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Short Communication
Effects of Minocycline on H_2O_2-induced Cell Death and Interleukin-8 Production in Human Small Airway Epithelial Cells

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Abstract

Background and Objective: The antibiotic minocycline (MINO) is known to have anti-inflammatory activity. This study aimed to examine the effects of MINO on hydrogen peroxide (H_2O_2)-induced cell death and interleukin (IL)-8 production, which have been shown to increase during inflammation, using small airway epithelial cells (SAECs). **Materials and Methods:** The SAECs were pretreated with MINO (1, 5 and 10 μM) for 24, 48 or 72 h and subsequently exposed to H_2O_2 (100 μM) for 6 h. Cell viability was assessed using the WST-8 assay and levels of IL-8 protein were measured using an enzyme-linked immunosorbent assay (ELISA). Tukey's multiple comparison test was used for analysis of statistical significance. **Results:** Treatment of SAECs with 10 μM MINO or greater than 100 μM H_2O_2 significantly decreased cell viability. However, pretreatment with 1 μM MINO for 24 h significantly suppressed the H_2O_2-induced decrease in cell viability. In addition, IL-8 production increased in the H_2O_2 treated group as compared to the control group (not treated with H_2O_2), whereas IL-8 production decreased significantly following pretreatment with MINO (1, 5 or 10 μM) for 48 or 72 h. **Conclusion:** It was suggested that pretreatment of SAECs with low concentrations of MINO for 24 or 48 h has a cytoprotective effect and pretreatment for 48 or 72 h suppresses IL-8 production indicating a potential anti-inflammatory effect.

Key words: Minocycline, cytoprotective effect, cell death, interleukin-8, anti-inflammatory, small airway epithelial cells, immunosorbent

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Macrolides and tetracyclines possess anti-bacterial action and also exhibit distinct anti-inflammatory effects[1-4]. Chronic respiratory tract infections, in which oxidative stress is an important pathogenic factor[5], are characterized by a vicious cycle of infection and inflammation, with inflammatory cytokines, mainly interleukin (IL)-8, observed to increase in bronchial epithelial cell sat the infection site[1,6]. This precedes the development of cytotoxicity, including the death of bronchial epithelial cells[1,2].

Recently, the effect of clarithromycin (CAM), a macrolide antibiotic effective for diffuse panbronchiolitis (DPB), on H_2O_2-induced cytotoxicity in human small airway epithelial cells (SAECs) was investigated[7,8]. The results revealed that long-term treatment with low-concentration CAM inhibits the production of IL-8 and enhances anti-oxidant enzyme activity, e.g., glutathione peroxidase, which protects cells from H_2O_2-induced oxidative toxicity. On the other hand, minocycline (MINO) is used for the treatment of mycoplasma and chlamydia pneumonia, which are classified as diffuse lung diseases[9] like DPB. This therapeutic effect may involve anti-inflammatory actions other than the antibacterial activity possessed by MINO. That is, MINO has been found to have multiple non-antibacterial biological activities that are beneficial in experimental models of various diseases, including autoimmune diseases such as dermatitis, rheumatoid arthritis and inflammatory bowel disease[3,4]. The MINO also produces a neuroprotective effect[10,11] that may ameliorate conditions such as ischemia, traumatic brain injury and neuropathic pain as well as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, multiple sclerosis, spinal cord and its effects have been confirmed in experimental models of several neurodegenerative diseases[3,4].

Based on these findings, it is possible that MINO may have the same effect as CAM against chronic inflammatory airway diseases. However, there are no reports describing the direct effects of MINO on H_2O_2-induced cytotoxicity in bronchial epithelial cells[7, 8]. The purpose of this study was to examine the inhibitory effects of MINO on H_2O_2-induced cell death and IL-8 production in SAECs under the same conditions as long-term treatment of CAM at low concentrations previously demonstrated to show the cytoprotective effects on SAECs. Although it is not possible to directly translate a pharmacological effect in cells to treatment efficacy in clinical practice, the present study presented possible evidence that MINO therapy is effective against chronic inflammatory airway diseases through the inhibitions of cell death and IL-8 release in bronchial epithelial cells.

MATERIALS AND METHODS

The cultured cell study was conducted at the Faculty of Pharmaceutical Sciences, Hokkaido University of Science, Sapporo, Japan. The total period of this research was 7 months, from September, 2017 to March, 2018.

Materials: The MINO hydrochloride, H_2O_2 (30%) and the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium) assay system were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). A human IL-8 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bender Med Systems (Vienna, Austria). All other chemicals used in this experiment were of research grade.

Cell culture and drug treatments: Cell culture and treatments were according to methods described previously[7,8]. Briefly, a normal human small airway epithelial cell line (SAECs) and culture media were purchased from Lonza (Walkersville, MD, USA). Cells were seeded in 75 cm^2 filter vent flasks (Corning, NY, USA) and were grown to 80% confluence (3×10^6 cells/well) for each experimental condition at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were cultured in the presence or absence of MINO (1, 5 or 10 μM) for 24-72 h and were then stimulated with H_2O_2 (100 μM) for an additional 6 h. Pre-treatments with MINO were carried out with a different set of cells for each concentration and treatment duration. For stimulation of the cells with H_2O_2, the medium was changed to small airway basal medium (SABM) containing no supplements[12].

Cell viability: Cell viability was assessed using the cell counting kit-8 assay[13], WST-8. After treatment with MINO and H_2O_2, the SAEC medium was changed to SABM containing 10% WST-8 solution and the cells were incubated at 37°C for 2 h. Viable cells convert the WST-8 solution to an orange-colored formazan product with an absorbance at 450 nm. The optical density (OD) of the culture medium was measured at 450 nm with a spectrophotometric microliter plate reader (Bio-Rad, Hercules, CA, USA). The cell proliferation and viability were expressed as the ratio (%) of surviving cells to H_2O_2 untreated cells.

IL-8 measurement: After the treatments with MINO and H_2O_2, the culture media supernatants were collected and centrifuged to remove cell debris (12,000×g for 10 min). The IL-8 protein levels in the culture supernatants were analyzed using ELISA according to the manufacturer's instructions. Briefly, experimental samples were added into individual wells coated with human monoclonal anti-body specific for IL-8 and incubated for 3 h at room temperature. After 6 washes with phosphate-buffered saline (PBS) containing 0.1% Tween 20 to remove unbound protein, tetramethyl-benzidine was added to each well and incubated for 10 min at room temperature. The reaction was terminated by the addition of 1 M phosphoric acid. The color generated in each sample was determined by OD measurement at 450 nm using a spectrophotometric microliter plate reader. The IL-8 protein levels of unknown samples were calculated using a standard curve.

Statistical analysis: All data were expressed as Means±SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and differences, which were estimated by Tukey's multiple comparison test after the Goodness of fit test and Bartlett's test were considered to be statistically significant at $p<0.05$.

RESULTS

Effects of MINO and H_2O_2 on SAEC cell growth: Treatment of SAECs with 1 or 5 μM MINO for up to 72 h produced no significant change in cell viability. However, at a high concentration (10 μM), cell viability decreased significantly ($p<0.01$) compared to the control group indicating the cytotoxicity of MINO (Table 1). On the other hand, following H_2O_2 treatment (100, 300 and 500 μM) for 6 h, cell viability decreased in a concentration-dependent manner to 77.6±10.4, 65.9±6.4 and 26.4±1.5%, respectively. Furthermore, treatment with H_2O_2 for 24 h resulted in a stronger reduction in cell viability at 300 and 500 μM (Table 2).

Effect of MINO pre-treatment on H_2O_2-induced cell death in SAECs: Pre-treatment of SAECs with 1 μM MINO for 24 or 48 h prior to H_2O_2 (100 μM) treatment significantly ($p<0.01$ or $p<0.05$) suppressed the H_2O_2-induced decrease in cell viability compared to100 μM H_2O_2 treatment alone. Additionally, pre-treatment with 5 μM MINO for 24 h significantly suppressed the H_2O_2-induced decrease in cell viability ($p<0.01$). However, at a high concentration (10 μM) of MINO, the H_2O_2-induced decrease in cell viability was not suppressed at any time point assayed (Table 3).

Effect of MINO pre-treatment on H_2O_2-induced IL-8 protein release in SAECs: The exposure of SAECs to H_2O_2 (100 μM) caused an increase in supernatant IL-8 levels (significantly increased at 48 h) compared to the control group. Pre-treatments with 1 or 5 μM MINO for 48 or 72 h and with 10 μM MINO for up to 72 h prior to H_2O_2 treatment significantly ($p<0.01$ or $p<0.05$) decreased H_2O_2-induced IL-8 protein release compared to H_2O_2 treatment alone (Fig. 1).

Table 1: Effect of MINO on SAEC cell growth

	Cell viability (%)		
	MINO treatment time (h)		
Groups	24	48	72
Control cells	100.0±9.8	100.0± 6.8	100.0± 9.0
1 μM MINO	104.9± 8.6	95.4± 6.0	105.2± 8.6
5 μM MINO	85.4± 7.7	86.9± 6.1	91.2± 7.1
10 μM MINO	67.9± 8.5**	70.2±9.0**	69.7± 5.5**

Cell viabilities of the MINO untreated group (control cells) at each treatment time are expressed as 100%, data are expressed as Means±SD of six independent experiments, **p<0.01 vs. control cells

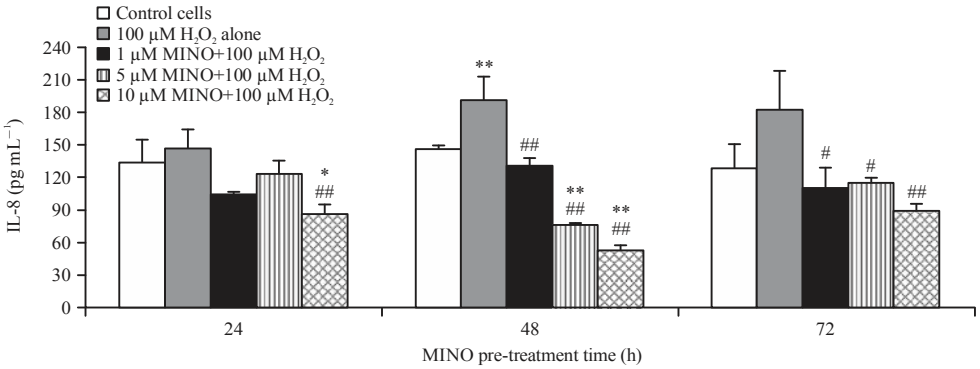

Fig. 1: Effect of MINO pretreatment on IL-8 production

Data are expressed as Means±SD of three independent experiments, *p<0.05, **p<0.01 vs. control cells, #p<0.05, ##p<0.01 vs. cells stimulated with H_2O_2 alone

Table 2: Effect of H_2O_2 on SAEC cell growth

	Cell viability (%)	
	H_2O_2 treatment time (h)	
Groups	6	24
Control cells	100.0±12.4	100.0±10.8
100 μM H_2O_2	77.6±10.4**	76.2±3.5**
300 μM H_2O_2	65.9±6.4**	57.7±5.3**
500 μM H_2O_2	26.4±1.5**	21.4±0.8**

Cell viabilities of the H_2O_2 untreated group (control cells) at each treatment time are expressed as 100%, data are expressed as Means±SD of six independent experiments, **p<0.01 vs. control cells

Table 3: Effect of MINO pre-treatment on H_2O_2-induced cell death in SAECs

	Cell viability (%)		
	MINO pre-treatment time (h)		
Treatment groups	24	48	72
Control cells	100.0±8.5	100.0±7.6	100.0±11.0
100 μM H_2O_2 alone	62.5±2.3**	77.1±5.6**	81.3±3.6*
100 μM H_2O_2+1 μM MINO	94.6±9.8##	94.2±6.8#	91.4±3.5
100 μM H_2O_2+5 μM MINO	84.3±9.6##	77.7±5.9**	85.3±5.3
100 μM H_2O_2+10 μM MINO	64.7±5.3**	66.1±8.4**	70.4±9.0**

Cell viabilities of the MINO and H_2O_2 untreated group (control cells) at each pre-treatment time are expressed as 100%, data are expressed as Means±SD of six independent experiments, *p<0.05, **p<0.01 vs. control cells, #p<0.05, ##p<0.01 vs. cells stimulated with H_2O_2 alone

DISCUSSION

The results of this work showed that pre-treatment of SAECs with low concentrations (1 or 5 μM) of MINO for 24 or 48 h significantly suppressed the H_2O_2-induced decrease in cell viability and pre-treatment for 48 or 72 h significantly decreased IL-8 protein release.

The MINO concentrations of 1, 5 and 10 μM used in this study were deemed reasonable because an adult taking 200 mg of MINO per day would exhibit blood levels of approximately[14] 1-5 μM. In addition, a 2-4 fold higher concentration (2-20 μM) was detected in human sputum and bronchial secretion fluid[15]. However, in a study examining the anti-tumor effect of MINO on mouse epithelial ovarian cancer cells[16] and human leukemia cells[17], MINO decreased cell viability, with IC_{50} values of 10-30 μg mL^{-1} (about 22-66 μM). Therefore, it was suggested that the cytotoxic concentration of MINO obtained in this study was lower than those reported values due to differences in experimental conditions, such as the cell type used and the evaluation method of cell viability. On the other hand, during inflammatory conditions in the lower respiratory tract, neutrophil-derived H_2O_2 in the respiratory tract viscous fluid is reported to reach a concentration of 50 μM[18]. Since H_2O_2 is also produced directly from bronchial epithelial cells following stimulation with pollutants such as bacteria and lipopolysaccharide[19], it was proposed that the H_2O_2 concentration (100 μM) used in this study reflects conditions observed during chronic inflammatory airway diseases.

Inflammation is the immune system's response to harmful stimuli such as pathogens, damaged cells, toxic compounds, reactive oxygen species or radiation[20] and uncontrolled inflammation finally leads to a loss of tissue function or cell viability at the tissue or cell level[21]. It has been shown that MINO causes a concentration-dependent protective effect on cell viability after oxidative stress[22,23]. Also in SAECs of this study, MINO suppressed cell death due to H_2O_2 (100 μM)-induced oxidative stress at low concentrations (1 and 5 μM). However, since cytotoxicity was observed at10 μM MINO, the decrease in cell viability induced by H_2O_2 may be further decreased depending on the MINO concentration in excess of the toxic threshold. Accordingly, when the MINO pre-treatment time is prolonged, evidence of cytotoxicity appears at low MINO concentrations, notably at 5 μM and the inhibitory effect of MINO on H_2O_2-induced cell death may be attenuated.

In regards to cell viability, the greatest effect was obtained by pretreatment with MINO for 24 h; however, the effect on IL-8 production released during the inflammatory process as an inflammatory cytokine[1,6,24] was inconsistent with this result. It was thought that inflammatory cytokines other than IL-8 might be involved in the mechanism of MINO's cytoprotective effect. Because MINO suppresses the activation of nuclear transcription[25] factor (NF)-κB, it is possible that the production of IL-1, IL-6 or tumor necrosis factor (TNF)-α induced by NF-κB is also suppressed. In this study, IL-8, which is considered the principal inflammatory mediator[1,6] was used as an index; however, the expression pattern of each cytokine varies depending on the MINO and H_2O_2 treatment conditions[25,26]. Therefore, it is possible that there will be a difference in the relationship between the production of respective cytokines and cell viability. Nevertheless, pretreatment with 10 μM MINO significantly reduced the production of IL-8 at all time points assayed. The reason for this observation may be that the cytotoxicity from 10 μM MINO reduced the number of IL-8-producing SAECs. It is known that reactive oxygen species such as H_2O_2 regulate the DNA binding activity of nuclear transcription factors such as NF-κB, altering gene expression and activating a number of major signaling pathways leading to cell death[27].

Therefore, compounds with antioxidant activity are considered to be effective for the inhibition of cytotoxicity and cell death caused by oxidative toxicity. Similar to α-tocopherol, MINO contains a multi-substituted phenol

ring in its chemical structure and shows reactive oxygen species scavenging ability[23]. It was suggested that the direct anti-oxidant activity of MINO contributes, at least in part, to the suppression of H_2O_2-induced cell death and IL-8 production in SAECs.

CONCLUSION

This study showed that pre-treatment of SAECs with low concentrations (1 or 5 μM) of MINO for 24 or 48 h suppresses cell death after H_2O_2 treatment (100 μM) and pre-treatment for 48 or 72 h inhibits IL-8 release. Therefore, it was expected that MINO may also exhibit cytoprotective and anti-inflammatory effects in bronchial epithelial cells.

SIGNIFICANCE STATEMENT

This study revealed possible cytoprotective effects on H_2O_2-induced cell death and IL-8 protein release in SAECs after pretreatment with MINO, which can be beneficial for the treatment of chronic airway inflammatory diseases. This study will assist researchers in uncovering a critical area of inflammatory-mediated bronchial epithelial cell death through oxidative stress that has previously been difficult to assess. Previous studies have not explored anti-inflammatory activities after pretreatment with MINO on chronic airway inflammatory diseases but instead focused on dermatitis, rheumatoid arthritis, central nerve system (CNS) diseases, neuropathic pain, ischemia, inflammatory bowel disease and cancer. Thus, new therapeutic regimens that effectively combat chronic airway inflammatory diseases may be developed.

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