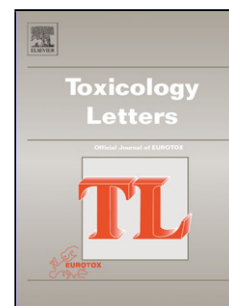


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Trimellitic anhydride facilitates transepithelial permeability disrupting tight junctions in sinonasal epithelial cells

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**Trimellitic anhydride facilitates transepithelial permeability disrupting tight junctions
in sinonasal epithelial cells**

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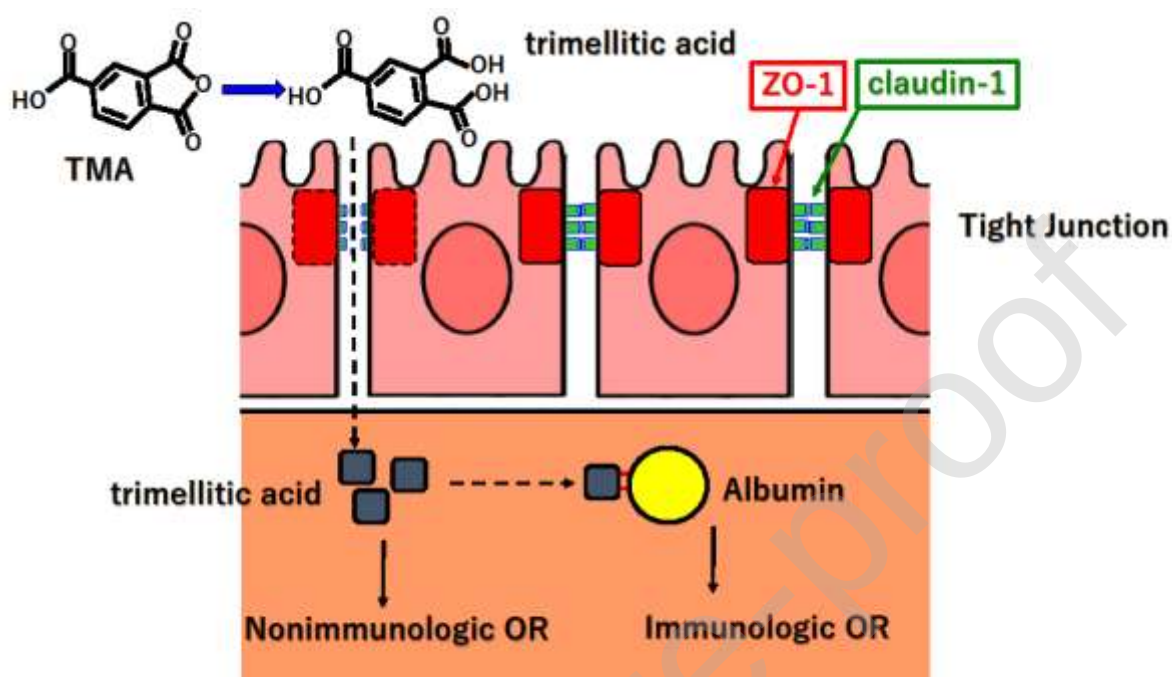
Running head: TMA disrupts tight junctions

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Graphical abstract



HIGHLIGHTS

- TMA attenuates transepithelial electrical resistance and increases paracellular permeability.
- ZO-1 and claudin-1 are disrupted by TMA in a dose-dependent manner.
- Trimellitic anhydride shows cytotoxicity only after prolonged incubation.

ABSTRACT

Trimellitic anhydride (TMA) is a chemical agent classified as a low molecular weight (LMW) agent causing occupational rhinitis (OR) or asthma. Although TMA is recognized as a respiratory sensitizer, the direct and non-immunologic effects of TMA remain unclear. Air-liquid interface (ALI) cultured human nasal epithelial cells (HNECs) derived from control subjects were treated with TMA, followed by measurement of the transepithelial electrical resistance (TEER), paracellular permeability of fluorescein isothiocyanate (FITC)-dextran and immunofluorescence of tight junction proteins claudin-1 and zonula occludens-1 (ZO-1). The cytotoxicity of TMA was evaluated by lactate dehydrogenase (LDH) assay. TMA at concentrations of 2 and 4 mg/ml significantly reduced the TEER within 10 minutes ($p=0.0177$ on 2 mg/ml; $p<0.0001$ on 4 mg/ml). The paracellular permeability of FITC-dextran was significantly increased upon challenge with 4 mg/ml TMA for 3 hours ($p=0.0088$) and 6 hours ($p=0.0004$). TMA treatment induced a reduction in the fluorescence intensity of claudin-1 and ZO-1 in a dose-dependent manner. LDH assay revealed 4 mg/ml TMA induced cytotoxicity only after 6 hours incubation, while 1 or 2 mg/ml TMA caused no cytotoxicity. Our results suggest that TMA has a potential to penetrate the epithelial barrier by disrupting claudin-1 and ZO-1, indicating an important role for sensitization and OR development.

Keywords

air liquid interface cell culture; occupational rhinitis; tight junctions; trimellitic anhydride

INTRODUCTION

Occupational rhinitis (OR) is an inflammatory disorder of the nasal lining attributable to conditions of a specific work environment. It should be distinguished from work-exacerbated rhinitis whereby pre or co-existent rhinitis worsens but is not caused by workplace exposure. OR has a significant effect on workers, with detrimental effects on productivity, performance and psychosocial functioning (Vandenplas et al., 2020). OR can be subclassified into immunologic and non-immunologic OR with both high molecular weight (HMW) and low molecular weight (LMW) agents implicated (Castano and Thériault, 2006; Siracusa et al., 2000). Immunologic OR is typically caused by HMW agents, such as flour and animal dander and the pathophysiology is considered to be the same as allergic rhinitis, mediated by antigen specific immunoglobulin E (IgE). In contrast to immunologic OR, non-immunologic OR mainly involves exposure to LMW agents and its pathophysiology is unclear. Several different LMW agents have been implicated in the development of OR including acid anhydride, diisocyanates, ammonium persulphate and platinum (Baker et al., 1990; Bernstein et al., 1993; Diab et al., 2009; Lysdal et al., 2014; Sastre et al., 2014; Zeiss et al., 1977).

Acid anhydrides such as trimellitic anhydride (TMA) are common chemical agents utilized in the production of paints, vinyl plasticizers and epoxy resins as hardening agents. An estimated 100,000 metric tons of TMA are produced every year worldwide, most of which are manufactured in the USA (Ghosh and Bernstein, 2020). Approximately 20,000 USA workers are estimated to be exposed to TMA in their work environments (Allergens, 1993).

Since nasal epithelial cells have been considered to form an initial protective layer against pathogens and physical or chemical stress, the epithelial barrier including tight junction (TJ) proteins could be the first target of TMA leading to OR. TJ proteins exist in the most apico-lateral region of the upper airway respiratory epithelium and seal the paracellular space to inhibit water evaporation and maintain solutes (FARQUHAR and PALADE, 1963). TJ disruption and resultant epithelial barrier dysfunction has been demonstrated in airway diseases such as allergic rhinitis, chronic rhinosinusitis, asthma or chronic obstructive pulmonary disease (Heijink et al., 2014; Soyka et al., 2012; Steelant et al., 2016; Xiao et al., 2011). Epithelial TJ disturbance not only increases paracellular permeability allowing external antigen invasion but also induces immune cell activation leading to the development of allergic rhinitis (Siti Sarah et al., 2020; Sugita and Kabashima, 2020)

TMAAs are typically considered non-immunogenic by themselves, however, they can conjugate with human serum albumin to form hapten-protein complexes and therefore have been well documented as a cause of immunologic OR and occupational asthma (OA) (Bernstein et al., 2011; Zeiss et al., 1982). Despite this, little is known about the direct and non-immunologic effects of TMAAs on the sinonasal epithelium. Given that TMA is a LMW substance, one would hypothesize that it would not possess protease activity, unlike an HMW substance. This study aims to explore the dose-dependent effects of TMA exposure on human nasal epithelial cells and their barrier function.

MATERIALS AND METHODS

Human subjects

The research protocol was approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital (reference HREC/15/TQEH/132 and 13604). All subjects gave written informed consent. Exclusion criteria were 18 years of age or younger, pregnancy, active smoker and patients with chronic rhinosinusitis.

Cell culture

Primary Human Nasal Epithelial Cells (HNECs) were obtained from brushings of the inferior turbinates in patients undergoing sinonasal or skull base surgery, using the protocol described by Ramezani et al (Ramezani et al., 2017b). Brushes were transferred into Roswell Park Memorial Institute (RPMI) medium (Gibco, Invitrogen, Melbourne, Australia) and cells were then collected after centrifugation (500 x *g* for 7 minutes). Cell samples were then depleted of macrophages in a 100- mm-diameter culture plate coated with anti-CD68 antibody (Dako, Glostrup, Denmark) for 20 minutes. Cells were grown in collagen-coated flasks (Thermo Scientific, Waltham, MA, USA) with Ex-medium (PneumaCult™-Ex Plus Basal Medium along with PneumaCult™-Ex Plus 50X Supplement, Hydrocortisone Stock Solution [StemCell Technologies, Vancouver, Canada] and penicillin/amphotericin B [Thermo Scientific, Waltham, MA, USA]). Once the cells reached confluence, they were dissociated by trypsin, then centrifuged (5 minutes, 400 x *g* and resuspended in Ex-medium. Then 7×10^4 HNECs were seeded in a volume of 100 μ L B-ALI medium into the collagen coated apical chamber of Transwell plates (BD Biosciences, San Jose, California, USA) and

500 μ L of B-ALI growth medium was added to the basal chamber in all wells and incubated for 3–4 days at 37 °C with 5% CO₂. Then, the apical medium was removed and 500 μ L B-ALI™ differentiation medium was added to the basal chamber. The cultures were fed every other day by adding B-ALI complete differentiation medium (PneumaCult™-ALI Basal Medium with PneumaCult™-ALI 10X Supplement, PneumaCult™-ALI Maintenance Supplement [STEMCELL, Vancouver, Canada] and penicillin/amphotericin B [Thermo Scientific, Waltham, MA, USA]) to the basal chamber. HNECs at air liquid interface (HNEC-ALI) were maintained for a minimum of 21 days for the development of tight junctions. All HNEC-ALI cultures were kept at 37°C in a humidified atmosphere of 5 % carbon dioxide in air (Ramezanpour et al., 2019).

Measurement of transepithelial electric resistance (TEER)

Trimellitic anhydride (TMA, Tokyo Chemical Industry, Tokyo, Japan) was diluted into acetone (0.2 g/ml) and the concentration was adjusted with 1× phosphate buffered saline (PBS) to a final concentration of 1, 2 and 4 mg/ml. The same amount of acetone as for the TMA dilution was added into PBS as a negative control (4% diluted acetone into PBS). These agents and 5% Triton as a positive control were added into the apical chamber onto ALI cultured HNECs for 3 hours and 6 hours. Epithelial integrity of treated cells was evaluated by sequential Transepithelial Electrical Resistance (TEER) (Ω/cm^2) measurement using an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL, USA) as described (Hu et al., 2019).

Permeability assay

Epithelial permeability was measured by using 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, St. Louis, MO) after the last (3 or 6 hours) TEER measurement. FITC-dextran (3 mg/ml) was added to the apical chamber after 2 rinses of HNECs with PBS. The basolateral medium containing FITC-dextran was transferred into a clear bottom black 96 well plate in duplicate after 2 hours incubation at 37°C. FITC intensity of the basolateral medium was measured with a FLUOstar Optima 96-well fluorescence microplate reader (BMG Labtech, Ortenberg, Germany) (Ramezanpour et al., 2018).

Immunofluorescence staining

HNEC-ALI cultures were washed with PBS and fixed with 2.5 % formalin for 10 minutes on ice. The fixed cells were permeabilized with 1 % sodium dodecyl sulphate (SDS) for 10 minutes on ice and blocked with serum free blocker (Dako, Glostrup, Denmark) for 1 hour at RT. Then the samples were incubated with primary antibodies rabbit anti-claudin-1 and mouse anti-zonula occludens-1 (ZO-1) (Invitrogen, Carlsbad, CA, USA), 1:50 and 1:100 respectively, into PBS overnight at 4°C. The following day, cells were washed 2x with PBS and the cells were incubated with secondary antibodies donkey anti-rabbit Cy3 and donkey anti-mouse IgG Alexa Fluor 488 (both 1:200, Jackson ImmunoResearch Labs Inc., West Grove, PA, USA) for 1 hour at RT. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at RT following secondary antibody washing out. The cut membranes including stained cells were transferred onto glass slides and mounted with a drop of fluorescence mounting medium (Dako, Glostrup,

Denmark). Images from immunofluorescence slides were obtained by confocal laser-scanning microscope LSM700 (Zeiss Microscopy, Jena, Germany) with $\times 20$ magnification, and image processing was performed with ZEN Imaging Software (Carl Zeiss AG, Oberkochen, Germany). Tight junction (TJ) proteins (ZO-1 or claudin-1) were quantified as mean immunofluorescence intensity (MFI) using image stacks with 20 μm thickness composed of 21 sequential slices. Analysis of immunofluorescence intensity was performed by randomly evaluating three different images per membrane in a blinded manner (Tuli et al., 2021).

Cytotoxicity assay

After treatments were applied to the HNEC-ALI cultures the cytotoxicity was assessed using a lactate dehydrogenase (LDH) assay (Promega, Madison, WI, USA). 50 μl of supernatant from the basolateral chamber were added to a 96 well plate and incubated with 50 μl LDH reagent for 30 minutes in the dark at room temperature (RT). The reaction was finalized with stop solution and the absorbance was recorded with a FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany) at 490 nm. Results were presented as a percentage of negative control values (Ramezanpour et al., 2017a).

Statistical analysis

All values were expressed as mean $\pm$ standard error of the mean (SEM). Data were analyzed with statistical software (Graph Pad Prism 7, San Diego, CA). The difference between the two groups was analyzed by using the 2-tailed unpaired *t* test or Fisher's exact test. A one-

way analysis of variance (ANOVA) followed by a Dunnett's test was conducted for TEER measurement. Parameters were considered statistically significant when $P < 0.05$.

RESULTS

Subjects

HNEC-ALI cultures were established from a total of 13 donors (6 males, 7 females, aged 44.6 ± 4.1 years). Demographic and relevant information of the subjects is summarized in Table 1. In brief, no subjects had a history of chronic or acute rhinosinusitis, allergic rhinitis, asthma or other significant co-morbidities and as per the exclusion criteria, no subjects were smokers. Samples from 8 subjects were used for TEER, permeability assay and LDH assay and samples from 5 were used for immunofluorescence.

TMA impaired the nasal epithelial barrier in a dose-dependent manner.

TMA at concentrations of 2 and 4 mg/ml, but not 1 mg/ml, significantly decreased the TEER within 10 minutes of application compared to vehicle control ($p = 0.0177$ on 2 mg/ml; $p < 0.0001$ on 4 mg/ml) (Figure 1A). The TEER decline was irreversible and time-dependent. The paracellular flux of FITC-dextran across HNEC-ALI cultures was significantly increased upon challenge with 4 mg/ml TMA for 3 hours ($p = 0.0088$) and 6 hours ($p = 0.0004$), indicating an increase in paracellular permeability at this concentration. No change in paracellular permeability was seen at the lower concentrations of 1 and 2 mg/ml TMA (Figure 1B).

TMA incubation results in the cleavage of claudin-1 and ZO-1.

Immunofluorescence demonstrated tight junction and cellular disruption with reduced fluorescence at 2 and 4 mg/ml TMA but not at 1 mg/ml TMA (Figure 2A). No apparent alteration of the expression of ZO-1 (100.5 ± 2.8 %, $p > 0.05$) and claudin-1 (98.9 ± 3.3 %, $p > 0.05$) was observed in cells incubated with 1 mg/mL TMA compared to vehicle control. At 2 mg/ml of TMA the expression of ZO-1 was significantly reduced (70.7 ± 6.0 %, $p = 0.0007$) as was the expression of claudin-1 (41.7 ± 6.7 %, $p < 0.0001$). A further reduction of staining intensity of ZO-1 (34.4 ± 5.0 %, $p < 0.0001$) and claudin-1 (23.3 ± 5.0 %, $p < 0.0001$) was seen in cells treated with 4 mg/mL TMA. Figure 2B).

TMA treatment demonstrated cytotoxicity only after prolonged incubation.

Using an LDH assay, no cytotoxicity was demonstrated at any of the time points tested for 1 or 2 mg/ml TMA (Figure 3). 4 mg/ml TMA only showed cytotoxicity with incubation periods > 6 hours ($p = 0.0023$).

DISCUSSION

This is the first study to investigate the irritant effect of TMA on human nasal epithelial cell barrier. TMA was seen to reduce transepithelial resistance and increase paracellular permeability in a dose and time-dependent manner, without evidence of significant cytotoxicity. This may facilitate its ability to penetrate the submucosa and sensitize the immune system over time in exposed individuals.

TMA is produced as powder or flake and kept solid at room temperature. When heated to high temperatures, typically associated with industrial manufacturing, it can become aerosolized. Once inhaled, TMA is converted to trimellitic acid which can cause a localized non-immunologic irritant reaction at the cell-environment interface or lead to sensitization and later development of OR. Studies supporting TMA's role in the pathogenesis of non-immunologic OR, include those showing its direct effect on mast cell degranulation in the absence of IgE (Ogi et al., 2016). It is possible that through its initial action on disrupting the epithelial membrane's integrity, as well as its negative effect on paracellular tight junction activity, it facilitates its own submucosal penetration. Although most other high molecular weight aeroallergens such as papain, pollen and house dust mite have been shown to disrupt TJ proteins through their protease activity, the exact mechanism of how TMA disrupts the epithelium and TJs remains unknown (Gunawan et al., 2008; Henriquez et al., 2013; Iida et al., 2014; Tymoszek et al., 2016). A previous study by Li et al indicated that TMA exposure induces transient receptor potential vanilloid 1 (TRPV1) channel activation possibly responsible for respiratory exacerbation seen in asthma (Li et al., 2019). Another study also suggested the possible involvement of TRPV1 in the potential mechanism of TJ degradation in acidic conditions (Xu et al., 2013). These results suggest that acidic stress caused by trimellitic acid potentially leads to TJ disruption through TRPV1 activation.

Trans-epithelial passage of TMA into the submucosa, also allows it to interact and conjugate with other substances in this region such as albumin. This process converts TMA, which alone is not considered immunogenic, into highly immunogenic haptens-

protein complexes which can sensitize the immune system to further exposure. This process typically takes longer, between 1-5 years, and ultimately manifests as OR and eventually OA (Grammer, 2016). As the symptoms of rhinitis and/or conjunctivitis usually pre-date the onset of asthma symptoms in TMA sensitized workers (Grammer et al., 2002), these symptoms may therefore serve as the point of intervention, to identify and treat these workers before they develop TMA related asthma.

Although OSHA (Occupational Safety and Health Administration, USA) defines the permissible exposure limit (PEL) of TMA as 0.04 mg/mL, the exposure level varies from <0.6 to 1990 µg/mL in TMA factories (Bernstein et al., 2011; Zeiss et al., 1982). Our study indicates that the non-immunologic effects of TMA on the epithelial barrier, are dose-dependent, with changes in TEER observed at or above 2 mg/ml TMA, and additional changes in paracellular permeability at or above 4 mg/ml TMA, independent of the duration of exposure. Although this study did not reproduce physiological conditions completely as the solubilized TMA was used in this study instead of fume TMA, our results suggest that workers in factories where levels of TMA exceed certain concentrations are at risk of developing non-immunological OR in the short term, before becoming sensitized. In terms of developing immunologic OR and ultimately OA, studies suggest that the exposure period may play a more important role than exposure concentrations (Barker et al., 1998, 2000). This indicates that even at lower TMA concentrations than that cause TJ degradation could place workers at risk of TMA induced respiratory conditions in the long term, reinforcing the need for adequate personal protective equipment and appropriate ventilation and extraction systems even in factories with low exposure levels.

An obvious limitation of our study is its *in vitro* nature and the fact that TJ function is a highly complex process influenced by many physiological and pathological factors in the living organism (Schneeberger and Lynch, 1992). Furthermore, our study used solubilized TMA rather than the fume itself, to avoid exposure of personnel in our laboratory. This may influence the real-world relevance of our findings.

To conclude, our study demonstrates that TMA has a potential to penetrate the epithelial barrier by disrupting claudin-1 and ZO-1. We consider that this dose-dependent TJ disruption plays an important role in the development of TMA related non-immunologic OR and sensitization. Awareness of environmental TMA levels and early screening of workers for rhinitis type symptoms may reduce the development of the more severe form of immunologic OR and OA.

FOOTNOTES

Abbreviations

ANOVA; analysis of variance, ALI; air-liquid interface, DAPI; 4', 6-diamidino-2-phenylindole, FITC; fluorescein isothiocyanate, HMW; high molecular weight, HNEC; human nasal epithelial cell, IgE; immunoglobulin, LDH; lactate dehydrogenase, LMW; low molecular weight, MFI; mean immunofluorescence intensity, OA; occupational asthma, OR; occupational rhinitis, PBS; phosphate-buffered saline, PEL; permissible exposure limit, RT; room temperature, RPMI; Roswell Park Memorial Institute, SDS; sodium dodecyl sulphate,

SEM; standard error of the mean, TEER; transepithelial electric resistance, TJ; tight junction, TMA; trimellitic anhydride, TRPV1; transient receptor potential vanilloid 1, ZO; zonula occludens

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Author Contributions

K.O., S.L. and M.R., conducted the biological experiments; A.P. and P.-J.W. collected the clinical samples; A.P., P.-J.W., and S.V. designed the study; C.C., S.J. and S.V. analyzed the data; S.F., S.V. and A.P. drafted the manuscript; all authors critically reviewed the manuscript and approved the final version.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE LEGENDS

Table 1. Patient characteristics on each experiment.

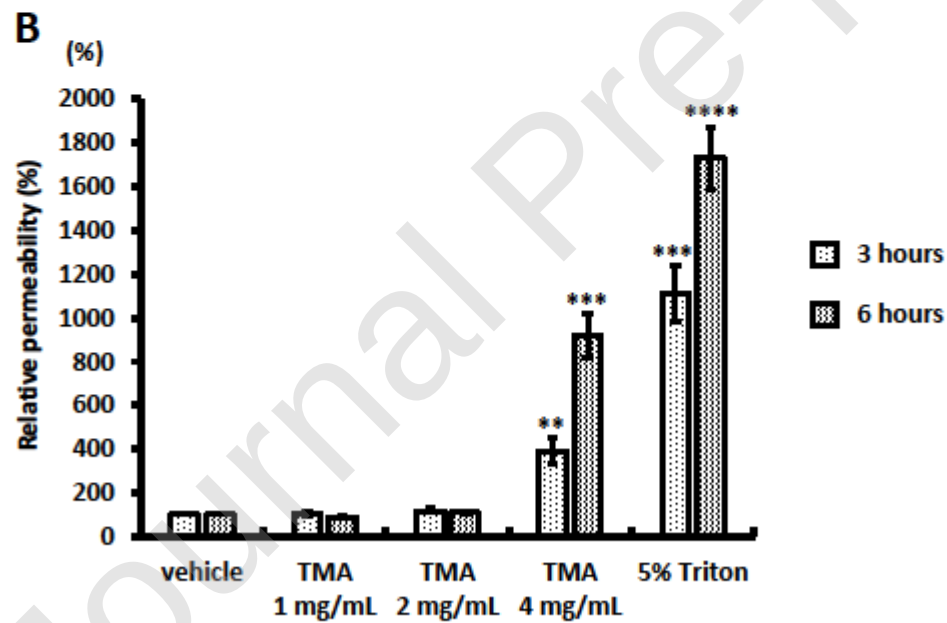
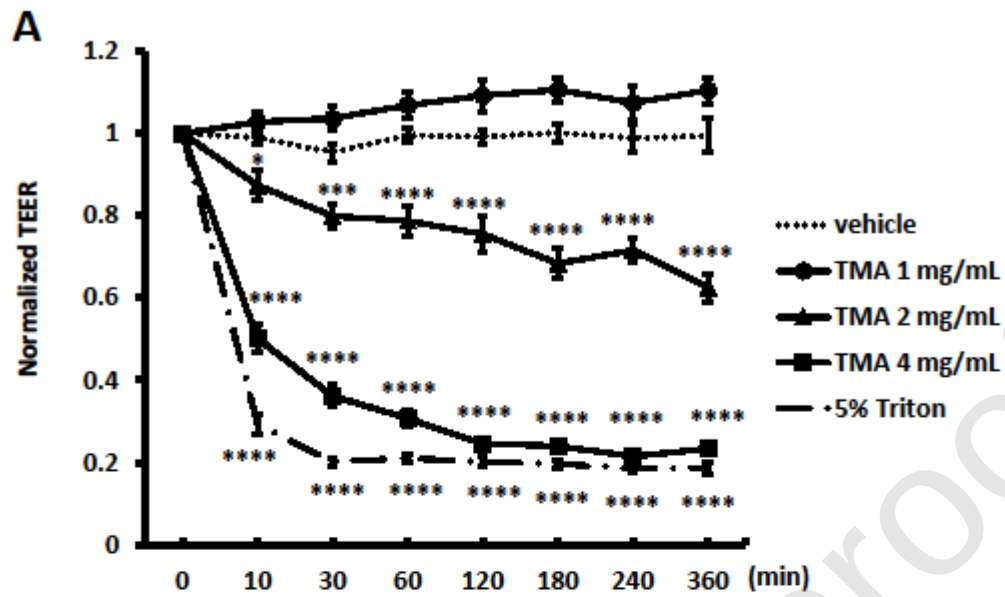
The values are shown as numbers or mean $\pm$ SEM. The statistical significance was evaluated by Student t test or Fisher's exact test. IF=immunofluorescence. TEER = transepithelial electrical resistance.

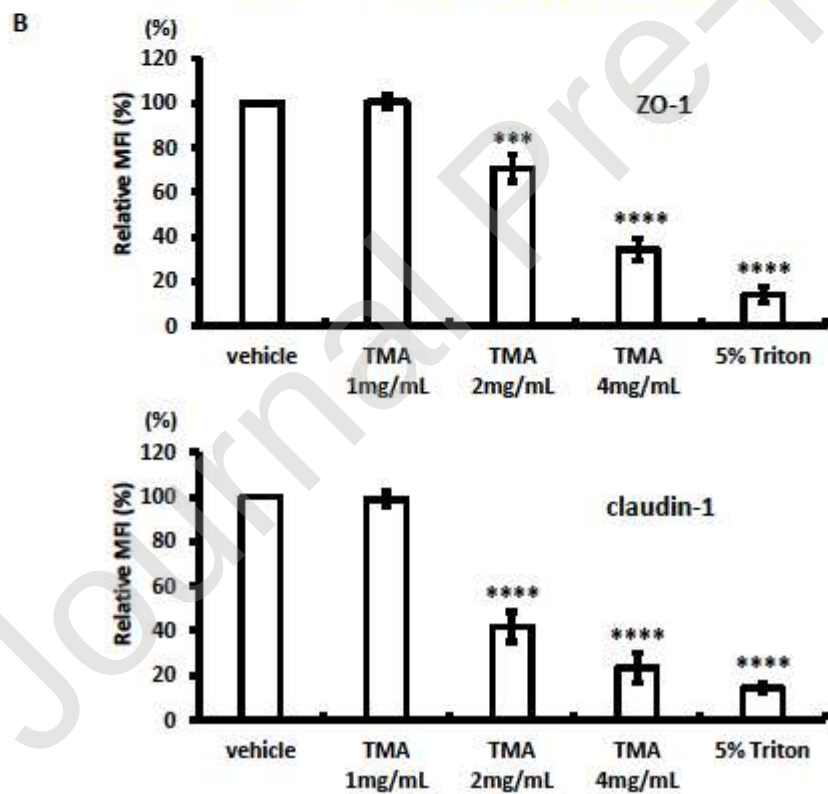
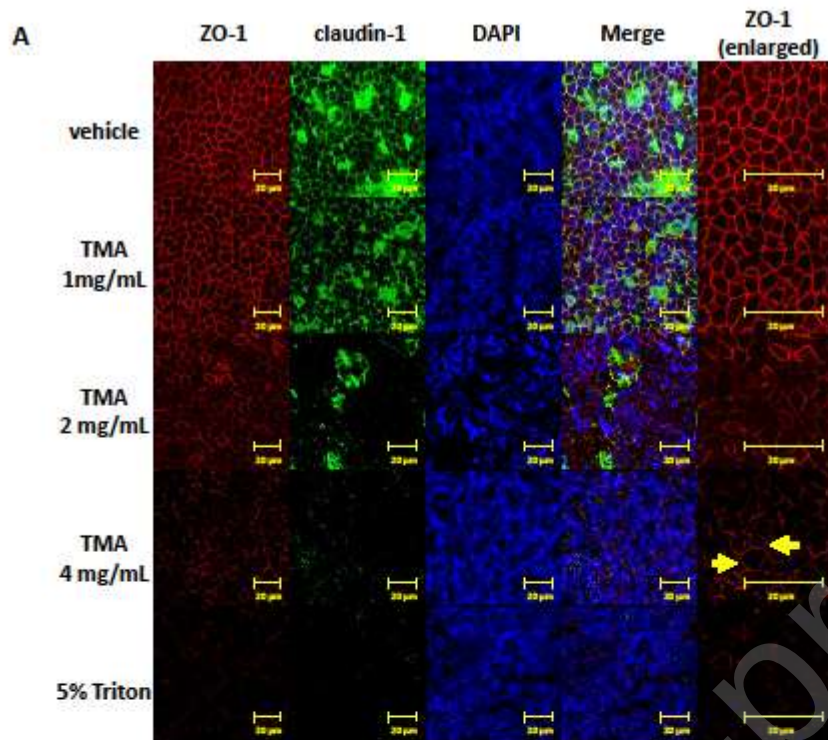
Figure 1. The dose-dependent effect of TMA on epithelial integrity. ALI cultured cells were incubated with varying concentrations of TMA (1, 2, 4 mg/mL) for 3 hours ($n=8$) and 6 hours ($n=8$). Vehicle (4% acetone in PBS) was used as the negative control. 5% Triton was used as the positive control. (A) TEER change was recorded by sequential measuring with voltmeter over time. (B) Paracellular permeability was assessed with FITC-dextran after final TEER measurement at 3 hours and 6 hours incubation with TMA. Values are means $\pm$ SEM; $n = 8$ replicates. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$, one-way ANOVA followed by Dunnett's test.

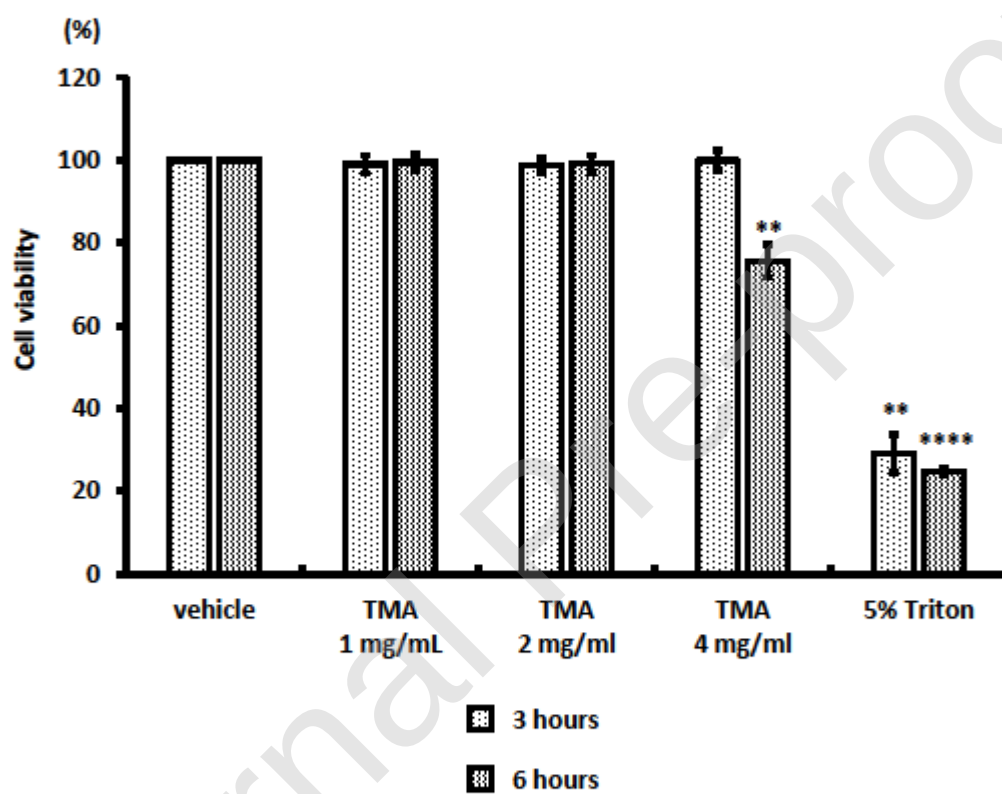
Figure 2. The dose-dependent effect of TMA on ZO-1 and claudin-1. ALI cultured cells were double-stained for ZO-1 and claudin-1. (A) The representative images of immunofluorescence of TJ in ALI cultured HNECs. Vehicle (4% acetone in PBS) was

used as the negative control. 5% Triton in PBS was used as the positive control. Red, ZO-1; green, claudin-1; blue, DAPI. The yellow arrows indicate the dislocation of ZO-1 induced by TMA treatment. Images were taken with $\times 20$ magnification and displayed as Z stacks with a 20- μm slice thickness. (B) The quantitative analysis of mean immunofluorescence intensity (MFI) of ZO-1 and claudin-1 normalized with DAPI. The quantification in each time point was normalized to vehicle control. The values are means $\pm$ SEM; $n = 5$ replicates. $**P < 0.01$, $****P < 0.0001$, one-way ANOVA followed by Dunnett's test.

Figure 3. TMA treatment does not affect cell viability on ALI cultured HNECs. The medium in the basolateral chamber was collected after 3 and 6 hours for each treatment and LDH released into the medium was measured by LDH assay. Vehicle (4% acetone in PBS) was used as the negative control. 5% Triton in PBS was used as the positive control. The relative cell viability of each treatment was normalized to that of cells incubated with the vehicle as a negative control. Values are means $\pm$ SEM; $n = 8$ replicates. $****P < 0.0001$, one-way ANOVA followed by Dunnett's test.







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Item	TEER Permeability assayLDH assay(Figure 1, 3)	IF(Figure 2)	<i>p</i> -value
Number of Subjects	8	5	
Age	43.4 ± 6.2	46.6 ± 4.0	N.S
Male/Female	3/5	3/2	N.S
allergic rhinitis	0	0	N.S
asthma	0	0	N.S
smoking	0	0	N.S