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Effect of resveratrol in acute cochlear damage caused by 3-nitropropionic acid

3-nitropropionic acid 에 의한 급성 와우 손상에서 resveratrol 의 효과

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ABSTRACT

Introduction: 3-Nitropropionic acid (3-NP), a mitochondrial toxin, induces the acute hearing loss by impairing mitochondrial energy generation. Resveratrol is known to have potent protective effects against ototoxicity from various sources as an antioxidant. The aim of the present study is to investigate the protective effect of resveratrol against 3-NP-induced acute cochlear damage.

Methods: Fifteen male Sprague-Dawley rats were divided into 3 groups. In group A, the 3-NP (500 mM, 4 μl, intratympanically) was injected, and in group B, only resveratrol (10 mg/kg, 5 days, intraperitoneally) was administered. In group C, resveratrol pretreatment (10 mg/kg, 5 days, intraperitoneally) was performed before 3-NP injection. The auditory brainstem response (ABR) was recorded at 8, 16, and 32 kHz before and after treatment. And, 4 weeks after treatment, the histological analysis of cochleae from each group was performed to investigate changes of cochlear cells.

Results: After 3-NP exposure (group A), ABR thresholds were significantly elevated compared to those treated with only resveratrol (group B) which showed normal findings. However, resveratrol pretreatment before 3-NP

exposure (group C) led to a significant decrease of ABR thresholds. Histological analysis of group A revealed loss of fibrocytes in the spiral ligament (SL), hair cells and supporting cells in the organ of Corti, stellate fibrocytes and interdental cells in the spiral limbus, and spiral ganglion (SG) cells, whereas group B and C demonstrated normal findings. In addition, the thickness of stria vascularis in group A and C were decreased compared to that in group B, and that of group A was the thinnest. When investigating the cell counts of the fibrocytes in SL and the ganglion cells in SG area, those of group A was significantly decreased compared to those in group B and C.

Conclusions: These results suggest that resveratrol may have a protective effect against 3-NP-induced acute cochlear damage.

Keywords: Resveratrol, 3- nitropropionic acid, ototoxicity

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LIST OF ABBREVIATIONS

3-NP : 3-nitropropionic acid

ABR : Auditory brainstem response

OC : Organ of Corti

SG : Spiral ganglion

SL : Spiral ligament

SV : Stria vascularis

Introduction

Mitochondria produce ATP, the primary source of cellular energy, and their acute damage may induce an energy failure-related acute hearing loss. 3-nitropropionic acid (3-NP) known to be a mitochondrial toxin causing an oxidative damage, is a neurotoxin produced by some fungal species and an irreversible inhibitor of succinate dehydrogenase. (1) A recent study showed that the rat treated with 3-NP in the cochlea had a severe hearing loss by the cellular degeneration in the cochlear lateral wall. (2) In addition, the possible source of oxidative stresses was thought to be the mitochondrial dysfunction. (3)

Resveratrol is a kind of natural polyphenolic compound found in various food products, with particularly high levels present in grape skins (50–100 μg/g) and red wine (1.52 mg/l). (4) It is well known to have antioxidative and anti-inflammatory effect. (5, 6) Some researchers reported that resveratrol modulates the lipid and lipoprotein metabolism, inhibits platelet aggregation, and also has immunomodulatory properties and vasorelaxing activity. (7) On otologic field, there were some reports about its antineoplastic activity and hearing preservation against the ototoxic agents or noise. (4, 8-12)

The present study was performed to investigate whether resveratrol pretreatment can protect the cochlea against the acute cochlear damage that occurs after local administration of 3-NP.

Materials and methods

Animal preparation and care

This study was carried out under approval of the Institutional Animal Care and Use Committee of Seoul National University Hospital Biomedical Research Institute. (IACUC 13-0234-C0A2-1) Fifteen 4-week-old male Sprague-Dawley rats were prepared. Light and dark periods, room temperature, and humidity were controlled strictly. The external ear canals and tympanic membranes of all animals were examined by a researcher and there was no evidence of otitis externa, tympanic membrane perforation or middle ear infection in any rats.

Drug preparation

Resveratrol (Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in saline (pH 7.4) at 10 mg/ml. As an ototoxic agent, 3-NP (Sigma, St. Louis, MO, USA) was dissolved in saline (pH 7.4) at 500 mM.

Treatment

Fifteen rats were randomly divided into three groups, each group was composed of 5 rats. As one rat in group C was dead, further studies were

performed with 14 rats. In group A (n=5), only 3-NP was administrated. In group B (n=5), only resveratrol was administrated and animals in group C (n=4) were treated with both 3-NP and resveratrol.

Animals of group A were treated with 500 mM of 3-NP intratympanically using 1 cc syringe with 26 G spinal needle under microscopy. For animals of group B and C, 10 mg/kg of resveratrol was administrated intraperitoneally for 5 days. At the third day of administration, 500 mM of 3-NP was injected intratympanically for animals of group C.

Auditory brainstem response (ABR) measurement

ABR was recorded before and 1, 2 and 4 weeks after administration. Each animal was anesthetized with Tiletamine/Zolazepam (1:1) (10 mg/kg) and xylazine (5 mg/kg). External auditory canals were examined before ABR recording. Tone-burst sounds (8, 16, and 32 kHz) were measured by SmartEP (Intelligent Hearing Systems version 3.30 program, Intelligent Hearing Systems, Miami, FL). Hearing threshold was defined as the lowest stimulus intensity level that showed a reliable waveform in the ABR trace by the visual inspection of one investigator.

Histopathology

Four weeks after administration, the rats were sacrificed under deep

anesthesia. Cochleae of all groups were harvested. After overnight fixation with 10% formaline in 0.1 M phosphate-buffered saline, decalcification was done with 10% ethylenediamine tetraacetic acid in 0.1 M phosphate-buffered saline. After rinsing with 0.1 M phosphate-buffered saline, the tissues were prepared as a frozen block and cut into 10 μm thick sections. Hematoxylin and eosin staining was performed for histological anaylsis.

Histological examination was done 4 weeks after administration in the lateral wall, organ of Corti (OC), and spiral ganglion (SG) area of all groups. Under the microscopic examination, the quantitative analysis was done for 5 samples per each area of each group. The average thickness of stria vascularis (SV) was measured in the manner described by Wangemann et al. (13) The thickness was determined by the average of three points determined evenly in the whole SV. The cell counting was done for the fibrocytes in the spiral ligament (SL) and SG cells in the SG area. The unit areas were 10,000 μm^2 and 2,500 μm^2, respectively. The cell count was determined by the average of cell counts in two unit areas per one sample.

Olympus BX51 microscope was used for microscopic examination and measurement of the thickness of SV and cell counting.

Statistical analysis

Data were analyzed with SPSS version 15.0 (SPSS Inc., Chicago, IL). The

Mann-Whitney U test and Kruskal-Wallis 1-way analysis were used to examine differences of ΔABR thresholds, the thickness of SV, and the cell counting among groups. A value of $p < 0.05$ was regarded as statistically significant.

Results

ABR threshold shift

ABR was measured in 14 rats except for 1 died. In pretreatment examination, mean ABR threshold in each group was not significantly different at 8 and 16 kHz (p = 0.959 and 0.783, respectively) whereas at 32 kHz, mean ABR threshold in group C was significantly higher than group B. (Fig.1-1, p = 0.008) One week after administration of 3-NP and/or resveratrol, there were significant differences of mean ΔABR thresholds in group A and C in comparison with those in group B in all frequencies. (Fig.1-2, $p < 0.001$, 0.001, and 0.002 for group A and B, and p = 0.006, 0.006, and 0.012 for group B and C in each frequency.) There was no significant difference between group A and C in all frequencies. Two weeks after treatment, mean ΔABR threshold at each frequency in group C showed a tendency of recovery. (Fig.1-3) Mean ΔABR thresholds at 8 and 16 kHz between group A and C were statistically significant (p = 0.013 and 0.025, respectively), however, not at 32 kHz. (p = 0.163) And the improvement was still limited and there was significant difference of ΔABR between group B and C at 8, 16, and 32 kHz. (p = 0.006, 0.015, and 0.026, respectively) Four weeks after treatment, further recovery of mean ABR threshold in group C was observed. (Fig.1-4) At 8, 16, and 32 kHz, there were significant differences of mean ΔABR threshold in group C in comparison with group A. (p = 0.006, 0.006, and 0.049,

respectively) And, there were no significant differences between group B and C in all frequencies. (p = 0.145, 0.387, and 0.437, at 8, 16, and 32 kHz)

Histological analysis

Histological examination was done in second cochlear turns among all groups. In lateral wall of cochlea, group A revealed loss of all types of fibrocytes in the SL and the atrophy of SV, while group B showed normal findings and group C less severe findings than that of group A. (Fig.2-1, 2-2, 2-3) The average thickness of the SV was 8.0 μm, 21.5 μm, and 16.1 μm, respectively. There are statistically significant differences among groups. The SV in group C was significantly thinner than group B, however there was also a significant difference between the thickness in group A and C. (Fig.2-4) The average cell counts of the fibrocytes in the SL were 2.6, 17.1, and 15.8, respectively. Group A showed a statistically significant decrease of cell counts in the SL compared with group B and C. (Fig.2-5)

In the OC area, group A showed the loss of most hair cells and supporting cells in the OC and stellate fibrocytes and interdental cells of the spiral limbus. (Fig.3-1) On the other hand, group B demonstrated normal findings and most cells in the OC area in group C were preserved. (Fig.3-2, 3-3)

In the SG area, group A demonstrated the severe degeneration of SG cell. (Fig.4-1) In group B, normal SG cell population was observed. (Fig.4-2) In group C, the degeneration of SG cells was not as severe as that of group A.

(Fig.4-3) The average cell counts in the SG area were 19.6, 36.8, and 36.7, respectively. Group A showed a statistically significant decrease of cell counts in the SG area compared with group B and C. (Fig.4-4)

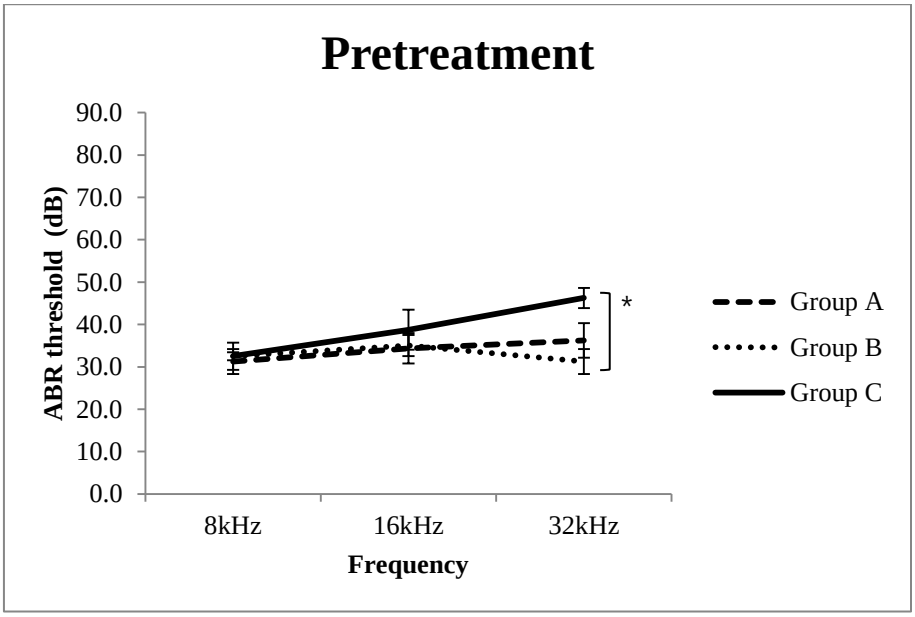

Fig. 1-1. Mean auditory brain response (ABR) thresholds before treatment in group A, B, and C. Mean ABR threshold in each group was not significantly different at 8 and 16 kHz (p = 0.959 and 0.783, respectively) whereas at 32 kHz, mean ABR threshold in group C was significantly higher than group B. (p = 0.008)

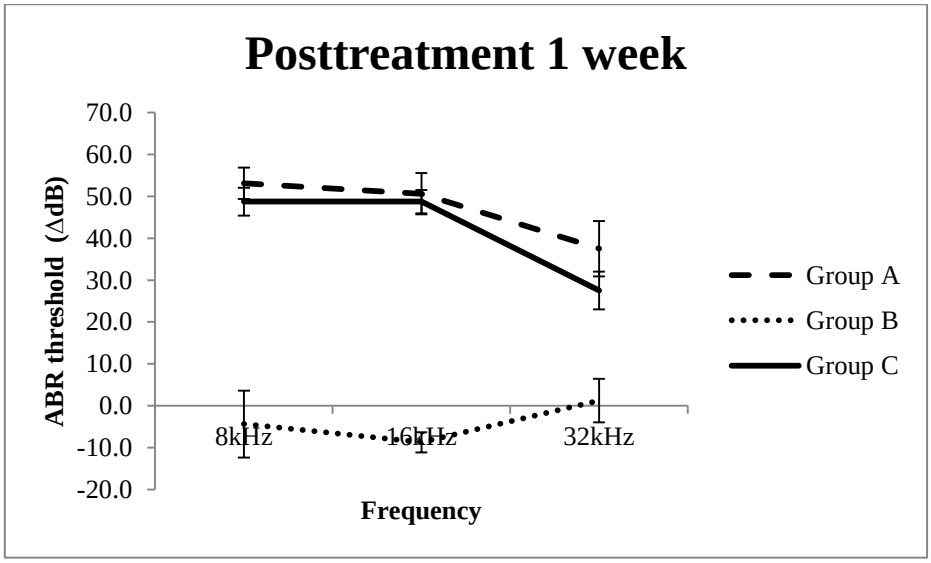

Fig.1-2. Mean Δauditory brain response (ΔABR) thresholds between pretreatment and 1 week after treatment in group A, B, and C. ABR thresholds in group A and C were significantly elevated. There were significant differences of mean ΔABR thresholds in group A and C in comparison with those in group B at 8, 16 and 32 kHz. ($p < 0.001$, 0.001, and 0.002 for group A and B, and $p = 0.006$, 0.006, and 0.012 for group B and C at each frequency) There was no significant difference between group A and C at all frequencies.

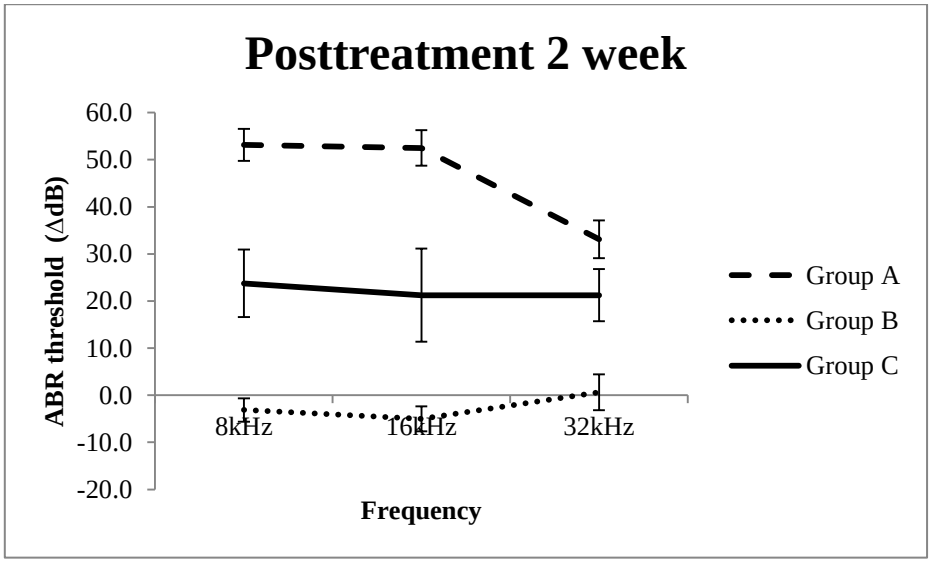

Fig.1-3. Mean Δauditory brain response (ΔABR) thresholds between pretreatment and 2 weeks after treatment in group A, B, and C. Mean ΔABR thresholds in group C were statistically smaller at 8 and 16 kHz compared with those in group A. (p = 0.013 and 0.025, respectively) Mean ΔABR thresholds between group B and C at 8, 16, and 32 kHz showed a significant difference. (p = 0.006, 0.015, and 0.026, respectively)

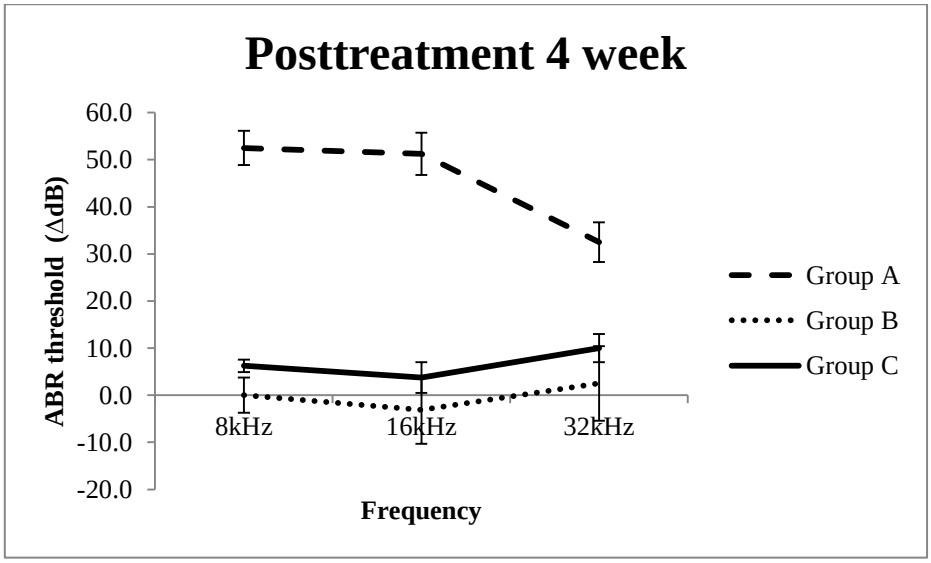

Fig.1-4. Mean Δauditory brain response (ΔABR) thresholds between pretreatment and 4 weeks after treatment in group A, B, and C. At 8, 16, and 32 kHz, there were significant differences of mean ΔABR thresholds between group A and C. (p = 0.006, 0.006, and 0.049, respectively)

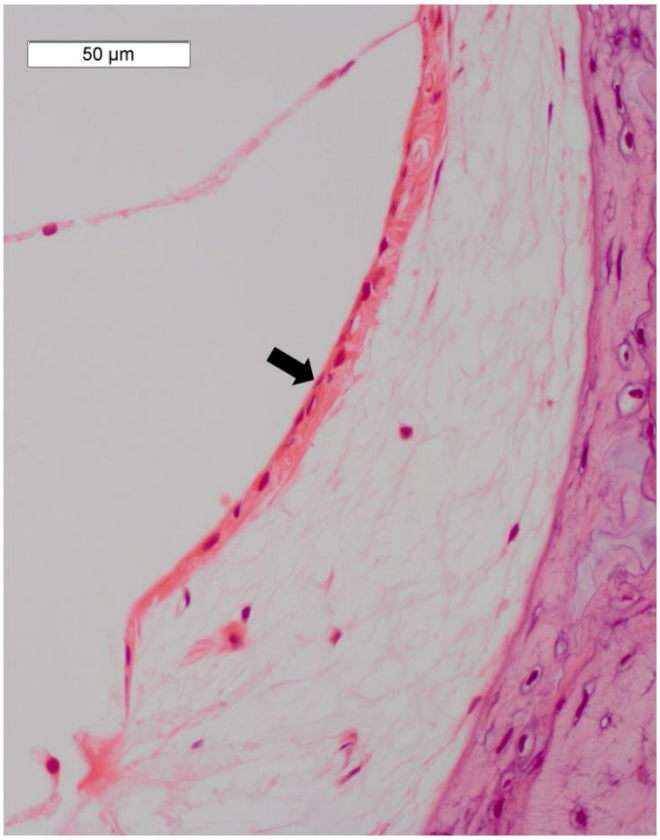

Fig.2-1. Group A : Hematoxylin and eosin-stained histological finding in the lateral wall of the second cochlear turn. Loss of all types of fibrocytes in the spiral ligament and the atrophy of stria vascularis are shown. (arrow)

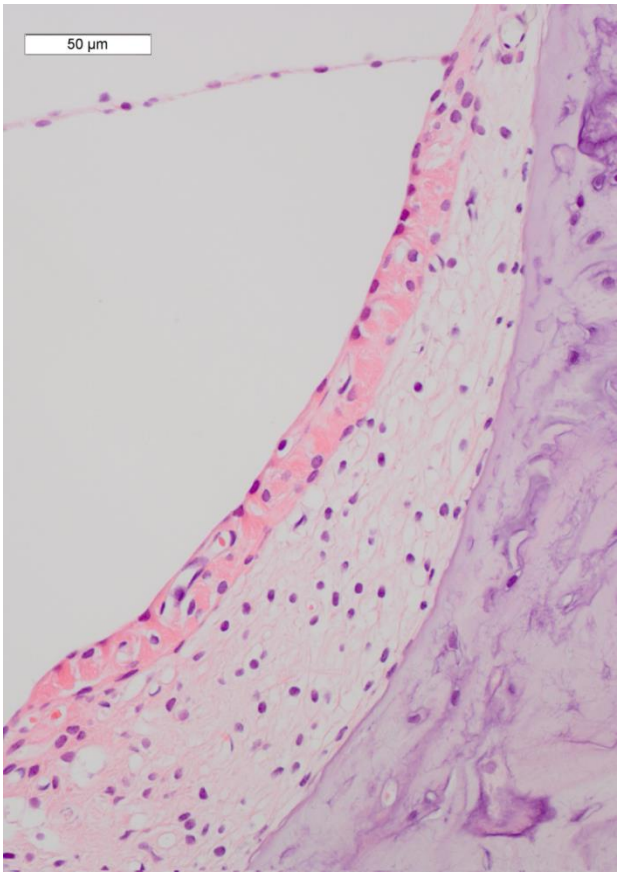

Fig.2-2. Group B : Hematoxylin and eosin-stained histological finding in the lateral wall of the second cochlear turn. The density of fibrocytes and the thickness of stria vascularis show normal findings.

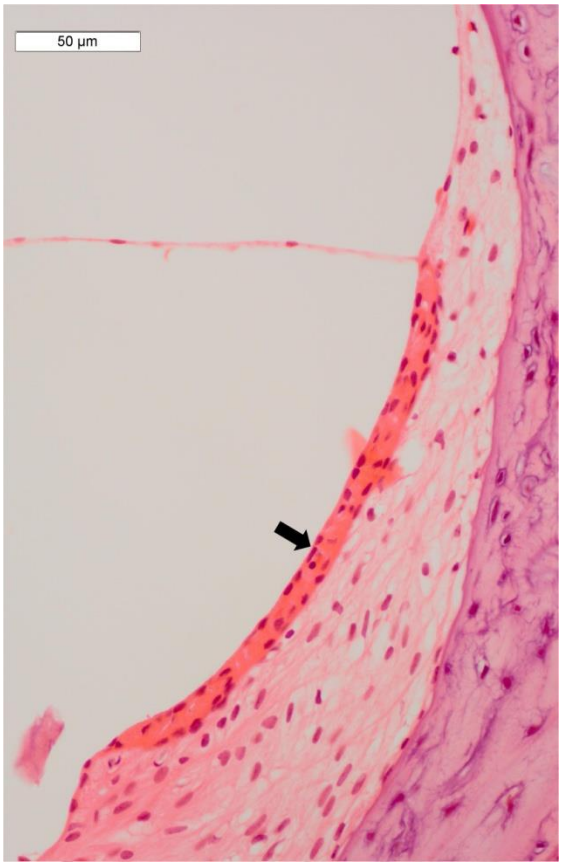

Fig.2-3. Group C : Hematoxylin and eosin-stained histological finding in the lateral wall of the second cochlear turn. The fibrocytes and stria vascularis demonstrate preserved findings compared with those of group A. (arrow)

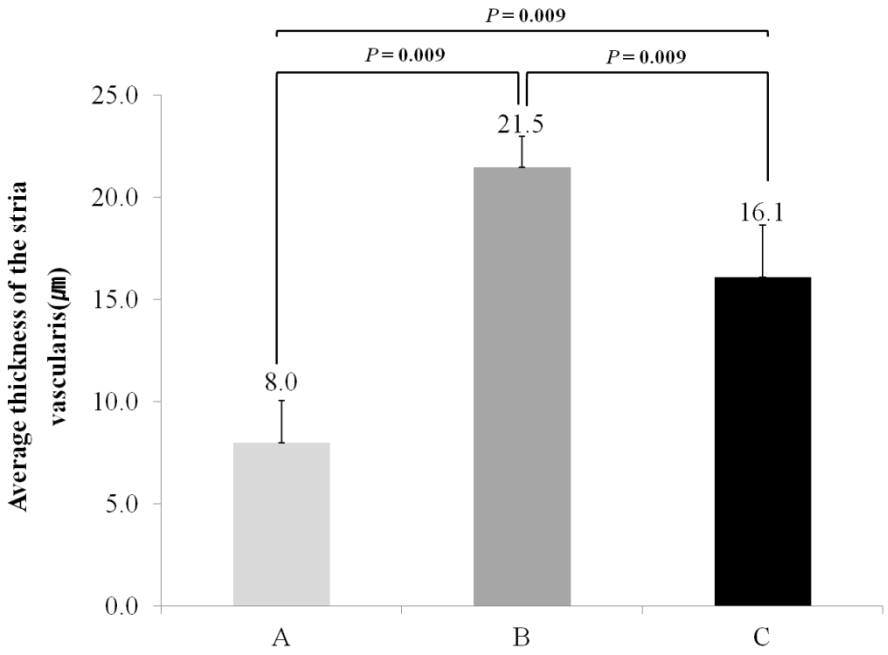

Fig.2-4. Average thickness of the stria vascularis among 3 groups. There are statistically significant differences among groups.

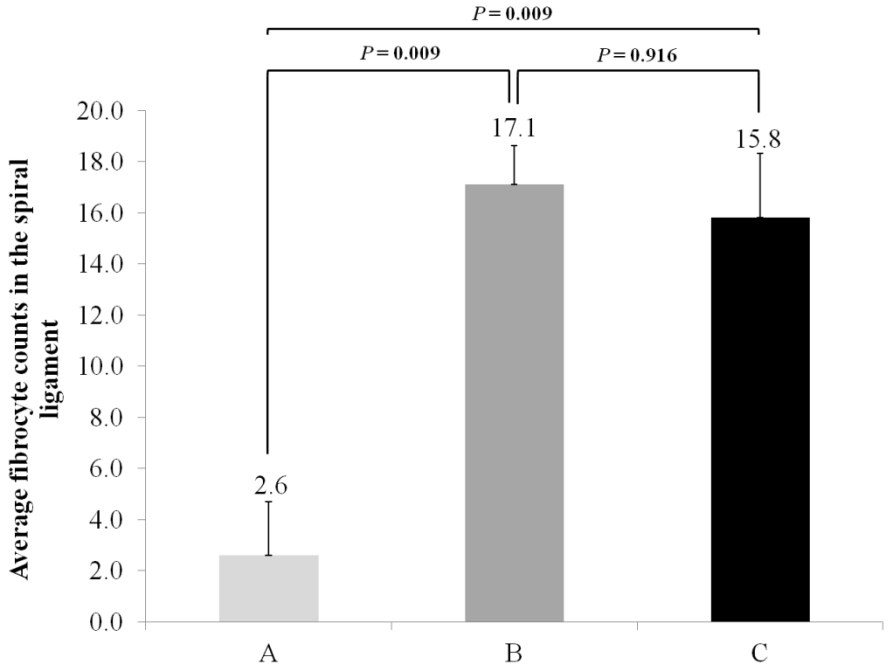

Fig.2-5. Average cell counts among 3 groups in fibrocyte region in the spiral ligament (SL). Group A showed a statistically significant decrease in cell counts than group B and C in the SL.

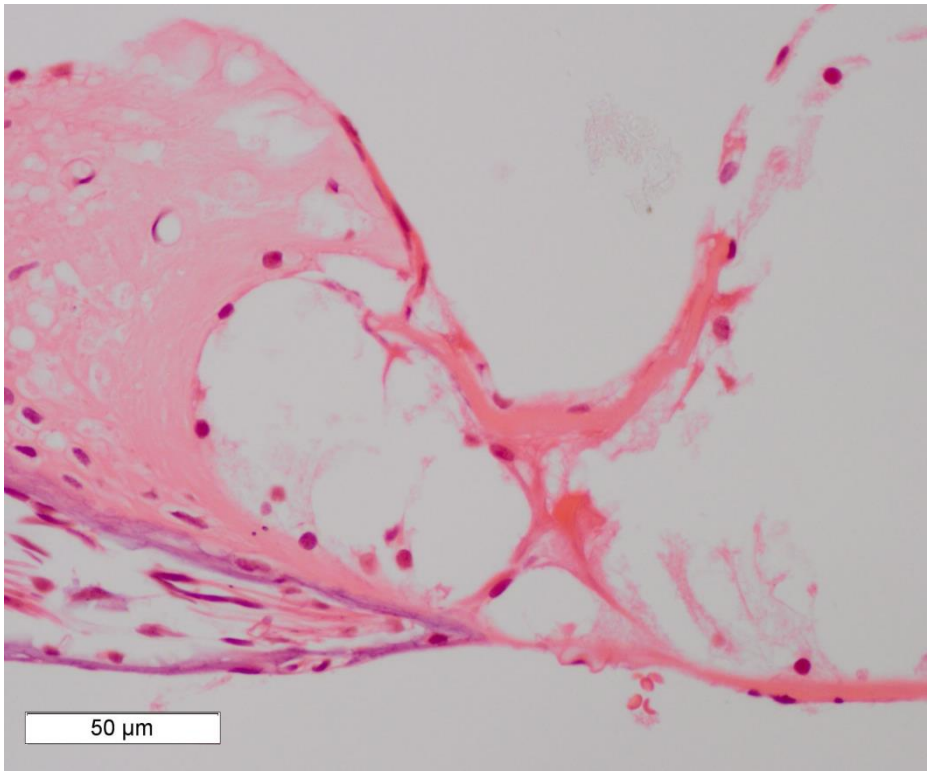

Fig.3-1. Group A : Hematoxylin and eosin-stained finding of organ of Corti (OC) in the second cochlear turn. Loss of most hair cells and supporting cells in OC and stellate fibrocytes in the limbic central zone of the spiral limbus is observed.

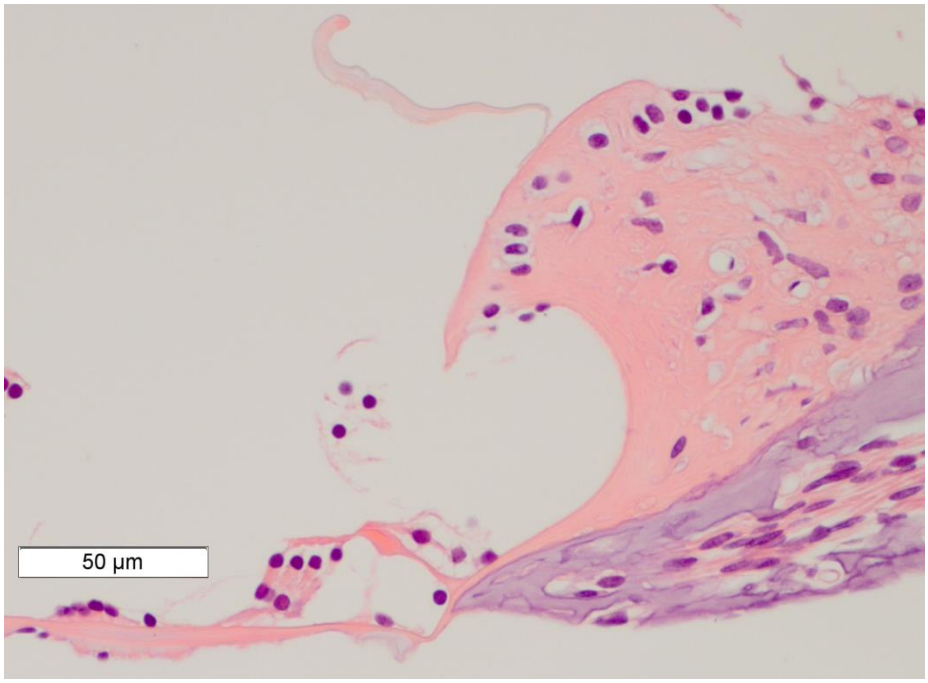

Fig.3-2. Group B : Hematoxylin and eosin-stained finding of organ of Corti in the second cochlear turn. Normal cell distribution is found.

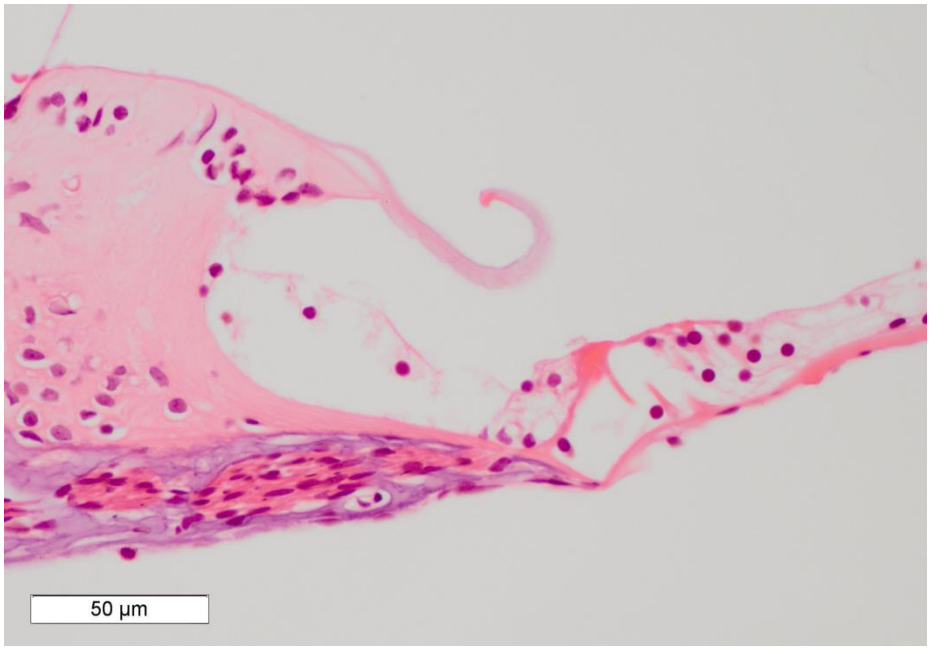

Fig.3-3. Group C : Hematoxylin and eosin-stained finding of organ of Corti (OC) in the second cochlear turn. Hair cells and supporting cells in OC and stellate fibrocytes in the limbic central zone of the spiral limbus show normal findings.

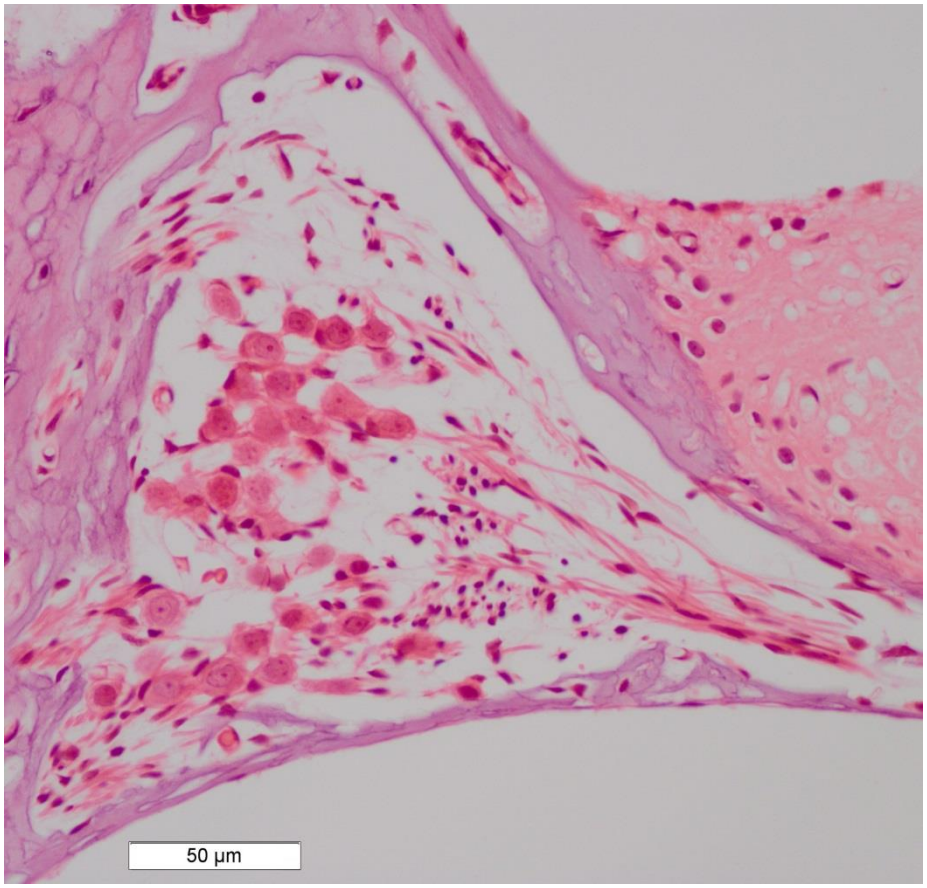

Fig.4-1. Group A : Hematoxylin and eosin-stained histological finding in the spiral ganglion (SG) cells of the second cochlear turn. Severe degeneration and loss of SG cells is shown.

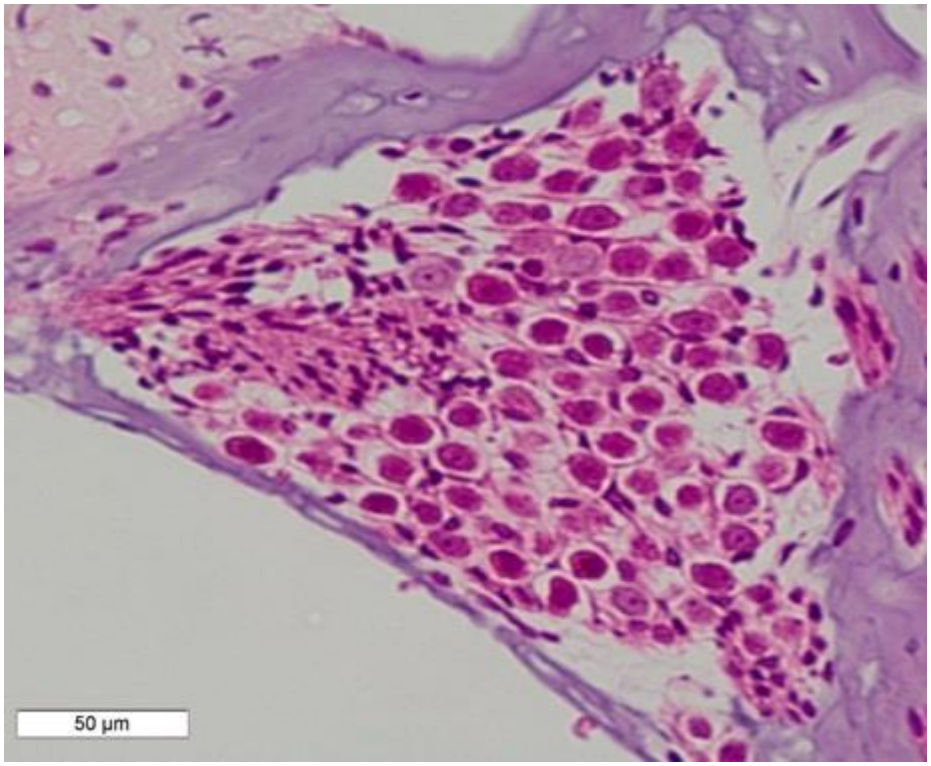

Fig.4-2. Group B : Hematoxylin and eosin-stained histological finding in the spiral ganglion (SG) cells of the second cochlear turn. Normal SG cell population is observed.

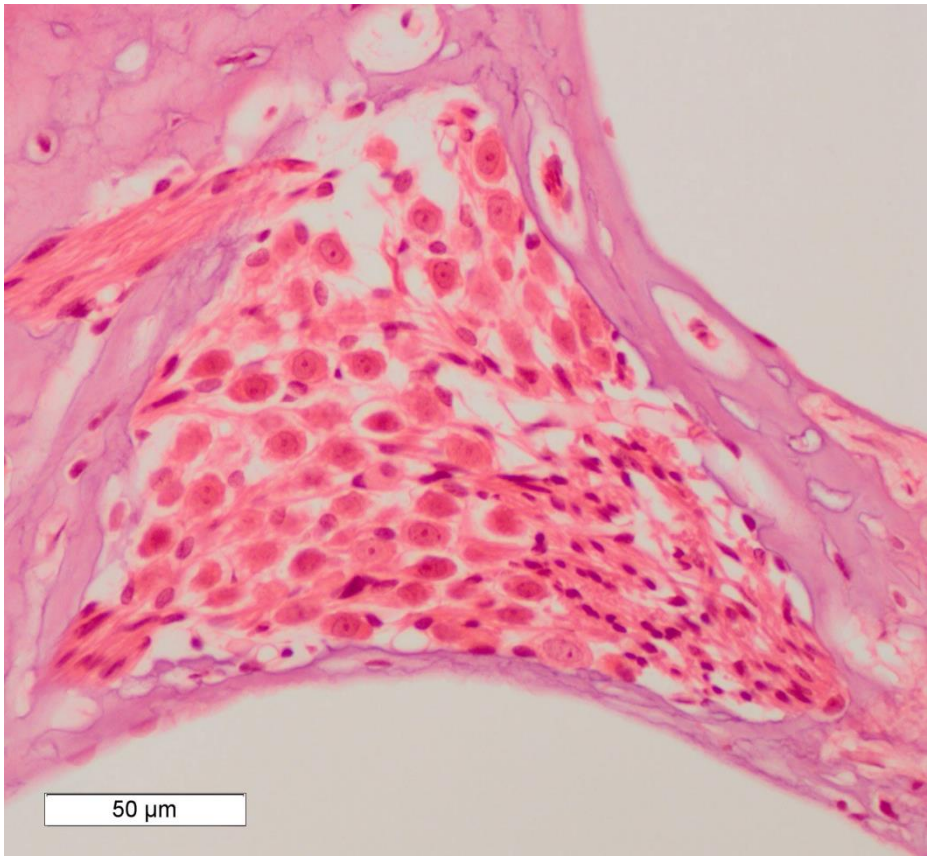

Fig.4-3. Group C : Hematoxylin and eosin-stained histological finding in the spiral ganglion (SG) cells of the second cochlear turn. Preservation of SG cells is observed.

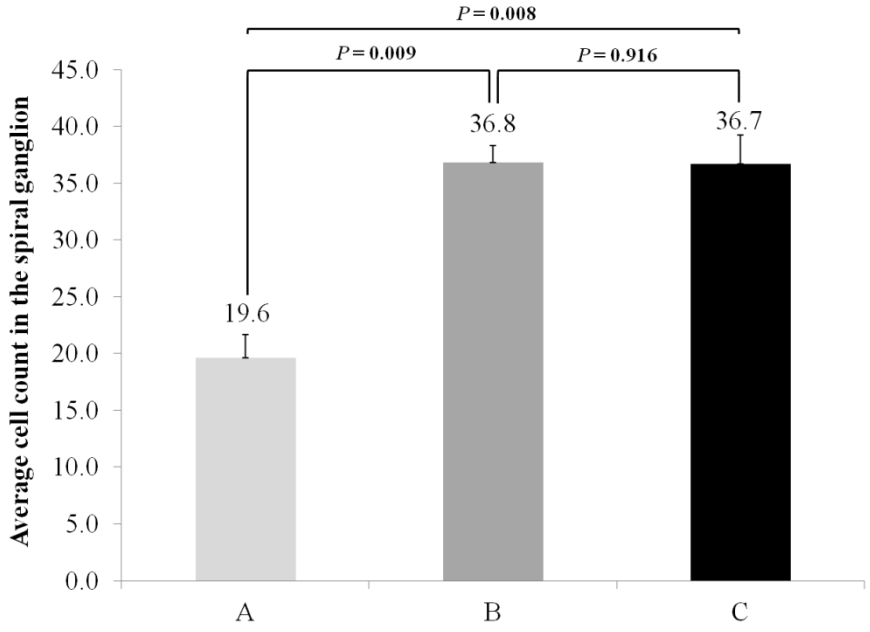

Fig.4-4. Average cell counts among 3 groups in spiral ganglion (SG). Group A showed a statistically significant decrease in cell counts than group B and C in the SG area.

Discussion

Homeostasis in the cochlear fluid and generation of the endocochlear potential are essential for the maintenance of cochlear function. Potassium ion (K^+) known to be the major charge carrier for the generation of the endocochlear potential requires changes of gradient for the ion channel activation. To maintain the K^+ gradient and its recycling, there are two independent gap junction systems in the cochlea. (14) One system is the connective tissue gap junction system. K^+ is taken up from perilymph by fibrocytes in the SL and diffuses into intermediate cells in the SV. The intermediate cells mainly generate the endocochlear potential by relieving K^+ into the intrastrial fluid. Then, K^+ in the intrastrial fluid is removed by the strial marginal cells to maintain K^+ gradient in the cochlea. The other system is the epithelial gap junction system. K^+ within the hair cells in the OC is taken up by supporting cells in the OC and then, moves toward the interdental cells in the spiral limbus and root cells within the SL. (15, 16) K^+ recycling is regulated by active K^+ transport. (17) Therefore, the ATP depletion induced by toxic agents including 3-NP and the resultant the disturbance of cochlear homeostasis and abolishment of endocochlear potential can cause a sensorineural hearing loss.

Various methods for the establishment of hearing loss animal model by the administration of 3-NP have been introduced. (2, 15, 17-23) Initially, the approach through round window niche (2, 18, 21, 23) was tried and thereafter

approaches via round window membrane (17), cochleostomy (15), and posterior semicircular canal (19) were reported. Recently, the intratympanic injection of 3-NP also showed the significant increase of hearing thresholds. (20, 22) The intratympanic injection has some advantages compared with other administration methods. That is, the injection technique is simple and minimally invasive, therefore the mechanical damage to the intracochlear structure may be prevented. However, if the intratympanic administration of 3-NP is decided, the delivery of the protective agent using the same administration route should be avoided because 3-NP induces the severe proliferation of mucosa in the middle ear cavity.

The present study also showed the thinning of SV and degeneration of the fibrocytes in the SL, hair cells and supporting cells in OC, and ganglion cells in the SG area. By the diffusion of 3-NP into the cochlea, acute mitochondrial dysfunction and the resultant histological changes in the cochlea can be induced. (18) Fibrocytes in the SL were known to be the major susceptible structure to 3-NP exposure. (18) And, some authors reported that 3-NP also induced the loss of outer hair cells in the OC and/or the degeneration of SG cell. (15, 19, 24) Changes of endolymphatic potential in the area were thought to be the cause for cellular degenerations. (19)

Recently, there were some studies reporting the protective effect of resveratrol against several ototoxic stimuli. (5, 11, 25-27) Seidman et al. suggested that resveratrol had a protective effect on noise-induced hearing loss. (11, 25) Other researchers have reported that resveratrol showed the protective effect

against ototoxicity induced by cisplatin. (4, 26, 27) The main mechanism of protection by resveratrol suggested in some studies was thought to be the anti-oxidative property of resveratrol by the down-regulation of Cyclooxygenase-2 expression. (25) In previous some studies, resveratrol was administrated before exposure to ototoxic stimuli (11, 27), whereas other authors administered resveratrol both before and after exposure to the stimuli (4, 25, 26). In the present study, 3-NP was injected in the middle of administration periods (5 days) of resveratrol to evaluate the protective effect of resveratrol against acute cochlear damage by 3-NP as well as its preventive effect.

The main route into the cochlea of intratympanically delivered ototoxic agents is thought to be through the round window. Therefore, the hearing loss induced by ototoxic agents will mainly affect high frequency ranges. In this study, however, the ABR threshold at the high frequency after 3-NP injection was not severe compared to those at 8 and 16 kHz. High dose of ototoxic agents exceeded a maximum limit of hearing thresholds and there was no significant difference in degrees of hearing loss at each frequency. (18, 19) The present study showed the big shift of ABR thresholds at 8 and 16 kHz in group C treated with resveratrol and 3-NP injection. This finding may be explained by that the restoration of ABR threshold at the high frequency may be disturbed due to more toxic effect of 3-NP around the round window membrane.

The present study investigated the functional restoration and structural preservation of the cochlea after administration of resveratrol in 3-NP-induced

hearing loss. One week after administration of 3-NP and resveratrol, group C showed elevated ABR threshold. However, two and four weeks after administration, this group revealed a progressive recovery of ABR thresholds. By the analogy from this result, resveratrol may play a role of cellular protection or restoration in the cochlea. The effect of resveratrol administration after ischemic damage was also reported in the neurovascular injury model. (28)

With the administration of resveratrol, the fibrocytes in SL, the hair cells in OC, and the ganglion cells in SG area showed almost normal findings. And, cell counting analysis in the area also demonstrated consistent results. Therefore, when considering with results of ABR and histological analysis, resveratrol seems to initiate main restorative action for the cellular function between 1 and 4 weeks after intracochlear diffusion of 3-NP. The present study also showed that the thickness of SV in group C was more normal than in 3-NP only group. However, this finding in group C was significantly thinner than the thickness in group B, resveratrol only group. The SV is one of main structures responsible for endolymphatic homeostasis and ototoxic stimuli to the SV is known to induce a temporary reduction of endolymphatic potential and transient hearing loss even without the damage of the OC. (29, 30) And, it was reported that the residual shrinkage of SV after damage persists even after the endolymphatic potential recovers. (29) In the present study, the atrophic finding in the SV in group C may be a form recovered from severe atrophy like in group A to some degree or it is being recovered

continuously.

Although there was no study with 3-NP and resveratrol in the otological field before this study, some authors have reported the neuroprotective effect of resveratrol against a neurodegenerative model in brain induced by 3-NP in neurological field. (3, 31, 32) In the Huntington's disease model induced by 3-NP, they suggested that the motor and cognitive impairment could be reversed with repeated administration of resveratrol for 8 days.

For further evaluation, immunocytochemistry and/or molecular biologic analysis for the investigation of the detailed protective mechanism of resveratrol is needed. Because resveratrol is present in various kinds of foods like red wine and grape skin, and peanut, the clinical application of resveratrol may be very easy and effective if the therapeutic value of resveratrol is proved in the future.

Conclusion

Administration of resveratrol showed a functional and morphological restoration from 3-NP-induced acute cochlear damage. Resveratrol may play a role of cellular protection from the cochlear damage.

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국문초록

서론: 미토콘드리아 독소로 알려진 3-nitropropionic acid (3-NP)는 미토콘드리아의 에너지 생성 기능의 손상에 의한 급성 난청을 유발한다. Resveratrol은 항산화물질로서 다양한 종류의 이독성 손상으로부터 강력한 보호효과를 가지는 것으로 알려져 있다. 이 연구의 목적은 3-NP로 인해 유발된 급성 와우 손상에 대한 resveratrol의 보호 효과를 관찰하는 것이다.

재료 및 방법: 15마리의 수컷 Sprague-Dawley rat을 세 개의 군으로 분류하였다. A군에서는 3-NP (500 mM, 4 μl) 를 고실내 주입하였고, B군에서는 resveratrol (10 mg/kg) 을 5일간 복강 내 주입하였다. C군에서는 3-NP 고실내 주입 전 resveratrol 전처치 (10 mg/kg, 5일, 복강내 주입) 을 시행하였다. 약물 주입 전후로 8, 16, 32 kHz에서 청성뇌간반응을 측정하였다. 와우의 세포변화를 관찰하기 위하여 약물 주입 4주 후, 각 군에서 와우의 조직학적 분석을 시행하였다.

결과: 3-NP를 투여한 A군의 청성뇌간반응역치는 정상소견을 보인

B군에 비해 의미있게 증가한 소견을 보였다. 그러나 3-NP 고실내 주입 전 resveratrol을 투여한 C군에서는 청성뇌간반응역치의 유의한 감소가 관찰되었다. A군의 조직학적 검사에서 나선인대의 섬유세포, 코르티 기관의 유모세포와 지지세포, 나선연의 성상 섬유세포와 치간세포, 나선신경절 세포수의 감소가 관찰되었으나 B군과 C군에서는 정상소견을 보였다. 또한 A군과 C군에서의 혈관조의 두께는 B군에 비해 감소된 소견을 보였으며, 그 중에서도 A군의 혈관조 두께가 가장 얇았다. 나선인대의 섬유세포와 나선신경절의 신경절세포수는 A군이 B군 및 C군과 비교하여 의미있게 감소했다.

결론: 본 연구결과 resveratrol은 3-NP로 인한 급성 와우 손상에 대해 보호효과를 가진다.

주요어: Resveratrol, 3- nitropropionic acid, 이독성

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