFDA for 20 minutes at 33°C in the dark. Fluorescence in the cell washed again with HBSS was then measured using a Perkin-Elmer VICTOR 3 luminescence spectrometer (Perkin Elmer, Waltham, MA, USA) at excitation and emission wavelengths of 485nm and 535nm each.

Nucleus/Cytoplasm Fractionation

HEI-OC1 cells were cultured for 24 hours and then treated with 10 ng/ml TNF-α for 24 hours with or without pretreatment with 1 mM ALA for 1h. Nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (CER) kits from Thermo Fisher Scientific (Waltham, MA, USA). In brief, cells in 100 mm dishes were washed with ice-cold PBS and were lysed with ice-cold CER I and CER II lysis buffer. Furthermore, the cells were centrifuged at 13,000 rpm for 5 minutes at 4°C to give rise to nuclei (pellet fraction) and cytosol (supernatant fraction). Next, the nuclei pellets were incubated with ice-cold NER buffer for 40 minutes and centrifuged at 13,000 rpm for 10 minutes at 4°C to give nuclei supernatant fraction.

Western Blot Analysis

The cells were washed with PBS, lysed with RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail, and centrifuged at 12,000 rpm for 25 minutes. Then, quantitated the proteins using the BCA Protein Assay kit from Thermo Fisher Scientific (Waltham, MA, USA). The quantitated proteins (20 μg) were fractionated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (GE Healthcare Biosciences, Uppsala, Sweden), and blocked with 5% skim milk/TBS-T for 1 hour. The blocked membranes were incubated with primary antibodies at 4°C overnight, followed by washing three times with TBS-T and incubating with secondary antibodies for 1 hour at room temperature. After washing three times with TBS-T, membranes were treated with enhanced chemiluminescence detection reagents from Dongin LS (Seoul, Korea), and protein bands were detected using Image-Quant LAS 500 biomolecular imager (GE Healthcare Biosciences, Boston, MA, USA).

Reverse Transcription quantitative polymerase chain reaction (RT-qPCR)

HEI-OC1 cell's total RNA was extracted using RNeasy Mini Kits from QIAGEN (Hilden, Germany). The cDNA was synthesized from 100ng of total RNA using an AMPIGENE cDNA Synthesis kit from ENZO (Farmingdale, NY, USA). After then, qPCR was performed using a TB Green Premix Ex Taq II from TaKaRa Bio, Inc (Kusatsu, Japan) and specific primers. The PCR conditions were as follows: 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 15 seconds. The GAPDH was used as a reference gene, and primer sequences were as follows:

SOD1[1] 5'-GGTGAACCAGTTGTGTTGTCAG-3' (forward)
SOD1 5'-ATGAGGTCCTGCACTGGTACAG-3' (reverse),
GCLC[2] 5'-ACACCTGGATGATGCCAACGAG-3'(forward),
GCLC 5'-CCTCCATTGGTCGGAACTCTAC-3' (reverse),
NQO1[3] 5'-GCCGAACACAAGAAGCTGGAAG-3' (forward),
NQO1 5'-GGCAAATCCTGCTACGAGCACT-3' (reverse),
HMOX1[4] 5'-CACTCTGGAGATGACACCTGAG-3' (forward),
HMOX1 5'-GTGTTCCTCTGTCAGCATCACC-3'(reverse),
GAPDH[5] 5'-TGCACCACCAACTGCTTAGC-3' (forward)
GAPDH 5'-GGATGCAGGGATGATGTTCT-3' (reverse).

Quantification of GSH, GSSG, and GSH/GSSG ratio

To quantify the GSH and GSSG and determine the GSH/GSSG ratio in the HEI-OC1 cells using a GSH/GSSG Ratio Detection Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Briefly, cultured HEI-OC1 cells treated with TNF-α with or without a

[1] SOD1, Superoxide Dismutase 1
[2] GCLC, Glutamate-Cysteine ligase catalytic subunit
[3] NQO1, NAD (P)H quinine oxidoreductase 1
[4] HMOX1, hemeoxygenase 1 gene
[5] GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

1mM of ALA were washed with cold PBS, lysed in 1X mammalian lysis buffer (0.5% NP-40/PBS), and centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was collected in the Eppendorf tube. Next, ice-cold trichloroacetic acid was added for deproteinization and cultured for 10 minutes in ice. Then centrifuged at 13,000 rpm for 15 minutes at 4°C. Sodium bicarbonate was added to neutralize the collected supernatant to pH 4—6. The supernatant reacted with the agent (GSH assay mixture or Total Glutathione assay mixture) and incubated for 15minutes in the dark and then measured fluorescence using Perkin-Elmer VICTOR 3 luminescence spectrometer (Perkin-Elmer, Waltham, MA, USA).

Statistical Analysis

Experimental data were represented as the means ± standard error (SE). Statistical analysis was performed with the Student's t-test or one-way analysis of variance (ANOVA) using Sigmaplot software (version 12.0; Jandel Scientific., San Rafael, CA, USA). Differences were considered statistically significant at a value of $p < 0.05$.

Results

α-Lipoic acid reduces ROS generation and has a cytoprotective effect from TNF-α-induced oxidative stress

Cell viability and ROS generations were observed 24 hours after being treated with different concentrations of TNF-α and ALA. TNF-α and ALA reduced cell viability and increased ROS generation significantly dose-dependent. In the TNF-α treated cells, cytotoxicity and ROS production increased markedly at 5 ng/ml or above (Figure 6A, 6B). In experiments of the ALA administration, at 1.5 mM or above, ALA could meaningfully affect cell viability and intracellular ROS (Figure 7A, 7B). So, in an experiment to measure the effect of ALA preconditioning in TNF-α treated cells, we set concentration to 10 ng/ml of TNF-α and 1 mM of ALA, respectively.

The cells pretreated with 1 mM ALA for 1 hour and followed 24-hour-cultured in 10 ng/ml TNF-α-containing media showed significantly better cell viability. ALA pretreated cells showed about 69.4±2.2% of cell viability, compared with 10 ng/ml TNF-α-only treated cells, which had 52.4±1.2% of cell viability ($p<0.05$) (Figure 8A). In addition, quantified ROS using a fluorescence dye, CM-H2DCFDA, was significantly reduced in the 1 mM ALA preconditioned cells compared with 10 ng/ml TNF-α alone treated cells; 2.01±0.13 in the TNF-α alone, 1.29±0.09 in the ALA preconditioned cell (Figure 8B).

α-Lipoic acid activated the Nrf2/KEAP1 signaling pathway

To determine ALA's ability to activate the Nrf2 antioxidant signaling pathway, the cytosolic, nuclear Nrf2, and cytosolic KEAP1 expression level was assessed using Western blot analysis. Cytosolic Nrf2 and KEAP1 expression was significantly reduced in the ALA preconditioning group compared to the control and TNF-α-only group; Cytosolic Nrf2 was 0.97±0.04 in the TNF-α alone, 0.72±0.05 in the ALA preconditioned cell; KEAP1 was 2.11±0.35 in the TNF-α alone, 0.60±0.09 in the ALA preconditioned cell (Figure 9A, 9B, 9C). Conversely, the nuclear Nrf2 markedly increased in the ALA preconditioning HEI-OC1 cells; 0.93±0.14 in the TNF-α

alone, 1.96±0.31 in the ALA preconditioned cell (Figure 9A, 9D). There were no significant changes in cytosolic GAPDH and nuclear Lamin B (Figure 9A).

mRNA expression of SOD1, GCLC, NQO1, and HMOX1 (Figure 10) and the protein levels of SOD1, γ-GCSc[6], NQO1, and HO-1[7] (Figure 10) showed significantly high in the ALA preconditioning HEI-OC1 cells, compared with TNF-α-only treated cells; mRNA expression of SOD1 was 1.01±0.10 in the TNF-α alone, 1.55±0.20 in the ALA preconditioned cell (Figure 10A), GCLC was 0.98±0.07 in the TNF-α alone, 1.70±0.12 in the ALA preconditioned cell (Figure 10B), NQO1 was 1.19±0.06 in the TNF-α alone, 3.25±0.03 in the ALA preconditioned cell (Figure 10C), and HMOX1 was 1.16±0.07 in the TNF-α alone, 1.71±0.18 in the ALA preconditioned cell (Figure 10D). The protein level of SOD1 was 1.05±0.07 in the TNF-α alone, 1.73±0.05 in the ALA preconditioned cell (Figure 10E,10F), γ-GCSc was 1.14±0.07 in the TNF-α alone, 2.59±0.47 in the ALA preconditioned cell (Figure 10E, 10G), NQO1 was 0.97±0.08 in the TNF-α alone, 1.69±0.15 in the ALA preconditioned cell (Figure 10E, 10H), and HO-1 was 1.03±0.05 in the TNF-α alone, 2.29±0.44 in the ALA preconditioned cell (Figure 10E, 10I).

α-Lipoic acid preconditioning up-regulates the intracellular redox potential

In the RT-qPCR and Western blot analysis, mRNA of GCLC and protein level of γ-GCSc were markedly higher in the ALA preconditioning HEI-OC1 cells (Figure 10B, 11B, 11E). Thus, the GSH level and the GSH/GSSG ratio were significantly higher in the ALA preconditioning HEI-OC1 cells than in control or TNF-α-only treated cells; the level of GSH was 14.7±0.33 μM in the untreated control, 5.60±0.50 μM in the TNF-α alone, and 21.97±0.51 μM in the ALA preconditioned cell (Figure 11A). GSSG was 0.92±0.16 μM in the untreated control, 2.34±0.21 μM in the TNF-α alone, and 1.65±0.09 μM in the ALA preconditioned cell (Figure 11B).

Glutathione disulfide (GSSG) is an oxidized form of two GSH molecules. So, the higher ratio of GSH to GSSG indicates intracellular redox potential. ALA preconditioning HEI-OC1 cells showed a significantly lower level of GSSG and an increased GSH to GSSG ratio; GSSG was

[6] γ-GCSc, γ-glutamylcysteinesynthetase heavy subunit
[7] HO-1, heme oxygenase 1

0.92±0.16 μM in the untreated control, 2.34±0.21 μM in the TNF-α alone, and 1.65±0.09 μM in the ALA preconditioned cell (Figure 12B), and GSH/GSSG ratio was 16.97±2.71 in the untreated control, 2.44±0.36 1in the TNF-α alone, and 13.41±0.61 in the ALA preconditioned cell (Figure 11C).

Discussion

Although numerous studies have been conducted over several decades, the exact mechanisms that cause sensorineural Hearing Loss (SNHL) are still not entirely understood. However, research has suggested that mitochondrial dysfunction and oxidative stress play a crucial role in various types of SNHL, including noise-induced hearing loss, age-related hearing loss, and ototoxic hearing loss [18, 107].

The mammalian mitochondria produce reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals[108]. It has a crucial role in apoptosis and necrotic cell death in developing several hearing loss pathologies[11]. In various sensorineural hearing loss, including age-related hearing loss, ototoxic drug-induced hearing loss, and noise-induced hearing loss, ROS-activated apoptotic cell death has been a significant cause[18]. In addition, the cochlea produced pro-inflammatory cytokines that could induce the migration of inflammatory cells from circulation to the cochlea [109]. ROS also activates mitogen-activated protein kinase (MAPK), accumulating pro-inflammatory cytokines in the cochlea and the apoptosis of the outer hair cells [110].

On the other hand, TNF-α, the critical regulator of inflammatory cytokine cascade, is generated by spiral ligament fibrocytes, outer hair cells (OHC), and supporting cells in the organ of Corti[31]. It promotes the infiltration of inflammatory cells and finally causes cochlear damage[111].

Additionally, TNF-α increases phosphorylated c-Jun N terminal kinase expression, cleaves caspase-3 forms an apoptotic factor cluster, and translocates the apoptotic marker endonuclease G to the nuclei of hair cells[112].

Moreover, TNF-α stimulates the generation of ROS that activates the apoptotic signaling pathway in auditory hair cells via mitochondrial caspase cascade[113].

As described above, oxidative stress and TNF-α are presumed to be the potent pathogenesis of hearing loss, numerous studies have been conducted over the years to determine whether hearing loss, particularly noise-induced and ototoxic hearing loss, can be prevented by pharmacological intervention that includes antioxidants and TNF-α blockers. Some of these studies have even proved the effectiveness of these interventions.

Antioxidants such as glutathione[94, 97], ebselen[114], N-acetyl, L-cysteine (L-NAC) [112, 115, 116], and acetyl-l-carnitine (ALCAR)[114, 116] have demonstrated a rescuing or preventive effect in noise-induced, age-related, and ototoxic hearing loss[117]. Ebselen has a similar mode of action to Glutathione peroxidase (GPX) and showed effective cytoprotection against noise exposure in guinea pigs and rats[115, 118]. ALCAR can prevent cell damage by improving mitochondrial membrane energetics[114, 116]. NAC, one of the most studied substances, reduced hair cell damage after noise exposure in animal models by scavenging free radicals, similar to the GSH pro-drug[116, 119].

Some experimental studies investigated the protective effect of polyphenol against cisplatin-induced ototoxicity [120] and noise-induced hearing loss[121]. In human studies, ALA has been shown to have a preventive effect on noise-induced hearing loss [122] and vitamin E in treating sudden idiopathic hearing loss[123].

In the cases of TNF-α blockers, such as etanercept, infliximab, and golimumab have been shown to improve hearing and reduce hearing impairment in cases of refractory autoimmune inner ear disease and noise-induced hearing loss in both animal models and human studies. [30, 50, 53]

ALA has been known for decades as an antioxidant that plays an essential role in mitochondrial activities[58]. It reacts with reactive oxygen species and protects cell membranes. Because of its potent antioxidant and anti-inflammatory activities, ALA benefits oxidative stress models like diabetes, ischemic cardiac diseases, or pregnancy-related problems[124]. It also suggested treatment for the prevention of vascular disease and chronic inflammation. In aspects of hearing, a study reported that ALA protects the outer hair cell and preserves stria vascularis and spiral ganglion neurons in a mouse with age-related hearing loss[112]. The other research about intra-tympanic ALA in a mouse model of acoustic trauma showed a similar rescue effect compared to intra-tympanic dexamethasone[103].

Some studies found ALA preconditioning promoted Nrf2 accumulation in the nucleus and the induction of HO-1 in a rat ischemic myocardial injury model. ALA has a neuroprotective effect in the ischemic stroke model by attenuating oxidative damage mediated by the Nrf2 pathway activation[125]. Nrf2 is an essential modulator of defense against oxidative stresses. Under

unstressed conditions, KEAP1, the repressor protein, blocks the transcriptional activity of Nrf2 by binging to Nrf2, and proteasomes degrade ubiquitinated Nrf2 in the cytoplasm. However, in oxidative stress conditions, cysteine residues in KEAP1 are modified, KEAP1-dependent Nrf2 ubiquitination is decreased and accumulated Nrf2. These stabilized Nrf2 translocated to the nucleus and bound to antioxidant-responsive elements (ARE), finally activating the transcription of factors for glutathione synthesis (Gclc, Cclm), xenobiotics (TXNRD1, PRDX1, and NQO1), heme metabolism (HMOX1), NAPDH generation, and phase II conjugation [126]. After noise exposure, the ABR threshold significantly deteriorated, and the expression of NQO1, HO-1, GCLC, GCLM, and TXNRD1 was markedly decreased in Nrf2 gene-deficient mice[127]. The level of GSH was also decreased. Also, compared to wild-type mice of the same age, a greater ABR threshold and faster loss rate of hair cells and SGN were shown in the Nrf2 knockout mice [128]. In the study of the preconditioning effect of intraperitoneal $CoCl_2$ injection in a noise-induced hearing loss mouse model, the expression of Prdx6 and the intracochlear levels of HIF-1α and Nrf2 significantly increased[129]. HO-1, a downstream protein of Nrf2, acts as a critical defender against cisplatin-induced oxidative hair cell damage by activating the Nrf2 signaling pathway [130]. Nrf2 increases the expression of mitochondrial glutathione (γ-L-glutamyl-L-cysteinyl glycine, GSH) and cellular NADPH [131]. GSH is also a crucial antioxidant enzyme that regulates diverse cellular functions. GSH exists in two different forms; the predominant form is the thiol-reduced, and the other one is the disulfide-oxidized form (GSSG). GSH is oxidized to GSSG by catalytic reactions, and GSSG is reduced to GSH by GSSG reductase using NSDPH [89]. Intense oxidative stress leads to depleting cellular GSH. Increasing GSSG and decreasing GSH mean an early phase of the apoptotic cascade. The intracellular GSH to GSSG ratio reflects the redox potential of the cell. The first step of GSH synthesis is catalyzation by glutamate-cysteine ligase (GCL), composed of catalytic and modifier subunits. So, γ-GCSc is the primary precursor of GSH and GCLC is a γ-GCSc coding gene [90]. GSH supplement showed a protective effect in the GSH-deficient guinea pigs from NIHL [94] and prevention against gentamicin ototoxicity via increasing cochlear GSH level [132].

In our study, cytotoxicity and ROS assays suggested that ALA preconditioning significantly reduces TNF-α-induced oxidative cell injuries. Results of Western blot and RT-qPCR analysis of the Nrf2 (Nuclear factor erythroid 2-related factor 2)/KEAP1 (Kelch-like ECH-associated

protein 1) signaling pathway-related proteins and genes showed that ALA preconditioning could protect the HEI-OC1 cell by up-regulating the antioxidant pathway.

In the cases of the Nrf2 downstream genes and protein - mRNA of SOD1, GCLC, NQO1, and HMOX1, and the protein levels of SOD1, γ-GCSc, NQO1, and HO-1 - reflect defense mechanism against to ROS and cellular redox potential, to quantify the ALA's possibility of preventive strategy for ROS induced hair cell death.

However, the limitation of in vitro studies such as this study is that it only measured the preconditioning effect due to the difficulties in determining the appropriate dosage and measuring pre- and post-treatment cytotoxicity. Therefore, further in vivo studies are required to estimate the treatment or rescue effects of ALA. Also, it is necessary to investigate whether the 1 mM ALA concentration used in this study is a safe and applicable dose for in vivo studies.

Conclusions

ALA preconditioning shows the cytoprotective effect on the HEI-OC1 cells from oxidative injury caused by treatment of TNF-α. As apoptosis of the hair cell caused by ROS from TNF-α and other factors is the crucial mechanism of hearing loss, it can be said that the cytoprotective effect of ALA administration shows the possibility of applying it to the prevention and treatment of hearing loss.

To investigate Nrf2 activation through ALA administration, we quantified KEAP1 protein, which is a regulator of the Nrf2, and SOD1, GCLC, NQO1, and HMOX1, which are downstream genes of the Nrf2 signaling pathway, and expressed proteins, SOD1, γ-GCSc, NQO1, HO-1. All the above mentioned genes and proteins were significantly increased in the ALA preconditioned group. GSH, another representative antioxidant, was also significantly increased in the ALA-treated HEI-OC1 cell. We concluded that administration of the ALA protected the cells by antioxidative action via activation of the Nrf2 signaling pathway and accumulation of the intracellular GSH. Based on this, further in vivo research is expected to lead to ALA could be another strategy for the prevention and treatment of sensorineural hearing loss.

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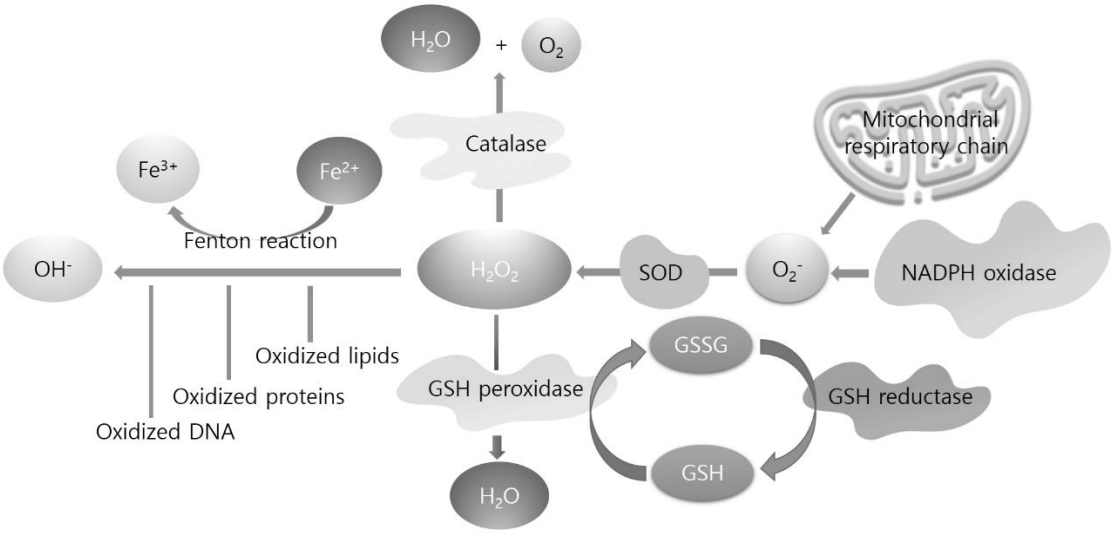
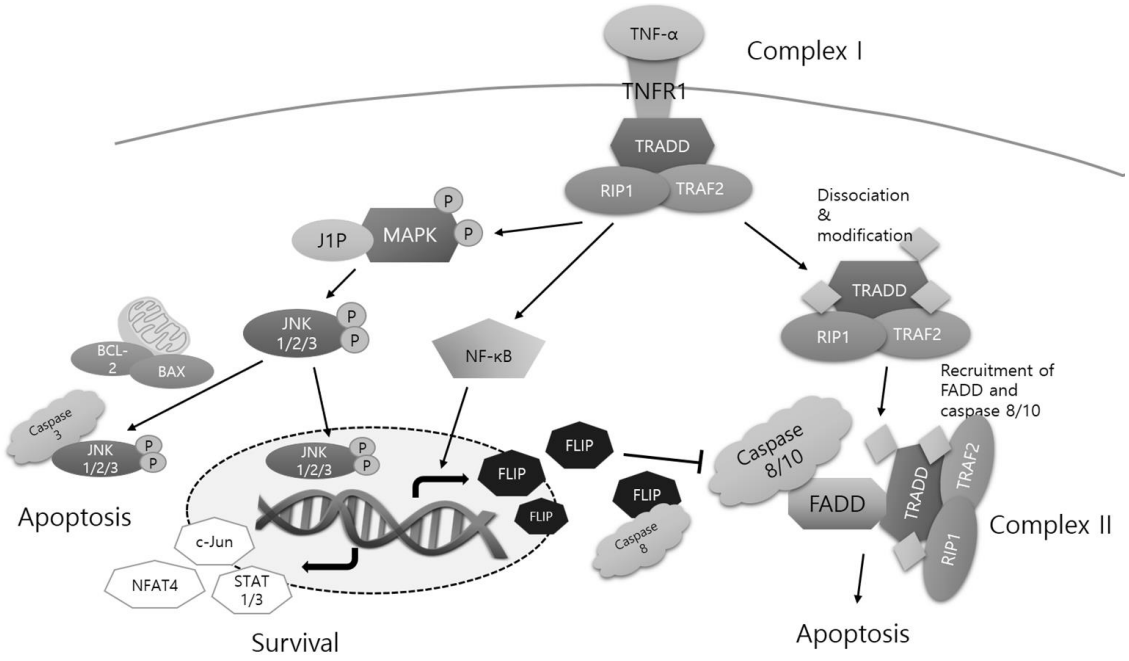
Figure 1. ROS generation and detoxification[2]



ROS are produced endogenously during oxidative phosphorylation in mitochondria. These ROS can also be created by interactions with external substances. NADP oxidase (NOX) is a membrane protein that contains binding regions for NADPH, FAD, and histidine residues, which produce superoxide. It can lead to the oxidation of lipids, proteins, and DNA, resulting in genomic insinstabilities. Adapted from 'Redox homeostasis dysregulation in noise-induced hearing loss: oxidative stress and antioxidant treatm -ent' by Zhou Y et al., 2023, J Otolaryngol Head Neck Surg. 52:78, Fig 1. p3. Copyright 2023 by Zhou \ Y et al.[2]

Figure 2. The TNF-α receptor 1-mediated apoptosis and survival signaling pathways[25, 33]



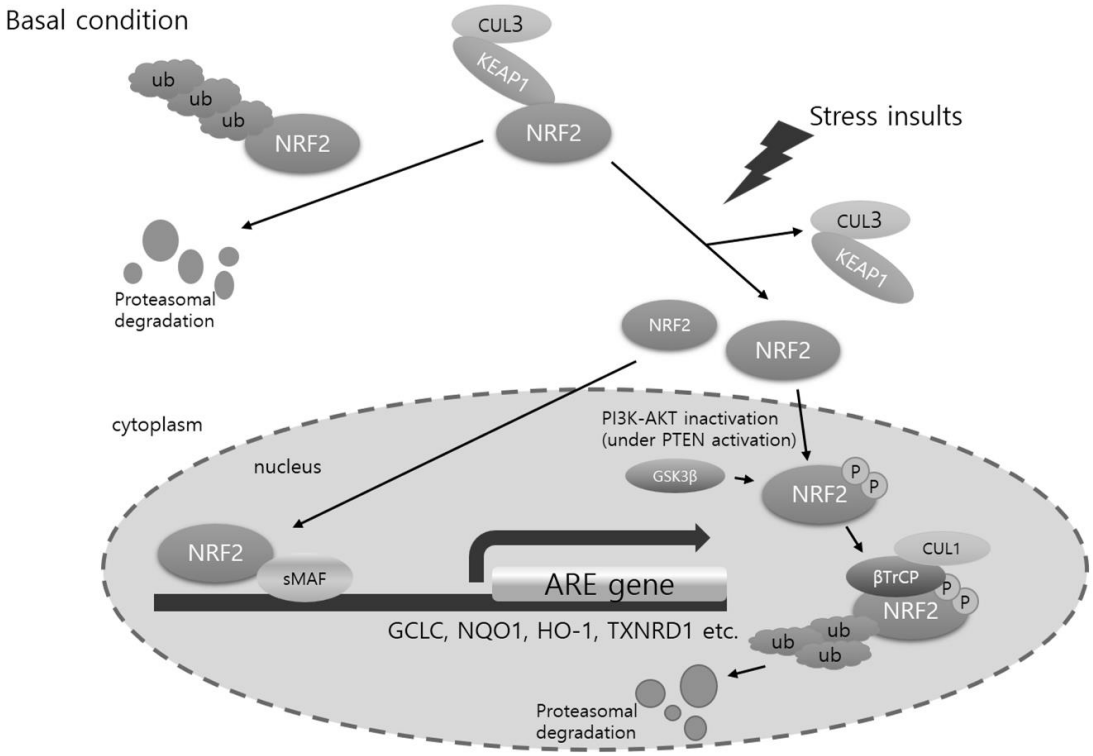
TNF-α binds to TNFR1, which leads to the formation of a complex (Complex I) consisting of the proteins TRADD, RIP1, and TRAF2. Complex I blocks apoptosis, promotes survival, and increases the production of inflammatory cytokines by activating NF-κB. It also enhances the production of $FLIP_L$, which blocks caspase 8. Oxidative stress activates MAPKKK, which leads to the activation of the scaffold protein JNK-interactive protein 1 (J1P). J1P binds to the MAPKKK and MAPKK family with JNK and activates JNK. The activated JNK promotes the transcription of cell survival genes. However, it can also dissociate and induce mitochondria-dependent apoptosis through the bcl2 and BAX (bcl2-associated X-protein). When TNFR1, TRADD, and RIP1 dissociate from TNFR1, complex II is formed through the liberated death domain (DD) of TRADD (and/or RIP1) binding FADD. Complex II recruits caspase-8/10 recruitment (forming complex II) and results in apoptosis. Adapted from 'Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes' by Micheau, O. and J. Tschopp, 2003, Cell 114, Fig 8. p8., Copyright 2003 by Micheau, O. and J. Tschopp[25], and 'unctional Roles of JNK and p38 MAPK Signaling in Nasopharyngeal Carcinoma' by Pua, L.J.W., et al., 2022, Int J Mol Sci, 23, Fig 2, p4. Copyright 2022 by Pua, L.J.W., et al.[33]

Figure 3. The chemical structure of optical isomers of ALA [1]

ALA has a single chiral center and an asymmetric carbon, which results in the formation of two optic al isomers: R- and S- ALA. The R isomer is naturally present while the S isomer is through chemical processes.

From 'Insights on the Use of α-Lipoic acid for therapeutic purpose' by Salehi B et al.,Biomolecules 2019. 9, Figure 1. p2. Copyright 2019 by Salehi B et al [1].

Figure 4. **The KEAP1-NRF2 signaling pathways[3]**

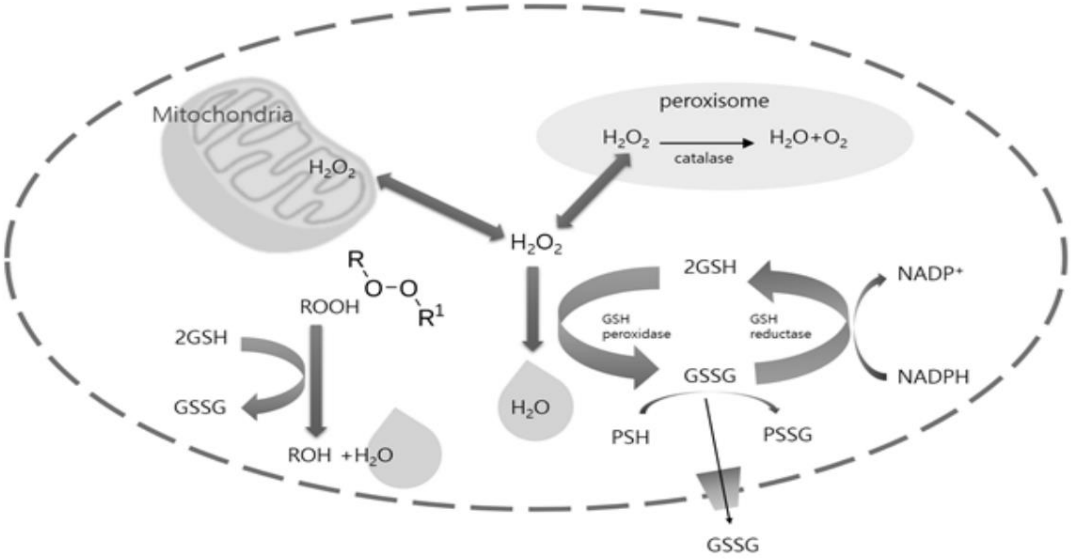


Under unstressed conditions, the NRF2 degradation is accelerated through KEAP1-CUL3 and βTrCP-CUL1 complex-related ubiquitination. When βTrCP recognizes NRF2, it is phosphorylated by GSK3β at two specific serine residues in the Neh6 domain. The phosphorylated NRF2 is ubiquitinated by the βTrCP-CUL1 ubiquitin E3 ligase complex and degraded by the proteasome system. The activation of the PI3K-AKT pathway inhibits the function of GSK3β.

In contrast, when exposed to oxidative stress, specific reactive cysteine residues in KEAP1 get modifi ed, which reduces the ubiquitin ligase activity of KEAP1. This results in the stabilization of NRF2, which then moves to the nucleus where it activates transcription of ARE (antioxidant reactive elements) genes.

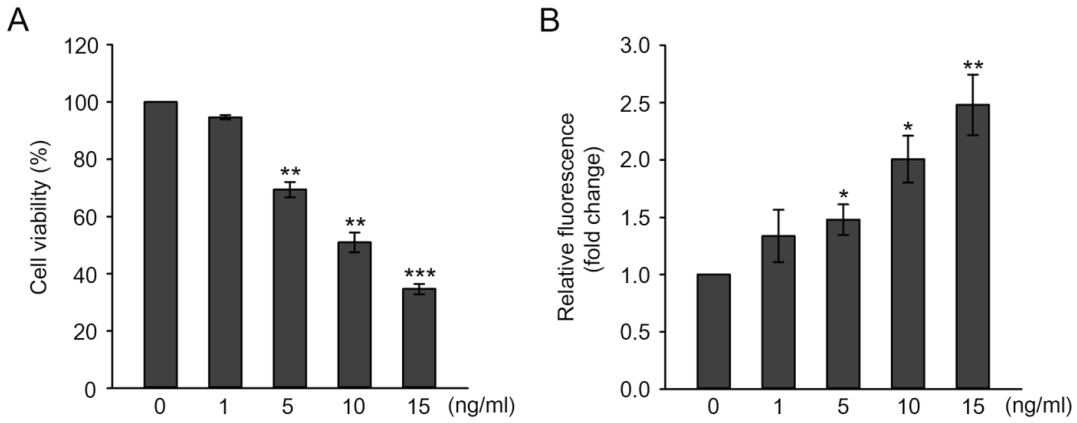
Adapted from 'The KEAP1-NRF2 System and Esophageal Cancer' by Hirose W et al., Cancer (Basel) 2022, 14., Figure 1. p3. Copyright 2022 by Hirose W et al[3].

Figure 5. Anti-oxidant function of GSH [92]



Under basal conditions, hydrogen peroxide is produced and can be metabolized by GSH peroxidase and catalase. GSSG is reduced back to GSH in a redox cycle with NADPH. Severe oxidative stress causes accumulation of GSSG due to reducing potential exhaustion. These accumulate GSSGs extracellular transport or form a mixed disulfide (PSSG) by reacting with a protein sulfhydryl (PSH) to maintain a redox equilibrium. Adapted from 'Regulation of glutathione synthesis' by Lu, S.C. 2009, Mol Aspects Med, 30, Fig 3, p4. Copyright 2009 Lu, S.C.[92]

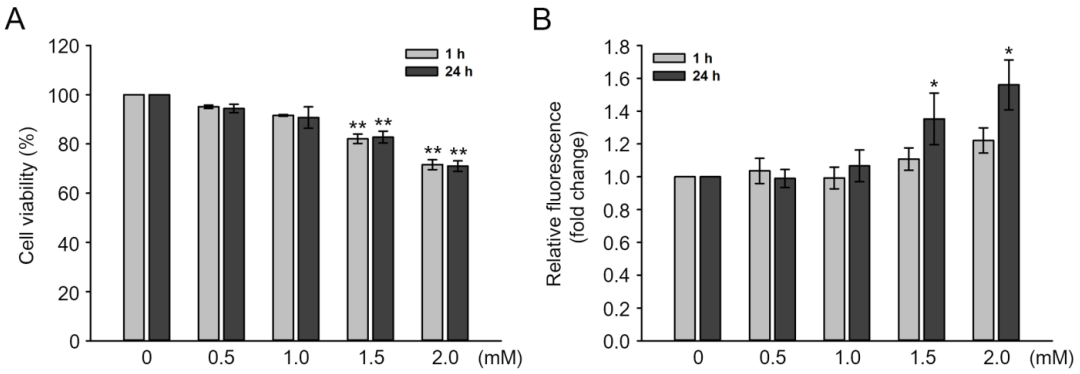
Figure 6. Effects of TNF-α on cell viability and ROS generation in HEI-OC1 cells



A. The values are expressed as the means ± SE for five independent experiments, expressed as a percentage of untreated control value. ** $p< 0.01$, *** $p< 0.001$ compared with untreated control.

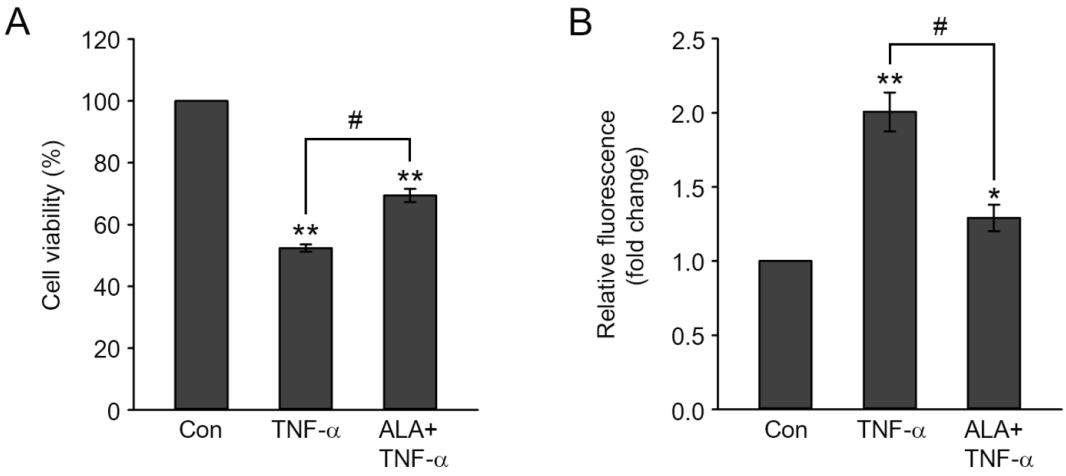
B. The values in a graph are represented as fold changes relative to the untreated control, expressed as means ± SE of three independent experiments. * $p< 0.05$, ** $p< 0.01$ compared with untreated control.

Figure 7. Effects of ALA on cell viability and ROS generation in HEI-OC1 cells



The values are expressed as the means ± SE for three independent experiments. * $p< 0.05$, ** $p< 0.01$, compared with untreated control.

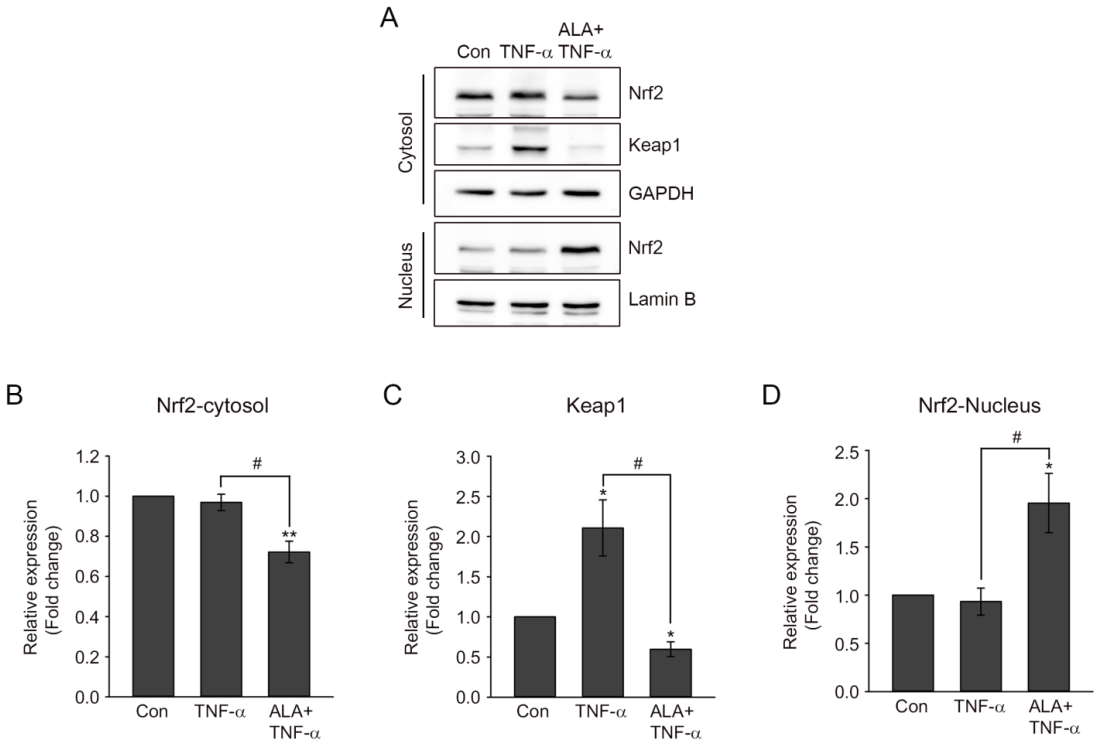
Figure 8. Protective effect of ALA on cell viability and ROS generation in TNF-α treated HEI-OC1 cells



A. The values are expressed as the means ± SE for four independent experiments as a percent age of untreated control value. $^{\#}p< 0.05$, ** $p< 0.01$, **; compared with untreated control, $^{\#}$; TNF-α only versus ALA pretreated cells.

B. The values in a graph are represented as fold changes relative to the untreated control, expr essed as means ± SE of three independent experiments. *,$^{\#}p< 0.05$, ** $p< 0.01$, *,**; compared with untreated control, $^{\#}$; TNF-α only versus ALA pretreated cells.

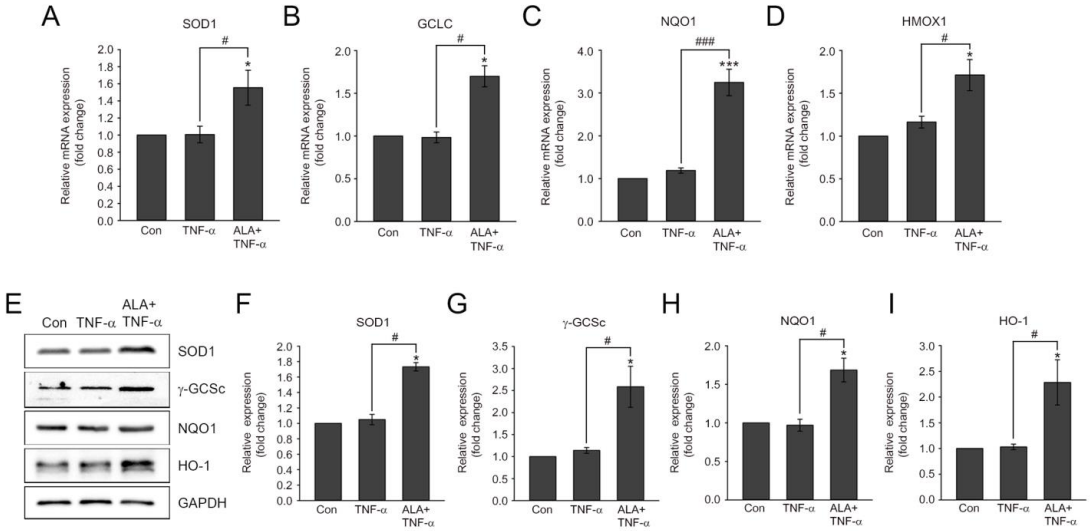
Figure 9. Effect of ALA pretreatment on Nrf2/KEAP1 signaling pathway



A. The protein expression of the Nrf2/KEAP1 signaling pathway was detected by western blot in the cytosol and nucleus of the HEI-OC1 cells.

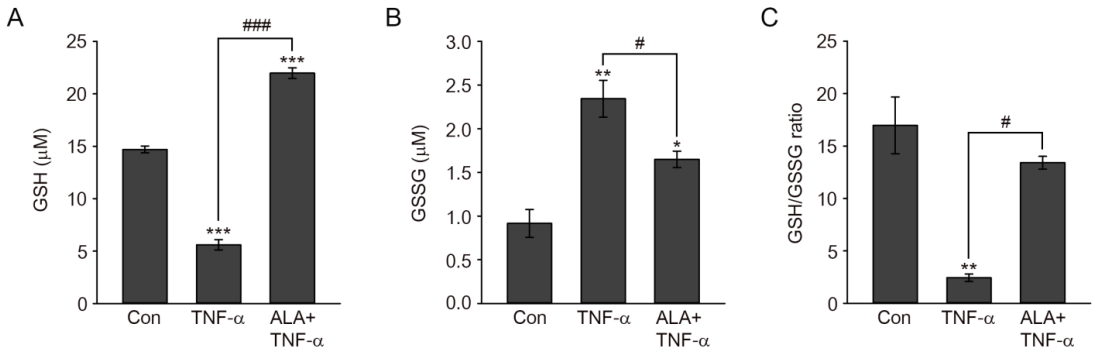
B-D. The values in a graph are represented as fold changes relative to the untreated control, expres sed as means ± SE of three independent experiments. *,$^{\#}p< 0.05$, ** $p < 0.01$, *,**; compared with untreated control, $^{\#}$; TNF-αonly versus ALA pretreated cells.

Figure 10. Effect of ALA pretreatment on Nrf2-regulated antioxidant genes and protein expression



The values in a graph are represented as fold changes relative to the untreated control, expressed as means ± SE of three independent experiments. $^{*,\#}p< 0.05$, $^{***,\#\#\#}p< 0.001$, *,***; compared with untreated control, $^{\#,\#\#\#}$; TNF-α only versus ALA pretreated cells. The mRNA level of SOD1 gene (A), GCLC gene (B), NQO1 gene (C), and HMOX1 gene (D). The protein levels of SOD1 (E,F), γ-GCSc (E,G), NQO1 (E, H), HO-1 (E, I), and the reference protein, GAPDH (E).

Figure 11. Effect of ALA pretreatment on GSH, GSSG, and GSH/GSSG ratio



Glutathione (A), GSSG (B) content of untreated, TNF-α only, and ALA preconditioned cells. Furthermore the GSH/GSSG ratio of each differently treated cell (C). Values are expressed as means ± SE for three independent experiments. $^{*,\#}p< 0.05$, ** $p< 0.01$, ***,$^{\#\#\#}p< 0.001$ *,**,***; compared with untreated control, $^{\#,\#\#\#}$; TNF-α only versus ALA pretreated cells.

국문요약

사회적 소음 노출 증가에 따른 소음성 난청, 인구 고령화로 인한 노인성 난청 및 항생제, 항암제, 진통소염제와 같은 이독성 약물의 사용이 늘어남으로써 함께 증가하는 이독성 난청 등 난청의 유병률 증가가 사회적 문제로 대두되고 있으나, 이러한 감각 신경성 난청의 경우 소음, 이독성 약물을 피하는 회피 요법이나, 중증 이상의 난청에서 보청기, 인공 와우 수술 등을 통한 청능 재활 요법을 제외하고는 마땅한 치료법이나 예방법이 없는 상황이다.

그래서 다양한 감각 신경성 난청 발생의 기전을 밝혀 예방과 치료에 적용하기 위한 많은 연구들이 있어왔고, 현재도 진행 중이다. 이러한 연구를 통해 감각 신경성 난청의 발생에서 공통적으로 확인된 것이 활성 산소 종에 의해 유발되는 내이 세포의 세포 자멸사와 TNF-α 의 증가로, 이후 항산화물질과 TNF-α 길항제를 감각신경성 난청에 적용하기 위한 노력도 지속되어왔다.

TNF-α 는 주요 염증 매개 물질로서 다양한 형태의 감각 신경성 난청의 발생 기전에서 염증 반응을 통한 세포 손상과 함께 활성 산소 종 생성을 촉진하며, 주요 항산화물질 중의 하나인 α-Lipoic acid (ALA)는 활성 산소 종에 의한 세포막 손상을 억제하여 이독성 난청, 노화에 의한 난청, 소음성 난청 및 돌발성 감각 신경성 난청 등 다양한 형태의 난청에서 내이 세포 보호 효과가 있음이 알려져 있다.

ALA 는 항산화제로서만이 아니라 염증 세포의 축적을 방지하여 세포 보호 효과가 있는 만큼 감각 신경성 난청의 발생 과정에서 ALA 의 투여가 감각신경성 난청에서 공통적으로 관찰되는 활성 산소 종 발생 및 TNF-α 의 과 발현을 억제할 수 있는 지와, 그 세포 보호 기전을 확인하는 것이 향후 난청의 예방과 치료에 긍정적인 역할을 할 수 있을 것으로 보인다.

본 연구에서는 마우스 내이 감각 유모 세포 주, House Ear Institute-Organ of Corti 1 (HEI-OC1 cell line, 에 TNF-α 처리를 통하여 활성 산소 종 발생을 측정하고, ALA 로 전 처리를 하여 항산화 기전 중 하나인 Nrf2/KEAP1 신호 전달 체계에 관여하는 유전 물질과 단백질, Glutathione (GSH) 및 산화 Glutathione (GSSG)를 정량하여 Nrf2 신호 전달 체계 활성화와

항산화 기전을 밝힘으로써 난청 발생의 예방에 적용할 수 있는 가능성을 확인하고자 하였다.

실험은 마우스 내이 감각 유모 세포(HEI-OC1)에 각각 다른 농도의 TNF-α, ALA 를 처리 후 배양하여 편광 분석을 통해 세포생존율, 활성 산소 종 발생을 측정하여 실험에 이용할 TNF-α, ALA 의 용량을 결정하였고, 대조 군과 TNF-α 단독, ALA 전 처리 후 TNF-α 에서 배양한 세포들의 세포 생존율, 활성 산소 종 발생 정도를 측정하여 ALA 전 처리의 세포 보호 및 활성 산소 종 감소 효과를 확인하였다.

또한 세 세포 군을 핵과 세포질로 분획화하여 Western blot 을 시행, 세포질과 핵 내의 Nrf2, 세포질 내 KEAP1 단백을, RT-qPCR 을 이용하여 Nrf2/KEAP1 신호 전달 체계의 유전물질인 SOD1, GCLC, HMOX1, NQO1 을, 그 발현 단백인 SOD1, γ-GCSc, NQO1, HO-1 을 Western blot 을 통해 정량, 비교하였다. 그리고 세 군에서 glutathione (GSH), 산화 glutathione (GSSG), 그리고 그 ratio 를 측정함으로써 ALA 의 세포 환원력을 함께 확인하고자 하였다.

ALA 전 처리를 시행한 세포 군에서 TNF-α 단독 군에 비해 약 17% 높은 세포 생존율과 약 64% 의 활성 산소 종 감소를 보였고, Nrf2/KEAP1 신호 전달 관련 유전자인 SOD1, GCLC, NQO1, HMOX1, 그 발현 단백질인 SOD1, γ-GCSc, NQO1, HO-1, 핵 내 Nrf2 모두 ALA 전 처리한 세포에서 유의하게 높게 측정되었다.
Nrf2 억제단백인 KEAP1 의 경우 ALA 전 처리 군에서 TNF-α 처리 군의 1/3 미만으로 낮았다. 또한 세포 내 glutathione (GSH)는 ALA 전 처리 군에서 TNF-α 단독 군의 약 4 배 정도로 높았고, glutathione 의 산화 형태인 GSSG 는 약 30% 낮게, GSH/GSSG 비는 ALA 전 처리 군이 약 6 배 높았다.

실험을 통해, ALA 가 TNF-α 에 의해 유발된 산화 스트레스에 대하여 활성 산소 종 생성 및 축적을 억제함으로써 세포 보호 효과를 나타내며, Nrf2/KEAP 1 신호 전달 체계의 활성화가 세포 보호 효과의 기전 중의 하나 임을 알 수 있었다. 또한 GSH assay 를 통해 ALA 전 처리가 세포 내 환원력을 증가 시키는 것 역시 세포 보호 효과의 기전으로 추정 해 볼 수 있겠다.

다만, 상기 결과는 HEI-OC1 세포 내 측정 결과이므로 동물 실험 및 임상 시험의 기초 자료로 이용될 경우 ALA 투약이 난청의 예방 및 치료법으로 적용 될 가능성에 대한 추가 연구와 근거 마련을 하는 데에 도움이 될 수 있을 것이다.