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Original Article

Altered Mitochondrial Functions and Morphologies in Epithelial Cells Are Associated With Pathogenesis of Chronic Rhinosinusitis With Nasal Polyps

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ABSTRACT

Purpose: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a complex inflammatory disease of the nasal and paranasal sinus mucosa. The disease is associated with mitochondrial dysfunction, structural changes in the mitochondria, and reactive oxygen species (ROS) generation. This study investigated whether there are functional and morphological changes in the mitochondria in the epithelial cells of nasal polyps (NPs) and *Staphylococcus aureus* enterotoxin B (SEB)-stimulated nasal epithelial cells.
Methods: In all, 30 patients with CRSwNP and 15 healthy subjects were enrolled. Mitochondrial ROS (mtROS) and changes in mitochondrial functions and structures were investigated in the uncinate tissue (UT) of healthy controls, the UT or NPs of CRSwNP patients, and human nasal epithelial cells with or without SEB stimulation.
Results: Oxidative phosphorylation complexes showed various responses following SEB stimulation in the nasal epithelial cells, and their expressions were significantly higher in the NPs of patients with CRSwNP than in the UT of controls. Generation of mtROS was increased following SEB exposure in nasal epithelial cells and was reduced by pretreatment with MitoTEMPO, which is used as an mtROS scavenger. In the tissues, mtROS was significantly increased in the NPs of CRSwNP patients compared to the UT of controls or CRSwNP patients. The expressions of fusion- and fission-related molecules were also significantly higher in SEB-exposed nasal epithelial cells than in non-exposed cells. In tissues, the expression of fission (fission mediator protein 1)- and fusion (membrane and mitofusin-1, and optic atrophy protein 1)-related molecules was significantly higher in the NPs of CRSwNP patients than in UT of controls or CRSwNP patients. Transmission electron microscopy revealed elongated mitochondria in SEB-exposed nasal epithelial cells and epithelial cells of NPs.

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Disclosure
There are no financial or other issues that might lead to conflict of interest.

Conclusions: Production of mtROS, disrupted mitochondrial function, and structural changes in nasal epithelial cells might be involved in the pathogenesis of CRSwNP.

Keywords: Rhinitis; mitochondria; reactive oxygen species; nasal polyps; *Staphylococcus*; *enterotoxin*; epithelial cells

INTRODUCTION

Chronic rhinosinusitis (CRS) is a common inflammatory disease of the nasal and paranasal sinus mucosa. Based on phenotype analyses, it can be subdivided into chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP).[1] CNP is often associated with lower airway disease and has numerous predisposing factors.[1] Although many studies have attempted to clarify the mechanisms of polyp generation, the exact etiology and pathophysiology have not yet been fully elucidated.[2,3]

The epithelial cells of the nasal and paranasal sinus mucosa are the primary sites involved in the response to various pathogens, leading to the production of various proinflammatory and inflammatory cytokines. Among the pathogens, *Staphylococcus aureus* enterotoxin B (SEB) is found in the nasal cavity and polyp tissues of CRSwNP patients and can induce the production of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress in epithelial cells in CRSwNP.[4,5] In addition, repetitive exposure to SEB induce eosinophilic inflammation and generates polyp-like lesions in a murine model.[4,6]

Mitochondria are intracellular organelles with a double membrane. Mitochondria function to generate energy as adenosine triphosphate (ATP) through oxidative phosphorylation. However, it is increasingly clear that mitochondria are not merely energy factories; they also regulate various cellular functions.[7,8] Major inflammatory and stress signaling networks are linked to mitochondrial dysfunction, structural change, and mitophagy signaling pathways.[7,8] Damage to the mitochondrial dynamics or mitophagy can lead to an increase in the release of ROS and result in inflammation.

Mitochondrial dysfunction and elevated ROS have been reported in a wide variety of allergic diseases, such as atopy, asthma, and allergic rhinitis.[9-12] Environmental pollutants and allergens can cause mitochondrial dysfunction by inducing ROS production and the ER stress response in airway epithelial cells.[7] House dust mite treatment in airway epithelial cells increases the contact between the stressed ER and mitochondria, leading to the assembly of dynamin-related protein 1 (Drp1, a mitochondrial fusion protein) at the point of contact. Drp1 assembly results in mitochondrial fragmentation and the production of proinflammatory cytokines.[7,13,14]

Mitochondrial ROS (mtROS) are produced as a byproduct of oxidative phosphorylation in the mitochondrial electron transport chain. Mitochondria are able to control ROS by manganese-dependent superoxide dismutase (Mn-SOD), which is a scavenger of mtROS; however, several other factors, including various pathogens and cigarette smoke, inhibit the function of Mn-SOD and lead to ROS production[8,15] Mitochondria are protected from oxidative stress via the dynamic regulation of their structures through fusion and fission.[9] Drp1 and fission mediator protein 1 (Fis1) are fission proteins that divide mitochondria.[16] Meanwhile, optic atrophy protein 1 (OPA1) on the inner membrane and mitofusin-1 (Mfn1) on the outer membrane are responsible for mitochondrial fusion.[9]

In this study, we hypothesized that mtROS and changes in mitochondrial structure and function may be associated with the pathogenesis of CRSwNP. We investigated whether there would be functional and morphological changes in the mitochondria in epithelial cells of nasal polyps (NPs) and SEB-stimulated nasal epithelial cells.

MATERIALS AND METHODS

Patients and tissue preparation

From January 2019 to January 2020, a total of 56 patients were enrolled. NP tissues and uncinate tissue (UT) was obtained from 30 patients who underwent endoscopic sinus surgery for CRSwNP. UT for the control group was collected from 15 patients who underwent dacryocystorhinostomy.

Patients who had taken oral or nasal glucocorticoids, antibiotics, or anti-leukotrienes within 4 weeks before surgery were excluded, as were those who had an upper respiratory tract infection, a recent history of smoking, or prior sinus surgery (**Table 1**).

Informed consent was obtained before human tissues were collected. The Institutional Review Board of Chungnam National University Hospital approved this study (approval No. CNUH 2018-12-025).

Reagents and nasal epithelial cells

SEB was purchased from List Biological Laboratories Inc. (Campbell, CA, USA) and MitoTEMPO antioxidant for mtROS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The human nasal epithelial cell line RPMI 2650 (KCLB10030; KCLB, Seoul, Korea) was cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum, penicillin (100 IU/mL) (Sigma-Aldrich), streptomycin (100 μg/mL) (Sigma-Aldrich), and 1% L-glutamine (Sigma-Aldrich). The cells were cultured at 37°C in 95% humidified air and 5% CO_2 in 75-cm^2 culture flasks.

Primary human nasal epithelial cells (pHNECs) were isolated from CRSwNP obtained from patients undergoing elective endoscopic sinus surgery. The specimens were digested in 5 μg/mL Dispase® (Roche, Indianapolis, IN, USA) and incubated at 37°C for 30 minutes. In addition, the epithelial layer was separated from the specimen, and trypsin-ethylenediaminetetraacetic acid (EDTA) was added before incubation for 10 minutes. The specimens in the trypsin-EDTA suspension were filtered through a 70-μm cell strainer (SPL, Pocheon, Korea) to remove any undigested tissue and washed twice with DMEM. Then, the tube was centrifuged at 1,300 RPM for 3 minutes. The obtained primary cells were cultured in airway epithelial cell growth media supplemented with a mixture of penicillin G (10,000

Table 1. Demographics of the study subjects

Group	Control	CNP
Subject	19	37
Gender (man:woman)	9:10	22:15
Mean age (yr)	54.4	53.5
Asthma	0	1
Atopy	0	0
Lund-Mackay CT score (mean)	0	14.9

CRSwNP, chronic rhinosinusitis with nasal polyps; CT, computed tomography.

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U/mL), streptomycin (10,000 μg/mL), and amphotericin B (25 μg/mL). Then the cells were placed in a humidified chamber and incubated at 37°C, 5% CO_2, and 95% air for 2 weeks. The second cell passage provided all of the cells used in this study.

The mtROS and mitochondrial membrane potential analyses

The mtROS activity was detected before and after SEB exposure in live cells using dihydroethidium derivatives MitoSOX red (Invitrogen, Carlsbad, CA, USA). RPMI 2650 cells were incubated with 5 μmol/L MitoSOX for 30 minutes at 37°C and 5% CO_2. The cells were imaged using an LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) and analyzed using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). MitoTracker Red CMXRos (Invitrogen) was used to stain live nasal epithelial cells before and after exposure to SEB (5 μg/mL) and/or MitoTEMPO (1 μM) for 48 hours, according to the manufacturer's protocol, to determine changes in the mitochondrial membrane potential.

Western blotting

Total protein concentration was determined using the Bradford assay (Cell Signaling Technology, Denver, MA, USA). Samples were resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to 0.45-μM polyvinyl difluoride membranes, and analyzed separately. After blocking with 5% skim milk at room temperature for 60 minutes, the blots were surveyed with primary antibodies. Expression of mitochondrial antioxidant was detected using anti-Mn-SOD (Abcam, Cambridge, MA, USA). We used anti-NDUFA9 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-SDHB (Abcam), anti-UQCRC2 (Abcam), anti-COXIV (Santa Cruz Biotechnology), and anti-ATP5A (Abcam) to detect the expression of oxidative phosphorylation complexes.

Immunofluorescence staining (IF) of NPs

We performed hematoxylin and eosin staining to identify eosinophils and non-eosinophils. Slides were first examined under 400× magnification, and the number of eosinophils was counted in 5 random high power fields (HPFs). Eosinophilic CRS was histopathologically defined when the eosinophil count was > 70/HPF.

Mn-SOD antibody (Abcam) was applied as the primary antibody for the IF of NP tissue. Alexa Fluor® 488-conjugated secondary antibodies were subsequently used at a concentration of 10 μg/mL against the primary antibodies and 4′,6-diamidino-2-phenylindole (Invitrogen) was used at a concentration of 300 nM for nuclear counterstaining. The slides were subsequently observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of the oxygen consumption rate (OCR)

The OCR was measured using a Seahorse XF24 analyzer (Seahorse Bioscience Inc., North Billerica, MA, USA). Control cells, and SEB-treated and SEB/MitoTEMPO-treated cells were seeded on XF-24 plates (1.5×10^4 cells in 200 μL growth medium per well) and incubated at 37°C/5% Co_2 for 48 hours. Three readings were taken after each addition of mitochondrial inhibitor. Oligomycin (2 μm/mL), carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 5 μM) and rotenone (2 μM) were used as the mitochondrial inhibitors and were injected at 15, 30, and 45 minutes after measuring the basal respiration. The OCR was automatically calculated by the Seahorse XF-24 software.

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Real-time quantitative polymerase chain reactions (RT-qPCR)

The messenger RNA (mRNA) expression levels of target genes were detected by RT-qPCR. The primers were purchased from GenoTech (GenoTech, Daejeon, Korea), and the primer sequences are listed in **Supplementary Table S1**.

Total RNAs from the tissues were extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. A similar amount of tissues and cells was used for each subject. AccuPower® RT PreMix (Bioneer, Daejeon, Korea) was used for complementary DNA (cDNA) synthesis according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed for cDNA synthesis using a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA expression was analyzed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories) with PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). All PCR assays were performed in triplicate. The relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Transmission electron microscopy

The tissue samples were fixed in a 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 hours, post-fixed in 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in graded ethanol and propylene oxide, and embedded in Epon-812. Ultra-thin sections made using an Ultracut E Ultramicrotome (Leica, Vienna, Austria), were stained with uranyl acetate and lead citrate and examined under a CM 20 electron microscope (Philips, Amsterdam, Netherlands).

Statistical analyses

Values are presented as histograms with the mean and standard error of the mean (SEM), or a scatter plot with mean and SEM. Differences between 2 groups were analyzed with a Wilcoxon matched-pairs signed rank test (2-tailed), while differences among more than 2 groups were analyzed using nonparametric Kruskal-Wallis tests (2-tailed) and 2-way analysis of variances. Correction for multiple comparisons was performed using the Bonferroni method. The Spearman's rank correlation coefficient was used to determine variable relationships. A P value of < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 22 (version 22.0.0.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism 6 (version 6.01; GraphPad Software, La Jolla, CA, USA).

RESULTS

Mitochondrial bioenergetics are altered in SEB-exposed nasal epithelial cells and NPs of patients with CRSwNP

To measure the bioenergetics of the mitochondria, we examined the protein expression of oxidative phosphorylation in enzyme complexes I-V in human nasal epithelial cells that were exposed to SEB and human NP tissues.

There were no significant differences in the expression of oxidative phosphorylation complex I (NDUFA9) following SEB treatment. Expression of oxidative phosphorylation complex II (SDHB) increased significantly with 0.1 μg/mL SEB stimulation and then showed a tendency to decrease with a higher dose. Expression of oxidative phosphorylation III (UQCRC2) enzymes increased significantly with 0.1, 1, and 5 μg/mL SEB treatment compared to the control. Expression of oxidative phosphorylation IV (COXIV) enzymes increased only with

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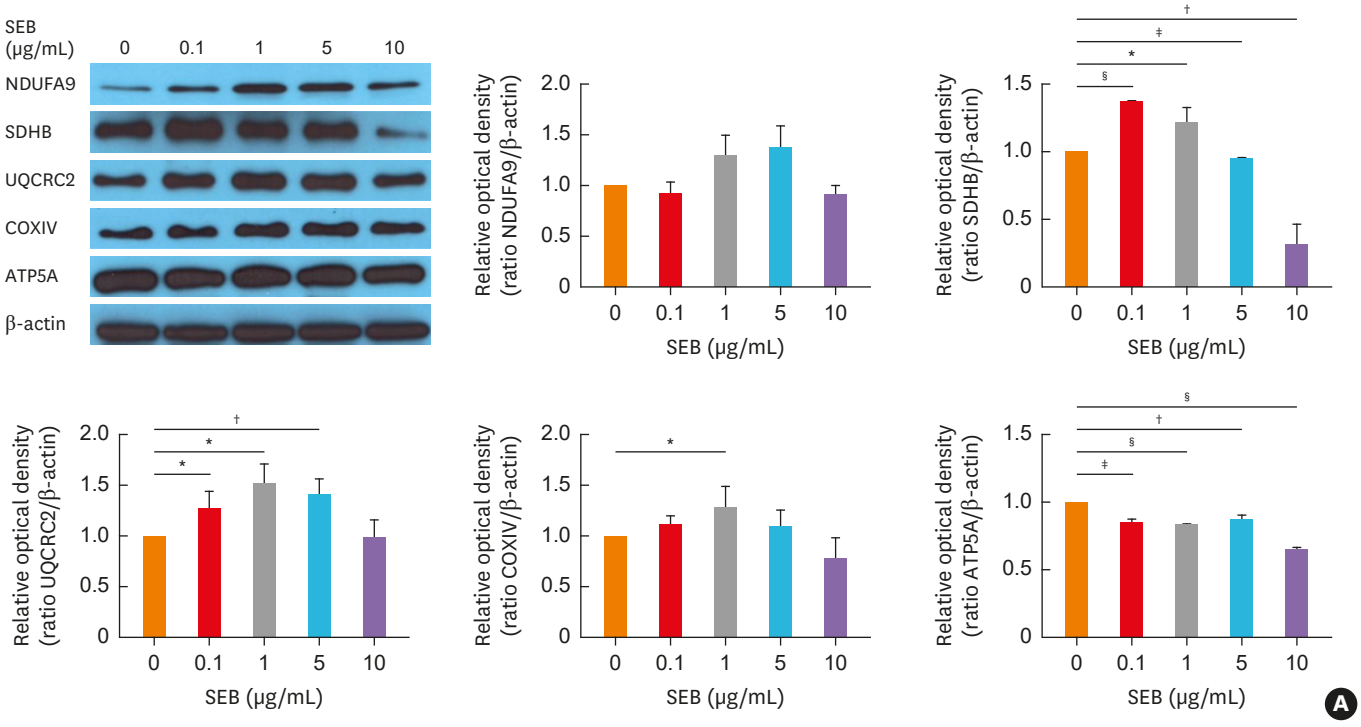
1 μg/mL SEB treatment compared to the control. Interestingly, the expression of oxidative phosphorylation V (ATP5A) enzymes significantly decreased with a given concentration of SEB (**Fig. 1A**).



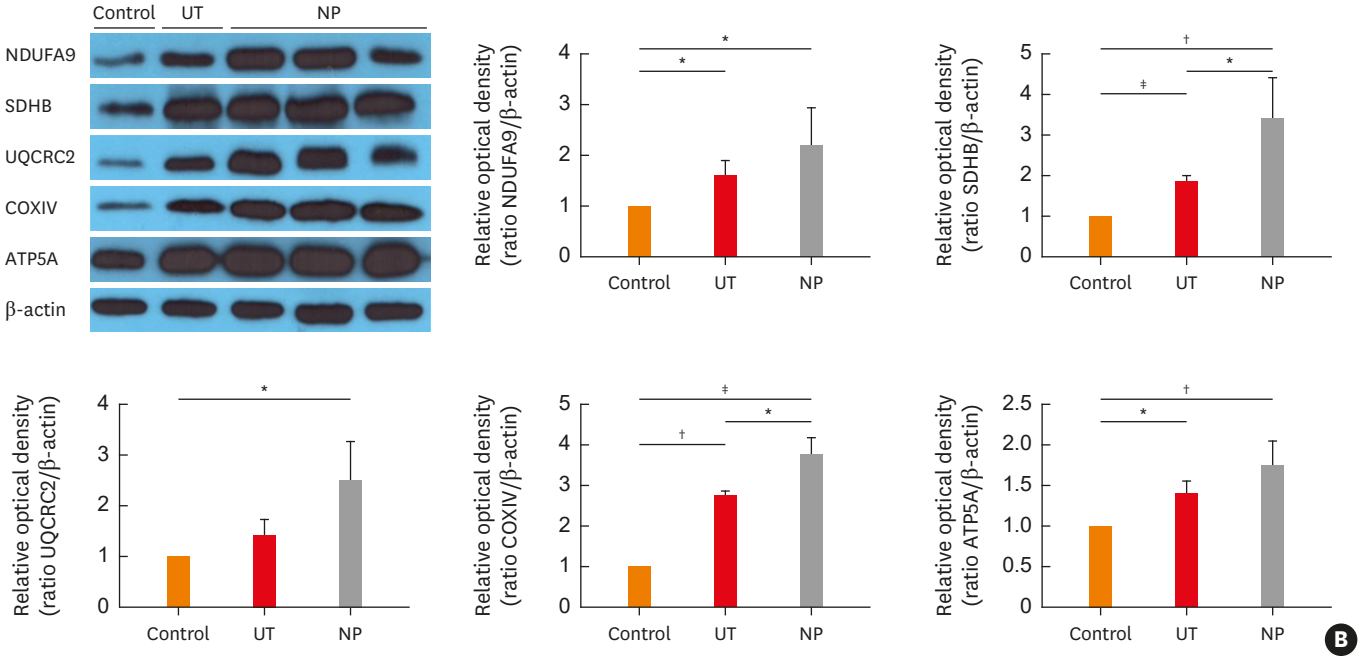


Fig. 1. Expression of oxidative phosphorylation complex enzymes and OCRs. (A) Oxidative phosphorylation complex I–V expression levels in RPMI 2650 cells exposed to different doses of SEB for 48 hours. (B) Oxidative phosphorylation complex I–V expression levels in NP tissue (control, n = 4; UT, n = 8; NP, n = 8). (C) OCRs in RPMI 2650 cells exposed to SEB (5 μg, 48 hours) and SEB (5 μg, 48 hours) with MitoTEMPO treatment (10 μmol/L).
OCR, oxygen consumption rate; SEB, *Staphylococcus aureus* enterotoxin B; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; UT, uncinate tissue; NP, nasal polyp.
$^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.005$; $^{\S}P < 0.001$.
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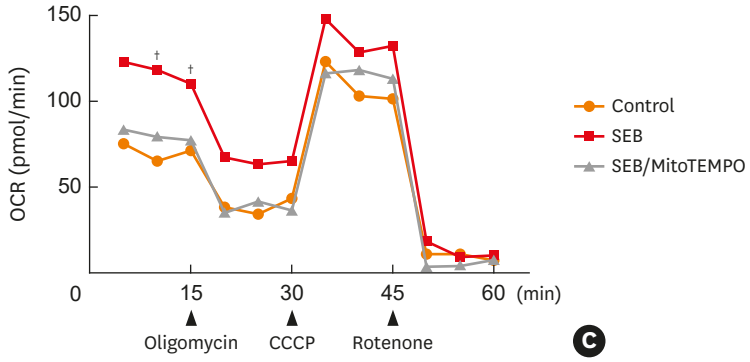

Fig. 1. (Continued) Expression of oxidative phosphorylation complex enzymes and OCRs. (A) Oxidative phosphorylation complex I–V expression levels in RPMI 2650 cells exposed to different doses of SEB for 48 hours. (B) Oxidative phosphorylation complex I–V expression levels in NP tissue (control, n = 4; UT, n = 8; NP, n = 8). (C) OCRs in RPMI 2650 cells exposed to SEB (5 μg, 48 hours) and SEB (5 μg, 48 hours) with MitoTEMPO treatment (10 μmol/L).
OCR, oxygen consumption rate; SEB, *Staphylococcus aureus* enterotoxin B; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; UT, uncinate tissue; NP, nasal polyp.
$^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.005$; $^{\S}P < 0.001$.

Expression of oxidative phosphorylation complexes was significantly higher in the tissues (UT and NP) in CRSwNP compared to the control, except for complex III (**Fig. 1B**). There were significant differences between UT and NP in CRSwNP for the expression of complexes II and IV, and expression of complexes II and IV were significantly higher in NP compared to UT in CRSwNP (**Fig. 1B**).

The OCR significantly increased with SEB treatment (5 μg/mL, 48 hours) compared to the basal status (control), and it significantly decreased with MitoTEMPO treatment in human nasal epithelial cells (**Fig. 1C**). Mitochondrial respiration decreased at a similar rate after treatment with oligomycin, which is a complex V (ATPase) inhibitor and represents ATP-linked respiration, regardless of SEB or SEB/MitoTEMPO treatment (**Fig. 1C**). After treatment with CCCP, which allows proton influx to the inner membrane, mitochondrial respiration reached maximal capacity in all groups. Following treatment with rotenone which is a complex I inhibitor, mitochondrial respiration was shut down (**Fig. 1C**).

The mtROS increases in SEB-stimulated human nasal epithelial cells and the epithelial cells of NPs

After stimulation with SEB (5 μg/mL, 48 hours), mtROS was measured in nasal epithelial cells (**Fig. 2A**) and pHNECs (**Fig. 2B**) using the MitoSOX immunofluorescence assay. The level of mtROS increased after SEB exposure and effectively reduced after treatment with MitoTEMPO, a scavenger of mtROS (**Fig. 2A and B**). Next, we examined the expression of Mn-SOD, an antioxidant for mtROS, on human nasal epithelial cells (**Fig. 2C**), pHNECs (**Fig. 2D**), and human NP tissues (**Fig. 2E**). Mn-SOD increased significantly in the cells exposed to SEB and reduced when cells were treated with MitoTEMPO (**Fig. 2C and D**). These findings suggest that mtROS increased after SEB exposure in the nasal epithelium and that Mn-SOD increases in response to the increase in mtROS. In human tissues, the level of Mn-SOD expression was significantly higher in NP in CRSwNP than in the control or UT in CRSwNP (**Fig. 2E**). In NP, elevated Mn-SOD expression mainly occurred in the epithelial layer (**Fig. 2F**).

SEB exposure induces mitochondrial aggregation

To investigate whether mitochondrial damage was induced by excessive oxidative stress, we investigated the mitochondrial membrane potential status using MitoTracker Red. Mitochondrial membrane potential was increased rather than depolarized after

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administration of SEB (**Fig. 3**). Treatment with MitoTEMPO to SEB-exposed cells showed slightly reduced mitochondrial membrane potential compared to cells exposed to SEB only (**Fig. 3**). The intracellular distribution of the mitochondria also changed following SEB exposure, and the mitochondria in most cells displayed a pattern of aggregation compared to the control. After treatment with MitoTEMPO, the aggregation decreased (**Fig. 3**).

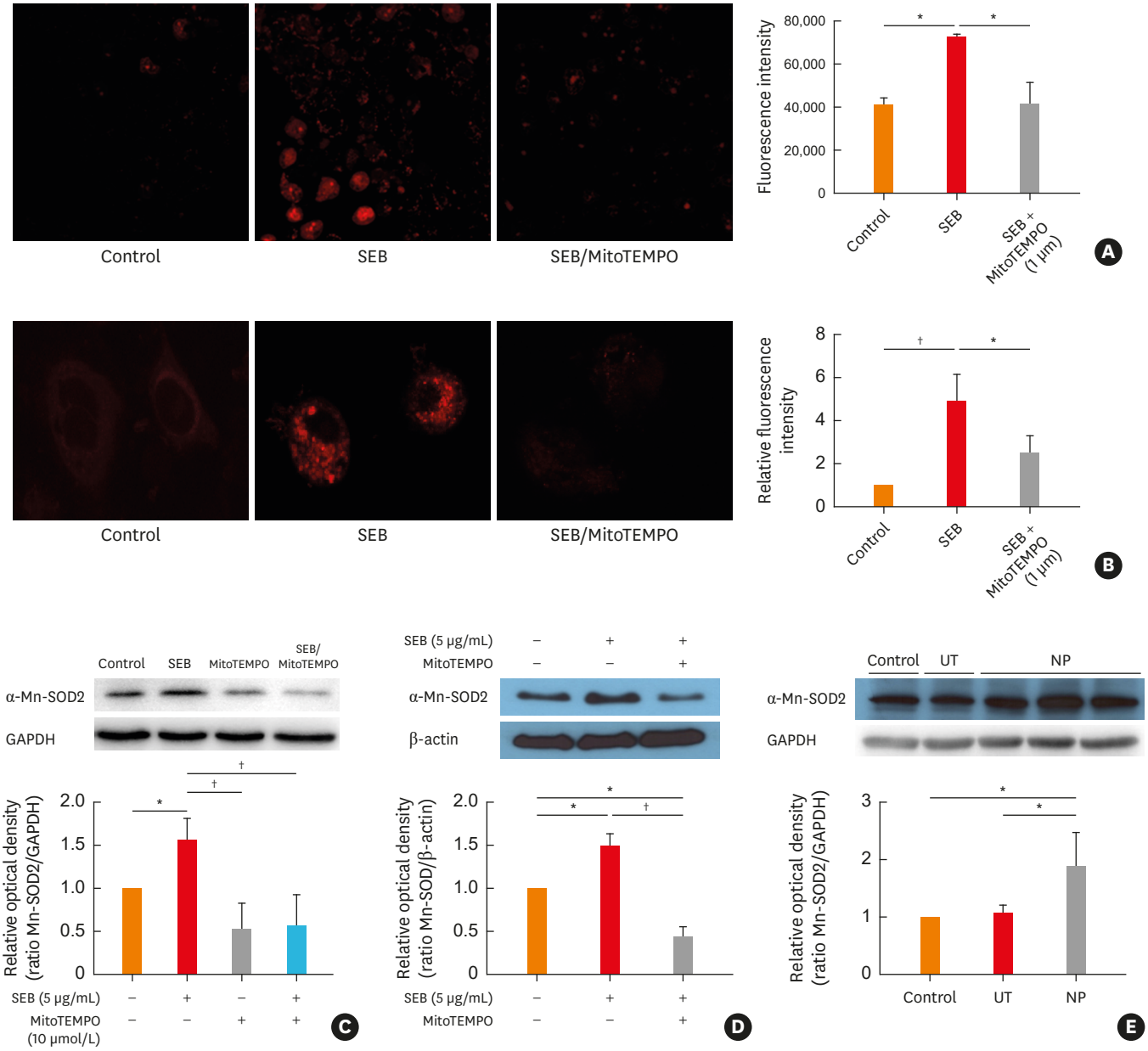

Fig. 2. The mtROS and Mn-SOD expression in nasal epithelial cells and NP tissue. Confocal images showing mtROS expression using MitoSOX after exposure to 0 and 5 μg of SEB (48 hours) on human nasal epithelial cells (RPMI 2650) (A) and pHNECs (B) with the presence and absence of MitoTEMPO (10 μmol/L). Mn-SOD expression levels in RPMI 2650 cells (C) and pHNECs (D) following SEB exposure with MitoTEMPO treatment. (E) Mn-SOD expression in NP tissues (n = 6). (F) Immunofluorescence image (×400) of Mn-SOD in NP tissue (control, n = 3; NE-NP, n = 4; E-NP, n = 4).
mtROS, mitochondrial ROS; SEB, *Staphylococcus aureus* enterotoxin B; Mn-SOD, manganese-dependent superoxide dismutase; NP, nasal polyp; pHNEC, primary human nasal epithelial cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; UT, uncinate tissue; DAPI, 4′,6-diamidino-2-phenylindole; NE-NP, non-eosinophilic nasal polyp; E-NP, eosinophilic nasal polyp.
*P < 0.05, †P < 0.01.

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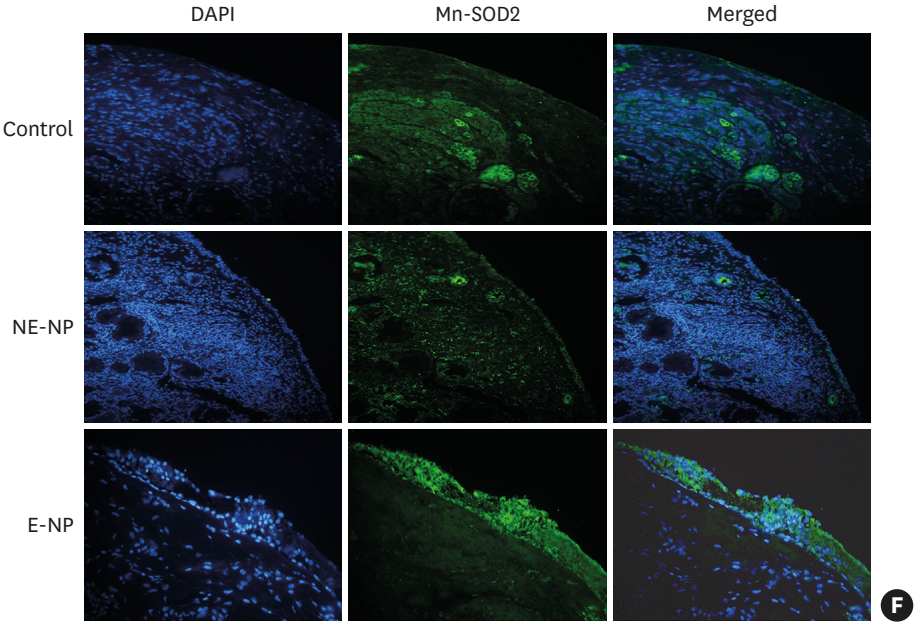

Fig. 2. (Continued) The mtROS and Mn-SOD expression in nasal epithelial cells and NP tissue. Confocal images showing mtROS expression using MitoSOX after exposure to 0 and 5 μg of SEB (48 hours) on human nasal epithelial cells (RPMI 2650) (A) and pHNECs (B) with the presence and absence of MitoTEMPO (10 μmol/L). Mn-SOD expression levels in RPMI 2650 cells (C) and pHNECs (D) following SEB exposure with MitoTEMPO treatment. (E) Mn-SOD expression in NP tissues (n = 6). (F) Immunofluorescence image (×400) of Mn-SOD in NP tissue (control, n = 3; NE-NP, n = 4; E-NP, n = 4).
mtROS, mitochondrial ROS; SEB, *Staphylococcus aureus* enterotoxin B; Mn-SOD, manganese-dependent superoxide dismutase; NP, nasal polyp; pHNEC, primary human nasal epithelial cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; UT, uncinate tissue; DAPI, 4′,6-diamidino-2-phenylindole; NE-NP, non-eosinophilic nasal polyp; E-NP, eosinophilic nasal polyp.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$.

This suggests that SEB affected mitochondrial activity rather than damaged the mitochondria because aggregations are representative of increased respiratory chain activity.[17]

Mitochondrial structures are dysregulated in SEB-stimulated human nasal epithelial cells and NPs

To assess whether SEB exposure induced changes in mitochondrial structures, mRNA expression of fission markers (Drp1 and Fis1) and fusion-related genes (Mfn1 and OPA1) were evaluated in human nasal epithelial cells using RT-PCR.

Expression of Drp1 significantly increased in a dose-dependent manner following treatment with SEB, and Fis1 expression also significantly increased compared to the control (**Fig. 4A**). Expression of both Mfn1 and OPA1 also significantly increased following SEB stimulation. Interestingly, the expression of Mfn1 significantly decreased after treatment with 10 μg/mL SEB compared to 1 μg/mL SEB (**Fig. 4A**).

The mRNA expression of Drp1 was significantly higher in the tissues (UT and NP) in CRSwNP compared to UT in the control. The expression of Fis1, Mfn1, and OPA1 was also significantly higher in NP in CRSwNP compared to UT in CRSwNP, or the control (**Fig. 4B**).

To assess whether the morphological changes in the mitochondria were followed by altered expression of the fusion and fission proteins, we examined the ultrastructure of mitochondria using a TEM. The mitochondria exhibited various sizes and morphologies in the epithelial cells of the control tissue (**Fig. 5A and B**). However, in the epithelial cells of NPs, we observed increased numbers of fragmented mitochondria with vacuoles (arrow in

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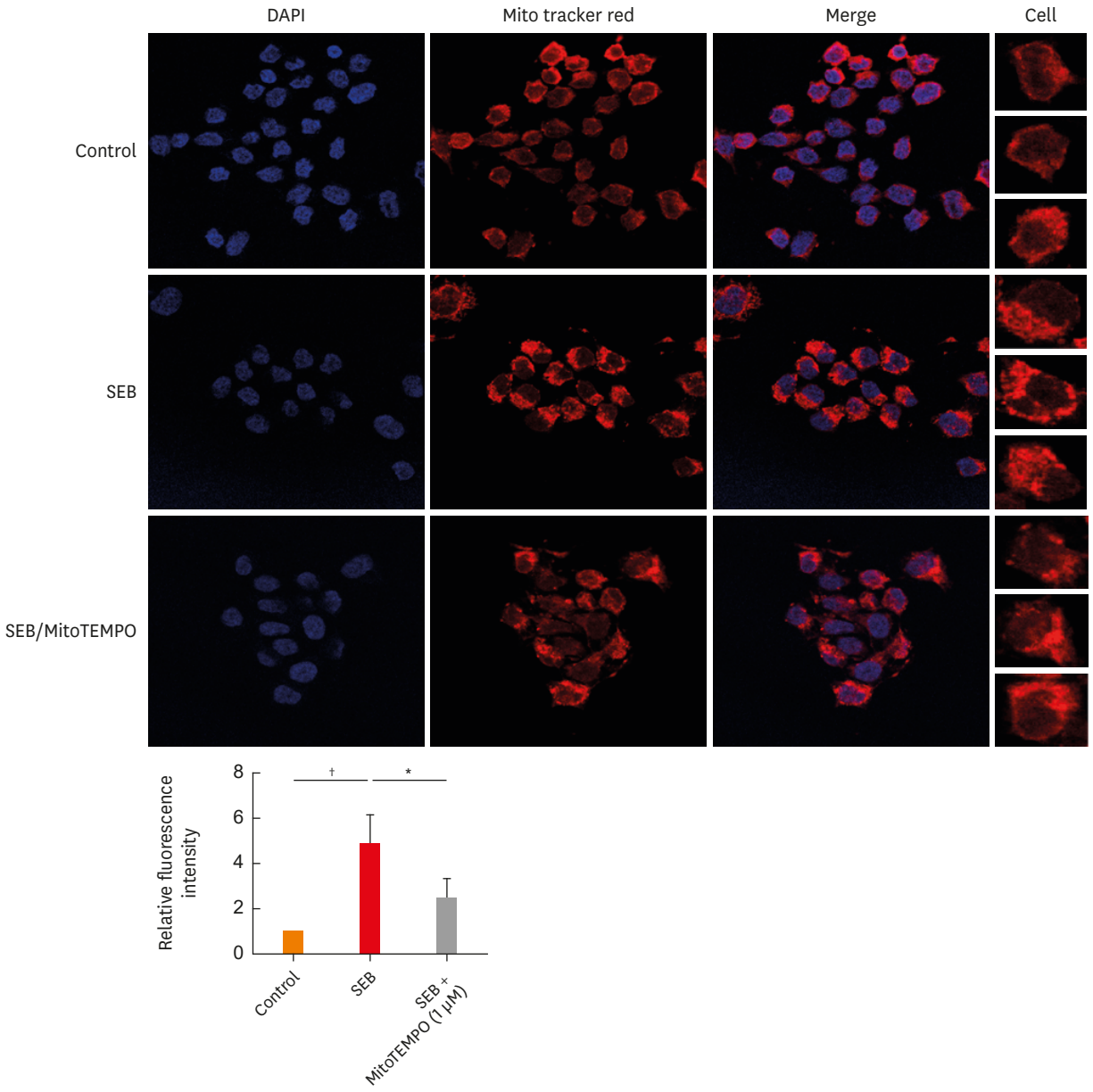

Fig. 3. The membrane potential of mitochondria in nasal epithelial cells. Confocal microscope image showing the membrane potential status of mitochondria using MitoTracker Red in RPMI 2650 cells exposed to 0 and 5 μg of SEB for 48 hours with or without MitoTEMPO treatment (10 μmol/L).
SEB, *Staphylococcus aureus* enterotoxin B; DAPI, 4′,6-diamidino-2-phenylindole.
*P < 0.05, †P < 0.01.

Fig. 5C) and mitochondria with unified elongated morphology (dotted line in **Fig. 5D**). In SEB-exposed human nasal epithelial cells, number of elongated mitochondria, which are indicative of a hyper-fusion state, also increased compared with control (**Fig. 5E and F**).

Mitochondrial turnover increases in SEB-stimulated human nasal epithelial cells and NPs

PTEN induced kinase 1 (PINK1), a marker of mitochondrial turnover, is activated for mitophagy when the mitochondria are damaged due to ROS or oxidative stress.[9] We found that oxidative stress increased in NP in CRSwNP and human nasal epithelial cells treated with

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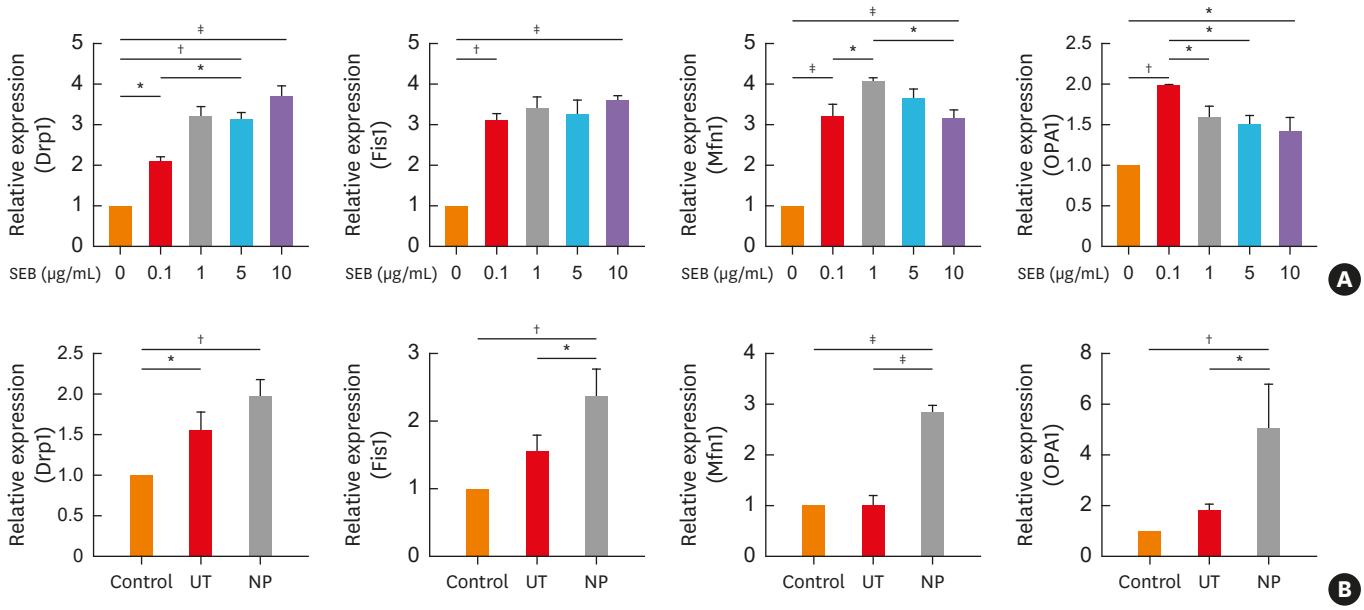

Fig. 4. The mRNA expression of fission and fusion markers. (A) The effects of SEB on mRNA expression of fission- and fusion-related genes in RPMI 2650 cells exposed to different doses of SEB. (B) The mRNA expression of fission- and fusion-related genes in NP tissue (control, n = 4; UT, n = 4; NP, n = 4).
SEB, *Staphylococcus aureus* enterotoxin B; mRNA, messenger RNA; NP, nasal polyp; UT, uncinate tissue; Drp1, dynamin-related protein 1; Fis1, fission mediator protein 1; Mfn1, membrane and mitofusin-1; OPA1, optic atrophy protein 1.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.005$.

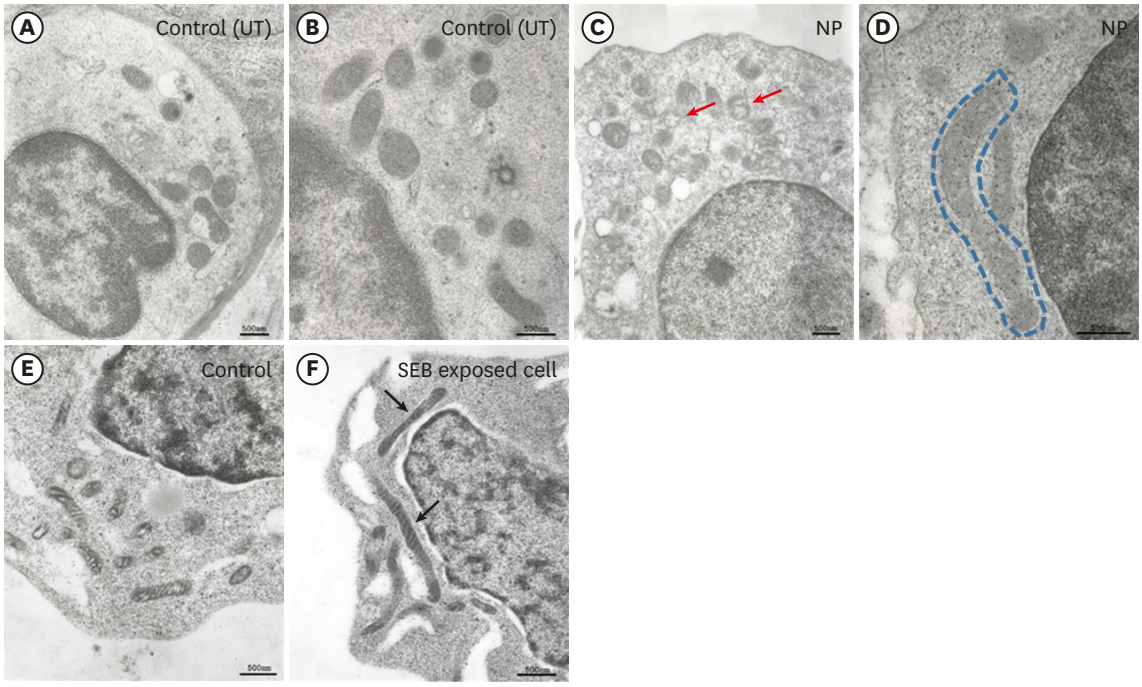

Fig. 5. Transmission electron micrograph of mitochondria. The mitochondria exhibited various sizes and morphologies in the epithelial cells of the control tissue (A, B). However, in the epithelial cells of NP, increased numbers of fragmented mitochondria with vacuoles (arrow in C) and mitochondria with unified elongated morphology (dotted line in D) were observed. In SEB-exposed human nasal epithelial cells, the number of elongated mitochondria (black arrow in F), which are indicative of a hyper-fusion state, was also increased compared with control (E, F).
SEB, *Staphylococcus aureus* enterotoxin B; UT, uncinate tissue; NP, nasal polyp.

SEB. Oxidative stress and excessive generation of ROS changed the function and morphology of the mitochondria. *PINK1* gene expression increased in human nasal epithelial cells (**Fig. 6A**)

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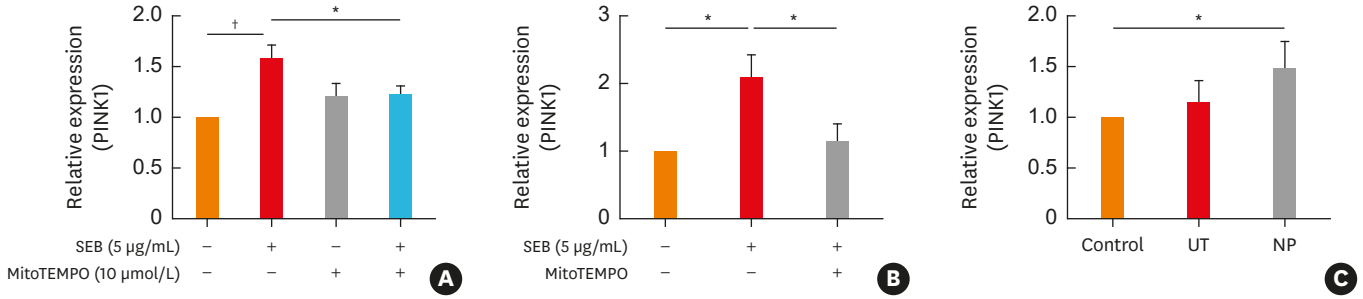

Fig. 6. PINK1 expression. PINK1 mRNA expression in human nasal epithelial cells (RPMI 2650) (A) and primary human nasal epithelial cells. (B) Exposure to SEB (5 μg, 48 hours) with or without MitoTEMPO treatment (10 μmol/L). (C) PINK1 mRNA expression in NP tissue (control, n = 4; UT, n = 5; NP, n = 5).
SEB, *Staphylococcus aureus* enterotoxin B; UT, uncinate tissue; NP, nasal polyp; PINK1, PTEN induced kinase 1; mRNA, messenger RNA.
*P < 0.05; †P < 0.01.

and pHNECs (**Fig. 6B**) treated with SEB but was abolished by MitoTEMPO treatment. NPs in CRSwNP showed similar results, and *PINK1* gene expression was significantly higher in NP in CRSwNP compared to control UT (**Fig. 6C**).

DISCUSSION

There is an increasing appreciation of the roles played by mitochondria beyond oxygen sensing and energy production. To date, few studies have explored mitochondrial changes in nasal and paranasal sinus diseases.[15,18] Despite the various etiologies related to the pathophysiology of nasal polyposis (including the microbiome, altered mucociliary clearance, epithelium-derived radicals, epithelial barrier disruption, and the host immune response), the precise role of mitochondria in nasal polypogenesis remains poorly understood.[1]

The mtROS is mainly generated as a byproduct of oxidative phosphorylation.[19] In lung epithelial cells, mitochondrial oxidative phosphorylation decreases during exposure to a cytotoxic dose of cigarette smoke, whereas exposure to a non-toxic dose of cigarette smoke increases mitochondrial biogenesis.[17,20] Asthma is also associated with reduced oxidative phosphorylation. A study on the bronchial epithelium of asthma patients showed decreased expression of COXIV (oxidative phosphorylation complex IV).[21] Expressions of oxidative phosphorylation complexes increased compared to control tissues in the present study. However, there were discrepancies between the results for SEB-stimulated human nasal epithelial cells and NPs, and oxidative phosphorylation complexes in human nasal epithelial cells showed a complicated expression pattern following SEB treatment. These results suggest that the bioenergetics of oxidative phosphorylation complexes are imbalanced following a relatively short period of SEB stimulation and that these complexes are inefficient at producing ATP, but are quick to produce mtROS, which induces oxidative stress in the cells.

Consequently, this functional change might affect mitochondrial dynamics to maintain homeostasis in the mitochondria.[22] However, NPs are the end result of recurrent active and persistent inflammation over a long period. For this reason, we assume that the expression of oxidative phosphorylation complexes in these polyps increased. Further studies may be needed to clarify our understanding of this phenomenon.

Once excessive ROS is generated, scavengers, such as vitamins C and E, glutathione, peroxidase, and SOD, clear the ROS to reduce oxidative stress.[23,24] Mn-SOD, a mitochondria-

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specific antioxidant, binds to the superoxide byproducts of the oxidative phosphorylation chain and converts them to hydrogen peroxide and diatomic oxygen. If the antioxidant capacity does not meet cellular requirement to eliminate the ROS, the increased ROS could influence the redox-sensitive cellular processes, and ultimately accelerate mitochondrial apoptosis.[23] Our data show that mtROS increased following exposure to SEB, and this is associated with the formation of NPs. The expression of Mn-SOD was also higher in the human nasal epithelial cell line and the epithelial cells of the NPs compared to the control tissues. Importantly, mtROS could modulate the structure and function of the mitochondria, creating a dysfunctional cycle and promoting disease pathophysiology.[25] These results indicate that an increase in mtROS might be the source of the oxidative stress that affects the pathophysiology of NPs.

MitoTEMPO was developed as a mitochondrial-targeted antioxidant. Increasing evidence has indicated the efficacy of MitoTEMPO as a potent and specific mitochondrial-targeted antioxidant for various diseases because it blocks downstream inflammatory processes.[26] We treated nasal epithelial cells with MitoTEMPO following the SEB challenge to investigate whether mitochondrial damage could be prevented by an mtROS-specific scavenger.

Any imbalance in the bioenergetics processes changes the mitochondrial shape, movement, and cellular interactions. Increased fusion and decreased fission create a hyper-fusion state that assists the cells in repairing cellular damage. Mitochondrial hyper-fusion provides a stress-resolving mechanism by creating elongated mitochondria, which are protected from degradation by mitophagy. The hyper-fusion state also showed increased efficiency in ATP synthesis.[27] Recently, hyper-fusion has been reported to be accompanied by nuclear factor-κB, which plays an important role in both the innate and the adaptive immune responses.[28] On the other hand, increased fission or decreased fusion causes fragmented mitochondria. Mitochondrial fragmentation is usually induced following severe cellular stress, and is a prior event to mitophagy and apoptosis, preventing the spread of damage in the mitochondrial network.[22] These spatiotemporal controls are important for the mitochondrial connection with other intracellular organelles such as the nucleus, ER, plasma membrane, and lysosome. Through these connections and interactions, mitochondria can influence cellular functions, including calcium ion regulation, protein production, turnover, and apoptosis.[14,29]

Cellular stress conditions change the balance between mitochondrial fusion and fission. In chronic obstructive pulmonary disease (COPD), Hoffman *et al.*[20] documented hyper-fusion while Hara *et al.*[16] reported mitochondrial fragmentation. The conflicting data suggest that mitochondrial dynamics depend on cell types and extrinsic stimuli due to their sensitive characteristics. In asthma, high expression of fission proteins has been reported.[20,30] Our data showed an increase in both fusion and fission markers in NPs, and also in the nasal epithelial cells following SEB exposure. These findings indicated that the dynamics of mitochondria in CRSwNP increased due to oxidative stress. To date, no definitive results have explained the mitochondrial dynamics involved in the airway epithelium response to oxidative stress.

Damaged or defective mitochondria are removed by mitophagy to maintain mitochondrial quality. PINK1 is a well-known mitophagy regulator, and its expression remains at low levels in healthy mitochondria.[9,31] The role of mitophagy in human diseases is ambivalent. In some diseases, mitophagy-related processes are pathogenic, whereas in other diseases, mitophagy plays a protective role.[31,32] PINK1 plays a protective role in neurons. On the other hand, the absence of PINK1 was thought to have a protective effect against mitophagy-dependent

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necroptosis in COPD. In acute lung injuries, *S. aureus* infection has been observed to increase PINK1 expression.[9,32] In our study, the expression of PINK1 was elevated in nasal epithelial cells after SEB administration. This result indicates that SEB-induced stress in nasal epithelial cells induced mitophagy. Similar findings were also observed in NPs. However, further studies are needed to determine the precise role of PINK1 in CRSwNP.

Our study demonstrates that functional and structural changes in mitochondria, including mtROS generation, Mn-SOD production, bioenergetics changes, and morphological changes, can be induced by SEB exposure *in vitro* and in the polyp tissues of CRSwNP patients. These findings indicate that such changes may be associated with the pathogenesis of CRSwNP, and future research is needed to validate the therapeutic potential of mitochondria-specific antioxidants targeting mtROS in relevant disease models.

SUPPLEMENTARY MATERIAL

Supplementary Table S1

Primer sequences used for real-time polymerase chain reactions

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REFERENCES

1. Kim DW, Cho SH. Emerging endotypes of chronic rhinosinusitis and its application to precision medicine. Allergy Asthma Immunol Res 2017;9:299-306.
PUBMED | CROSSREF
2. Kim DK, Eun KM, Kim MK, Cho D, Han SA, Han SY, et al. Comparison between signature cytokines of nasal tissues in subtypes of chronic rhinosinusitis. Allergy Asthma Immunol Res 2019;11:201-11.
PUBMED | CROSSREF
3. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European position paper on rhinosinusitis and nasal polyps 2012. Rhinol Suppl 2012;23:3 p preceding table of contents, 1-298.
PUBMED
4. Kim DW, Eun KM, Jin HR, Cho SH, Kim DK. Prolonged allergen exposure is associated with increased thymic stromal lymphopoietin expression and Th2-skewing in mouse models of chronic rhinosinusitis. Laryngoscope 2016;126:E265-72.
PUBMED | CROSSREF
5. Kim YM, Jin J, Choi JA, Cho SN, Lim YJ, Lee JH, et al. *Staphylococcus aureus* enterotoxin B-induced endoplasmic reticulum stress response is associated with chronic rhinosinusitis with nasal polyposis. Clin Biochem 2014;47:96-103.
PUBMED | CROSSREF
6. Kim DK, Jin HR, Eun KM, Mo JH, Cho SH, Oh S, et al. The role of interleukin-33 in chronic rhinosinusitis. Thorax 2017;72:635-45.
PUBMED | CROSSREF
7. Iyer D, Mishra N, Agrawal A. Mitochondrial function in allergic disease. Curr Allergy Asthma Rep 2017;17:29.
PUBMED | CROSSREF
8. Prakash YS, Pabelick CM, Sieck GC. Mitochondrial dysfunction in airway disease. Chest 2017;152:618-26.
PUBMED | CROSSREF
9. Yoo SM, Jung YK. A molecular approach to mitophagy and mitochondrial dynamics. Mol Cells 2018;41:18-26.
PUBMED
10. Aguilera-Aguirre L, Bacsi A, Saavedra-Molina A, Kurosky A, Sur S, Boldogh I. Mitochondrial dysfunction increases allergic airway inflammation. J Immunol 2009;183:5379-87.
PUBMED | CROSSREF

11. Kim SR, Kim DI, Kim SH, Lee H, Lee KS, Cho SH, et al. NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation. Cell Death Dis 2014;5:e1498.
PUBMED | CROSSREF
12. Kaminski MM, Sauer SW, Klemke CD, Süss D, Okun JG, Krammer PH, et al. Mitochondrial reactive oxygen species control T cell activation by regulating IL-2 and IL-4 expression: mechanism of ciprofloxacin-mediated immunosuppression. J Immunol 2010;184:4827-41.
PUBMED | CROSSREF
13. Siddesha JM, Nakada EM, Mihavics BR, Hoffman SM, Rattu GK, Chamberlain N, et al. Effect of a chemical chaperone, tauroursodeoxycholic acid, on HDM-induced allergic airway disease. Am J Physiol Lung Cell Mol Physiol 2016;310:L1243-59.
PUBMED | CROSSREF
14. Cahoon JM, Anathy V. Endoplasmic reticulum-mitochondrial interactions in house dust mite induced inflammation [dissertation]. [Burlington]: UVM College of Arts and Sciences; 2015.
15. Guo Z, Hong Z, Dong W, Deng C, Zhao R, Xu J, et al. $PM_{2.5}$-induced oxidative stress and mitochondrial damage in the nasal mucosa of rats. Int J Environ Res Public Health 2017;14:E134.
PUBMED | CROSSREF
16. Hara H, Araya J, Ito S, Kobayashi K, Takasaka N, Yoshii Y, et al. Mitochondrial fragmentation in cigarette smoke-induced bronchial epithelial cell senescence. Am J Physiol Lung Cell Mol Physiol 2013;305:L737-46.
PUBMED | CROSSREF
17. Ballweg K, Mutze K, Königshoff M, Eickelberg O, Meiners S. Cigarette smoke extract affects mitochondrial function in alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 2014;307:L895-907.
PUBMED | CROSSREF
18. Kim S, Kim MJ, Park DY, Chung HJ, Kim CH, Yoon JH, et al. Mitochondrial reactive oxygen species modulate innate immune response to influenza A virus in human nasal epithelium. Antiviral Res 2015;119:78-83.
PUBMED | CROSSREF
19. Mailloux RJ, McBride SL, Harper ME. Unearthing the secrets of mitochondrial ROS and glutathione in bioenergetics. Trends Biochem Sci 2013;38:592-602.
PUBMED | CROSSREF
20. Hoffmann RF, Zarrintan S, Brandenburg SM, Kol A, de Bruin HG, Jafari S, et al. Prolonged cigarette smoke exposure alters mitochondrial structure and function in airway epithelial cells. Respir Res 2013;14:97.
PUBMED | CROSSREF
21. Mabalirajan U, Dinda AK, Kumar S, Roshan R, Gupta P, Sharma SK, et al. Mitochondrial structural changes and dysfunction are associated with experimental allergic asthma. J Immunol 2008;181:3540-8.
PUBMED | CROSSREF
22. Youle RJ, van der Bliek AM. Mitochondrial fission, fusion, and stress. Science 2012;337:1062-5.
PUBMED | CROSSREF
23. Białas AJ, Sitarek P, Miłkowska-Dymanowska J, Piotrowski WJ, Górski P. The role of mitochondria and oxidative/antioxidative imbalance in pathobiology of chronic obstructive pulmonary disease. Oxid Med Cell Longev 2016;2016:7808576.
PUBMED | CROSSREF
24. Holecek V, Slípka J, Sobotová S, Slouka D, Rokyta R. Free radicals and antioxidants in otorhinolaryngology. Cesk Fysiol 2008;57:116-22.
PUBMED
25. Cloonan SM, Choi AM. Mitochondria in lung disease. J Clin Invest 2016;126:809-20.
PUBMED | CROSSREF
26. Jaffer OA, Carter AB, Sanders PN, Dibbern ME, Winters CJ, Murthy S, et al. Mitochondrial-targeted antioxidant therapy decreases transforming growth factor-β-mediated collagen production in a murine asthma model. Am J Respir Cell Mol Biol 2015;52:106-15.
PUBMED | CROSSREF
27. Tondera D, Grandemange S, Jourdain A, Karbowski M, Mattenberger Y, Herzig S, et al. SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO J 2009;28:1589-600.
PUBMED | CROSSREF
28. Zemirli N, Pourcelot M, Ambroise G, Hatchi E, Vazquez A, Arnoult D. Mitochondrial hyperfusion promotes NF-κB activation via the mitochondrial E3 ligase MULAN. FEBS J 2014;281:3095-112.
PUBMED | CROSSREF
29. Szymański J, Janikiewicz J, Michalska B, Patalas-Krawczyk P, Perrone M, Ziółkowski W, et al. Interaction of mitochondria with the endoplasmic reticulum and plasma membrane in calcium homeostasis, lipid trafficking and mitochondrial structure. Int J Mol Sci 2017;18:E1576.
PUBMED | CROSSREF

30. Rehman J, Zhang HJ, Toth PT, Zhang Y, Marsboom G, Hong Z, et al. Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. FASEB J 2012;26:2175-86.
PUBMED | CROSSREF

31. Billia F, Hauck L, Konecny F, Rao V, Shen J, Mak TW. PTEN-inducible kinase 1 (PINK1)/Park6 is indispensable for normal heart function. Proc Natl Acad Sci U S A 2011;108:9572-7.
PUBMED | CROSSREF

32. Ashrafi G, Schlehe JS, LaVoie MJ, Schwarz TL. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. J Cell Biol 2014;206:655-70.
PUBMED | CROSSREF