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Red ginseng aqueous extract improves mucociliary transport dysfunction and histopathology in CF rat airways

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Abstract

Background: We previously discovered that Korean red ginseng aqueous extract (RGAE) potentiates the TMEM16A channel, improved mucociliary transport (MCT) parameters in CF

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nasal epithelia *in vitro*, and thus could serve as a therapeutic strategy to rescue the MCT defect in cystic fibrosis (CF) airways. The hypothesis of this study is that RGAE can improve epithelial Cl^- secretion, MCT, and histopathology in an *in-vivo* CF rat model.

Methods: Seventeen 4-month old $CFTR^{-/-}$ rats were randomly assigned to receive daily oral control (saline, n=9) or RGAE (Ginsenosides 0.4mg/kg/daily, n=8) for 4 weeks. Outcomes included nasal Cl^- secretion measured with the nasal potential difference (NPD), functional microanatomy of the trachea using micro-optical coherence tomography, histopathology, and immunohistochemical staining for TMEM16a.

Results: RGAE-treated CF rats had greater mean NPD polarization with UTP (control = −5.48 +/− 2.87 mV, RGAE = −9.49 +/− 2.99 mV, $p < 0.05$), indicating, at least in part, potentiation of UTP-mediated Cl^- secretion through TMEM16A. All measured tracheal MCT parameters (airway surface liquid, periciliary liquid, ciliary beat frequency, MCT) were significantly increased in RGAE-treated CF rats with MCT exhibiting a 3-fold increase (control, 0.45+/−0.31 vs. RGAE, 1.45+/−0.66 mm/min, $p<0.01$). Maxillary mucosa histopathology was markedly improved in RGAE-treated cohort (reduced intracellular mucus, goblet cells with no distention, and shorter epithelial height). TMEM16A expression was similar between groups.

Conclusion: RGAE improves TMEM16A-mediated transepithelial Cl^- secretion, functional microanatomy, and histopathology in CF rats. Therapeutic strategies utilizing TMEM16A potentiators to treat CF airway disease are appropriate and provide a new avenue for mutation-independent therapies.

Keywords

Red ginseng; ginsenoside; mucociliary transport; mucociliary clearance; cystic fibrosis; CFTR; TMEM16A; TMEM16A potentiator; calcium-activated chloride channel; cystic fibrosis rat; nasal potential difference; micro-optical coherence tomography; functional microanatomy

INTRODUCTION

The primary phenotype associated with the loss of CFTR function in cystic fibrosis airway disease is a hyper-concentrated mucus gel that mucociliary transport (MCT) mechanisms struggle to remove. Chronic bacterial infection ensues, progressing to loss of lung function.[1] Treatments largely target the symptoms and physiologic manifestations of the underlying disease,[2] with the exception of CFTR modulators, which can partially rescue the function of mutated CFTR to improve clinical outcomes in genetically suited patients.[3] However, they cannot treat all patients nor restore lung function to normal levels. Effective, mutation-independent therapeutics are urgently needed.

Longstanding investigations into restoring Cl^- transport via alternative Cl^- channel pathways represent one such therapeutic strategy. The TMEM16A channel (anoctamin-1) was identified as the gene encoding the primary airway calcium-activating Cl^- channel in 2008.[4] Like CFTR; TMEM16A conducts both Cl^- and HCO_3^- across the airway epithelium and is expressed in both the surface epithelium and submucosal glands. TMEM16A is activated by the endogenous ligand, ATP, via the P2Y2 receptor leading to the release of intracellular Ca^{2+} as a second messenger. Initial excitement about this alternative Cl^-

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channel pathway to rescue the transport defect in CF was tempered when denufosol, a stable purinergic ligand that activates TMEM16A, failed to benefit CF patients, but rather induced adverse effects, such as cough.[5] The failure was attributed to rapid desensitization of the P2Y2 receptor and/or depletion of intracellular Ca^{2+} stores. Several other studies suggested TMEM16A maintains excessive mucus secretion during inflammatory airway disease and supports airway smooth muscle contraction,[6] leading some to advocate for inhibiting, not activating, it as a strategy for CF airway disease.

Korean red ginseng (*Panax ginseng Meyer*) has been utilized worldwide as alternative medicine, and its main bioactive components are steroidal saponins called ginsenosides.[7,8] Early research suggest the anti-inflammatory and neuroprotective character of these phytocompounds are applicable to a variety of conditions including respiratory, cardiovascular, and neurodegenerative disorders.[9–11] Our previous *in vitro* study demonstrated that the red ginseng aqueous extract (RGAE) was a significant potentiator of the TMEM16A channel and improved MCT parameters in wild type and CF murine epithelial cell culture models.[12] Evidence for potentiating activity was based upon stimulation of TMEM16A-mediated Cl^- secretion in the absence of cytosolic increase in Ca^{2+} and synergistic improvement in UTP-mediated Cl^- secretion as judged by whole cell patch clamp in human embryonic kidney cells expressing TMEM16A. Based on this previous study, we presumed RGAE could serve as a rescue mechanism in CF airways by potentiating the TMEM16A. In theory, a TMEM16A potentiator bypasses excessive stimulation of the purinergic Ca^{2+} signaling pathway and augments Cl^- secretion through the channel during endogenous activation, but without P2Y2 receptor desensitization. Another advantage of this compound is that, unlike the recently discovered TMEM16A potentiator ETX001,[13] ginsenosides are orally bioavailable.

We previously developed the $CFTR^{-/-}$ rat, which has dysfunctional MCT, goblet cells with distended mucus, hypertrophy of airway submucosal glands, and increased viscosity of respiratory secretions that first occur between 3 and 6 months, suggesting potential for ameliorating these abnormalities pharmacologically.[14–17] The hypothesis of this study is that orally administered RGAE will improve epithelial Cl^- secretion via TMEM16A, reverse the MCT defect in the airway, and normalize histopathology in the *in-vivo* CF rat model.

METHODS

$CFTR^{-/-}$ rat model

All animal use was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committees (IACUC) prior to initiation of the study. All experiments used Sprague Dawley $CFTR^{-/-}$ rats (Horizon Discovery Waterbeach, United Kingdom). This rat strain was bred and genotyped, as previously described.[18] In brief, heterozygote ($CFTR^{+/-}$) males and females were paired to generate wild type (WT) and knockout pups, and litters remained with lactating dams until 21 days of age. $CFTR^{-/-}$ rats were maintained on a standard rodent diet, with supplemental DietGel 76A (ClearH20, Portland, ME) and 50% Go-LYTLEY (Braintree Laboratories Inc. Braintree, MA) added to the water from weaning to reduce mortality from gastrointestinal obstruction.

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RGAE Feeding and Dosage

Four-month old CFTR$^{-/-}$ rats were chosen for this study. Seventeen rats were randomly assigned to receive daily oral placebo (saline, n = 9) or RGAE (Ginsenosides 0.4mg/kg/daily, n = 8) for 4 weeks. RGAE was provided by the Korea Ginseng & Tobacco Central Research Institute, and dosage of ginsenosides (0.4mg/kg/daily) was based on the daily recommended dosage for humans.[19,20] RGAE was produced from the roots of 6-yr-old red ginseng (Panax Ginseng Meyer) through a standardized manufacturing process. The general composition of RGAE is ash, 2.5%; total fat, 0.05%; total crude saponin, 70 mg/g; and total ginsenosides, 20 mg/g. Certifications of consistency and purity are provided through Current Good Manufacturing Practices Certifications, Hazard Analysis and Critical Control Points (HACCP), and International Organization for Standardization for Reliable Food Management Safety Systems certification (ISO 22000).

Outcome measures

1) Nasal potential difference measurement: Rats were anesthetized with ketamine (200 mg/kg) and xylazine (30 mg/kg) by intraperitoneal injection. The potential difference was measured using potassium chloride (KCl)/Calomel electrodes electrode and electronic data capture (AD Instruments, Sydney, Australia) as previously described for mice and humans.[21–26] Chemicals were obtained from Sigma (St. Louis, MO). Nasal cavities were perfused sequentially with the following pharmacologic agents: 1) Ringer's solution containing 148 mM NaCl, 4 mM KCl, 1.2 mM MgCl2, 2.25 mM CaCl2, 2.4 mM K2HPO4, 0.4 mM KH2PO4 (pH 7.4), and 100 μM amiloride - blocks epithelial Na^+ channels, as a means to isolate changes in short-circuit current (I_{SC}) secondary to effects on Cl^- channel activity); 2) a zero-Cl- -containing solution (NaCl in solution #1 replaced by 148 mM Na gluconate, 2.25 mM Ca gluconate, and 4 mM K gluconate; (pH 7.4) & forskolin (20 μM - activate CFTR by Cl^- gradient and elevating intracellular cAMP, which results in protein kinase A (PKA)-dependent phosphorylation of the CFTR regulatory domain), 3) INH-172 (10 μM) and GlyH-101 (10 μM) - CFTR inhibitors that permit determination of CFTR-dependent contributions to I_{SC}, and 4) uridine 5-triphosphate (UTP, 150 μM) – activates CaCC. Each condition was studied for 5 to 10 minutes until a stable signal was achieved. The activity was measured from the stable baseline to the highest point of hyperpolarization. Traces were interpreted in a blinded fashion.

2) Micro-optical coherence tomography (μOCT) image acquisition and analysis: Measurements of functional microanatomic parameters in *ex vivo* trachea tissue were performed using μOCT, a high-speed, high-resolution microscopic reflectance imaging modality.[27–32] Tracheae were excised and immediately placed on gauze soaked in Minimum Essential Media (Thermo Fisher Scientific, Waltham, MA), such that the apical surface of the trachea remained media free, and incubated under physiologic conditions (37°C, 5% CO_2, 100% humidity). Tracheae were allowed to equilibrate for 1 hour before imaging. Images were acquired at five controlled points along the tracheal length, using the cranial end as a reference. ASL and PCL were calculated by direct measurement of the visible depth within the image. To account for refractory properties of the liquid, layer thickness measurements were corrected for the refraction index of the liquid (n = 1.33). ASL and PCL depth were calculated from pixels to μm using a ratio of 0.82. CBF was evaluated by means

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of a time series of images by determining peak amplitude frequency in the temporal Fourier transform of areas demonstrating oscillatory behavior.[25,33–40] Mucociliary transport (MCT) rate was determined using the time elapsed, and distance traveled of native particulates in the mucus over multiple frames. For each trachea, images were acquired at standard distances along the ventral surface, with the optical beam scanned along the longitudinal direction. Statistics were performed for each individual replicate. All images were analyzed using ImageJ version 1.50i (National Institutes of Health, Bethesda, MD) and MATLAB® R2016a (The MathWorks, Natick, MA).

3) Maxillary sinus mucosa histology: After 4 weeks of treatment, rats were euthanized and heads were harvested. The nasal passages were flushed retrograde with 10% buffered formalin followed by immersion in formalin until processed. Specimens were provided to the UAB Comparative Pathology Laboratory (CPL) for sectioning and evaluation. Briefly, samples were decalcified for 4–5 days in Immunocal (Decal Chemical Corporation, Tallman, NY) solution, and rinsed thoroughly. Sections of the nasal cavity and maxillary sinuses were divided into 4 regions at specific anatomic sites as described previously.[41,42] Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The respiratory epithelium of the maxillary sinus at the mid-maxillary walls were measured for epithelial thickness and the density of goblet cells by 2 blinded judges. Additional slices were stained with Alcian Blue-Periodic Acid Schiff (AB-PAS) to examine intracellular mucus in the goblet cells of the nasal septum. Total mucus was calculated from the area normalized to the height of the epithelium. Sections were imaged on a Keyence All-in-One Fluorescence Microscope BZ-X800 with area calculations using the BZ-H4M/Measurement Application (Keyence, Osaka, Japan).

4) TMEM16A immunohistochemistry of CF rat lungs: Paraffin-embedded CF rat lung slices were subjected to immunohistochemical staining for TMEM16A (Anti-ANO1 primary antibody, rabbit polyclonal, CAT#: MBS176756 (MyBioSource, Inc., San Diego, Ca.) and visualized by 3,3'-diaminobenzidine (DAB) staining to detect TMEM16A expression. Nuclei were stained with hematoxylin. Data were analyzed with ImageJ Fiji software with semi-quantitative determination of protein expression using immunohistochemistry staining and analysis.[43] TMEM16A DAB staining intensities were normalized by the epithelial layer length for each sample to provide the value.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 9.0. Inferential statistics (mean+/− SD) were computed using 2-tailed unpaired t test. P values of less than 0.05 were considered significant. Statistics are presented as mean +/− standard deviation.

RESULTS

RGAE-treated CF rats exhibited no adverse effects

No adverse effects (e.g., decreased appetite, cough, breathing difficulty, or death) were noted during this study period. There was no statistical difference in weights before and after treatment between the two groups, although RGAE cohorts had less % of weight loss

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compared to controls (% of weight loss, control = 4.25 +/− 1.12 % vs. RGAE = 3.21 +/− 2.3, $p > 0.05$) during the study period.

RGAE increased UTP-mediated transepithelial Cl^- transport in vivo

To characterize the electrophysiology of the upper airway after 4 weeks of treatment of RGAE, transepithelial potential difference was monitored in response to a series of pharmacologic ion channel regulators (Figure 1). As expected, $CFTR^{-/-}$ rats showed no Cl^--dependent secretion when CFTR-mediated Cl^- transport was stimulated by Cl^--free Ringer's lactate with forskolin or blocked by CFTR inhibitors. However, $CFTR^{-/-}$ rats treated with RGAE had a much greater mean NPD polarization with UTP (control = −5.48 +/− 2.87mV, RGAE = −9.49 +/− 2.99mV, $p < 0.05$), indicating, at least in part, potentiation of UTP-mediated Cl^- secretion through TMEM16A.

RGAE improved the functional microanatomy of CF rat tracheas

We used μOCT to visualize and quantify the functional microanatomy of the trachea airway surface (Figure 2). Excised tracheas from CFTR KO rats who received RGAE had significantly greater ASL depth (control, 8.70+/−2.06 μm vs RGAE, 25.50+/− 18.04 μm; $p < 0.05$), PCL depth (control, 5.75+/−0.71 μm vs RGAE = 7.23+/− 1.08 μm; $p < 0.01$), and CBF (control, 7.22+/− 0.79 Hz vs RGAE = 8.75+/− 0.86 Hz; p<−0.01). The mean MCT[14] rate was also markedly improved 3-fold (control, 0.45+/−0.31 vs. RGAE, 1.45+/−0.66 mm/min, $p<0.01$).

Goblet cell abundance and epithelial height in the maxillary sinus mucosa were decreased in RGAE-treated CF rats

The two rat cohorts had normally developed epithelium consisting of a pseudostratified columnar epithelium comprised of both goblet and ciliated epithelial cells. As described previously, CF rats exhibit abnormal goblet cell histology with an excessive number distended with thick mucus when compared to wild type rats and thickened submucosa with mucus-filled submucosal glands by 3–6 months of age.[17,18] CF rats treated with RGAE had decreased goblet cell number (goblet cells/mm) and lacked the mucus-distended appearance that was present in the saline controls (control, 42.67+/− 11.68 vs. 29.48+/−11.64; $p<0.05$). The epithelial layer was also thinner in the CF rats treated with RGAE presumably due to the "loss" of distended goblet cells (control, 72.33+/−11.83 μm vs. RGAE, 56.50+/−8.64 μm, $p<0.01$) (Figure 3).

Intracellular mucus was decreased in RGAE-treated CF rat nasal septum epithelium

Similarly, when additional sections of the nasal cavities were stained with AB- PAS and examined for intracellular mucus, the RGAE-treated rats demonstrated a marked decrease in total intracellular mucus normalized to epithelial height in μm^2 per μm (control, 7.45+/−3.15 vs. RGAE, 4.27+/−2.75, $p<0.05$) (Figure 4).

TMEM16A expression was similar between RGAE-treated and control CF rats.

In order to assess whether RGAE might influence expression of TMEM16A protein as a potential reason for the observed effects, immunohistochemical staining for TMEM16A

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was performed on the CF rat lung slices of the rat cohorts. TMEM16A protein expression was not increased in RGAE-treated rats (0.0048+/−0.0033 vs. control, 0.0049+/−0.0030, p= 0.94) (Figure 5)

DISCUSSION

Ginsenosides are the active ingredients within Korean red ginseng with Rb1 recently identified to activate TMEM16A in guinea pig intestine.[44,45] In our previous *in vitro* study, RGAE significantly increased Cl^- transport in $CFTR^{-/-}$ murine nasal sinus epithelium and HEK cells expressing the TMEM16A in a Ca^{2+}-independent fashion indicating TMEM16A potentiation as the likely mechanism of action. Because RGAE significantly increased MCT parameters in $CFTR^{-/-}$ mice, this suggested a translational potential to improve MCT parameters in a CF animal model *in vivo*.[12] Indeed, the findings of the current study support this strategy. RGAE amplified UTP-mediated Cl^- secretion, increased all measures of functional microanatomy, and improved the appearance of maxillary sinus mucosa histology in $CFTR^{-/-}$ rats.[12] Similarly, total intracellular mucus was significantly decreased in CF rat nasal cavities treated with RGAE. Expression of TMEM16A was similar between RGAE and control rat lungs and thus RGAE did not confer its effect through overexpression of TMEM16A.

Thick mucus contributes to the pathophysiology of inflammatory airway diseases, including CRS, asthma, chronic obstructive pulmonary disease, and CF.[46–50] During inflammation, TMEM16A is known to be upregulated particularly in mucus producing cells, with only little expression in ciliated cells.[51] Combined with the clinical failure of earlier studies targeting TMEM16A through purinergic activation, interest has recently turned towards inhibiting TMEM16A in the airway to decrease mucus exocytosis.[5,52] In our study, RGAE-treated CF rats had significantly lower epithelial height, fewer identifiable goblet cells in the sinus mucosa, and overall decreased intracellular mucus in nasal epithelium. Thus, our results contradict reports that TMEM16A inhibition is required to reduce the goblet cell population.[53,54] We hypothesize that the decreased population of goblet cells, decreased intracellular mucus, and improved goblet cell and submucosal gland architecture is secondary to stimulation of mucus exocytosis during the 4 weeks of treatment, a finding that has been found to be defective in CF.[55] Combined with improved mucus clearance by augmented MCT, this would be expected to reduce goblet cell hyperplasia over time. While some postulate that mucus release by resolving mucus exocytosis has the potential to exacerbate cough and increase mucus expression into the airway, RGAE-treated CF rats experienced no adverse pulmonary events. Furthermore, we observed no ramifications of excess mucus release upon initial treatment. All MCT parameters increased in CF rat tracheas, including a 3-fold increase in MCT in rats ingesting RGAE. This finding suggests that either 1) exocytosis of mucus from goblet cells with entrapped mucus has no impact on clearance on the time scales we examined them; or 2) potentiation of TMEM16A Cl^- transport can overcome any deleterious impact of increased mucus load during an acute treatment period.

In the airways, the primary phenotype associated with the loss of CFTR function is a hyper-concentrated mucus gel that MCT mechanisms struggle to remove. Chronic bacterial

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infection ensues, progressing to loss of lung function.[6] Current treatments for CF largely target the symptoms and physiologic manifestations of the underlying disease,[7] with the exception of CFTR modulators, which can partially rescue the function of mutated CFTR to improve clinical outcomes in genetically suited patients.[8] However, they cannot treat all patients nor restore lung function to normal levels. Our discovery of an oral, bioavailable TMEM16A potentiator that rescues the anion secretory defect and CF-mediated MCT dysfunction can be complementary to current therapies and could target patients with no options for CFTR modulator therapy.

Importantly, a TMEM16A potentiator will only potentiate channels that are endogenously activated. In our study, UTP was administered during the NPD to observe how much activation can be achieved (hyperpolarization, change in transepithelial potential) with maximal stimulation relative to CF rats that did not receive drug. This is similar to measuring hyperpolarization in response to isoproterenol (CFTR activation) in CF patients in CFTR modulator trials.[2] Just as CF patients who are prescribed CFTR modulators do not need isoproterenol for the drug to have its observed effect, a TMEM16A potentiator would not require an exogenous activator of TMEM16A through P2Y2 signaling, like defenusol. Thus, the limitations of a treatment strategy with UTP and other P2Y2 analogues, including desensitization and chronic intracellular calcium release, are eliminated with use of a TMEM16A potentiator.

There are several limitations to the current study. While the CF rat has a mucus transport defect and abnormal histopathology, it does not develop spontaneous airway disease. The resolution of abnormalities of RGAE-treated CF rats may not be applicable in another *in vivo* model with concurrent or spontaneous airway infection and inflammation. Birket et al.[14] instilled with CF rat airways with a mucoid clinical isolate of Pseudomonas aeruginosa (PAM57-15) at 6 months and the animals subsequently developed bacterial persistence through 28 days post-infection. Since the model had airway mucus occlusion and lingering inflammation, it will be crucial to repeat these experiments post-infection to simulate overproduced and viscous mucus observed in human CF airways. Other properties of the airway surface, particularly the impact on mucin structure and function will also require evaluation. Moreover, it will be important to assay the pH of the mucus or to analyze HCO_3^- activity in the ASL. Although orally bioavailable, the absorption of ginsenosides are impacted by intestinal secretions, digestive enzymes, and gut bacteria.[56] To improve the bioavailability of these compounds, topical application of ginsenosides through inhalation or irrigation could improve the impact in the target area of the sinuses and lung. While the presumed mechanism of action is TMEM16A potentiation, further characterization of isolated ginsenosides in RGAE using single channel patch clamp analysis is warranted. Finally, other components of RGAE that are related to anti-inflammatory and anti-infective activity could be responsible for the intended effect and thus analysis of single ginsenosides will help determine specificity.

CONCLUSION

RGAE is the first known orally bioavailable compound capable of potentiating TMEM16A. Our *in vivo* study showed that RGAE significantly improves TMEM16A-mediated

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transepithelial Cl^- secretion, functional microanatomy of the airway, and maxillary sinus mucosa histopathology. A therapeutic strategy utilizing TMEM16A potentiators to treat CF airway disease is promising and provides a new avenue for mutation-independent therapies that target the Cl^- transport defect in CF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mutation-independent therapeutic strategies are needed for all CF patients.
- Korean red ginseng aqueous extract (RGAE) has TMEM16a potentiating properties.
- CF rats that ingested RGAE exhibited enhanced UTP-mediated Cl^- secretion.
- RGAE also improved Cl^- secretion, mucociliary transport (MCT), and histopathology.
- RGAE could serve as a therapeutic strategy to rescue the MCT defect in CF.

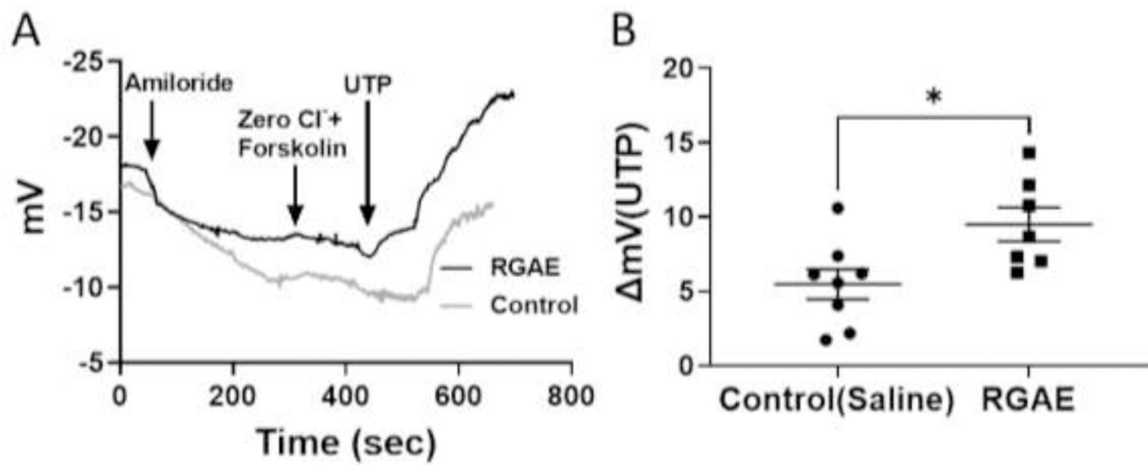

Figure 1.

Representative NPD tracings showing PD after serial administration of 1) amiloride (100 μM), 2)Cl^- free forskolin (20 μM), 3)INH-172 (10 μM) and GlyH-101 (10 μM), and 4) UTP (150 μM) (A), followed by summary data (B) in RGAE-treated and control CF rats. RGAE treatment caused NPD hyper-polarization with UTP (control = −5.96 +/− 2.3 mV, RGAE = −12.51 +/− 1.04 mV, $p < 0.05$; unpaired t test), indicating potentiation of UTP-mediated Cl^- secretion through TMEM16A.

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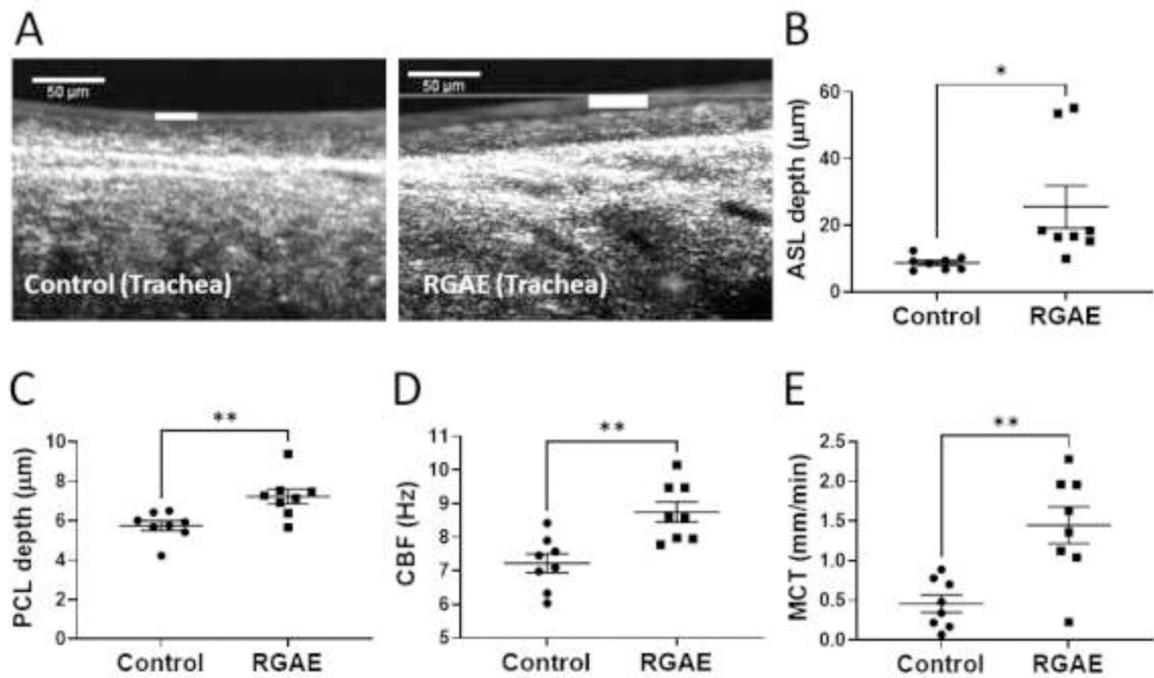

Figure 2.

Representative µOCT images (A) and summary data for ASL (B), PCL (C), CBF (D), and MCT (E) recorded in tracheas excised from control and RGAE-treated CF rats (*P<0.05, **P<0.01; unpaired t test). RGAE significantly improved all markers of functional anatomy (ASL – airway surface liquid, PCL – periciliary liquid, CBF – ciliary beat frequency, MCT – mucociliary transport, RGAE – red ginseng aqueous extract). White line = ASL depth. Representative µOCT videos are provided as supplementary data.

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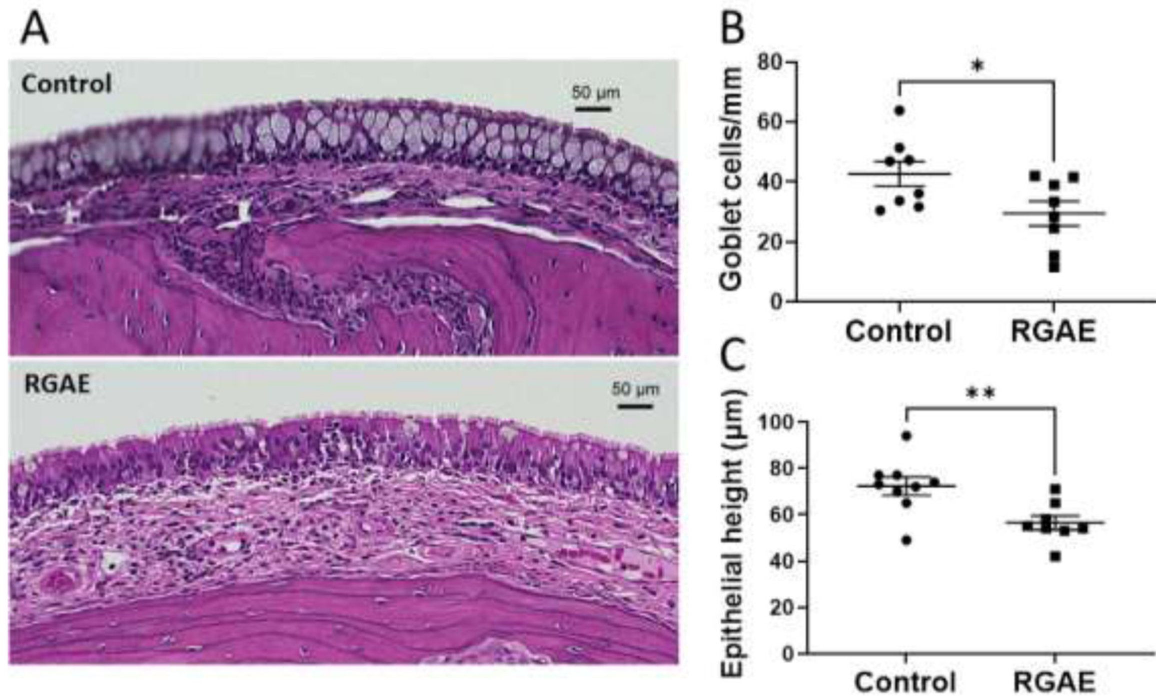

Figure 3.

Representative hematoxylin/eosin-stained maxillary sinus mucosa (A) of 10× H&E stained mucosa from control and ginseng-treated CF rats. Summary data regarding number of goblet cells (B) and epithelial height (C). RGAE-treated CF rats demonstrate marked improvement in histopathology (*P<0.05, **P<0.01, unpaired t test).

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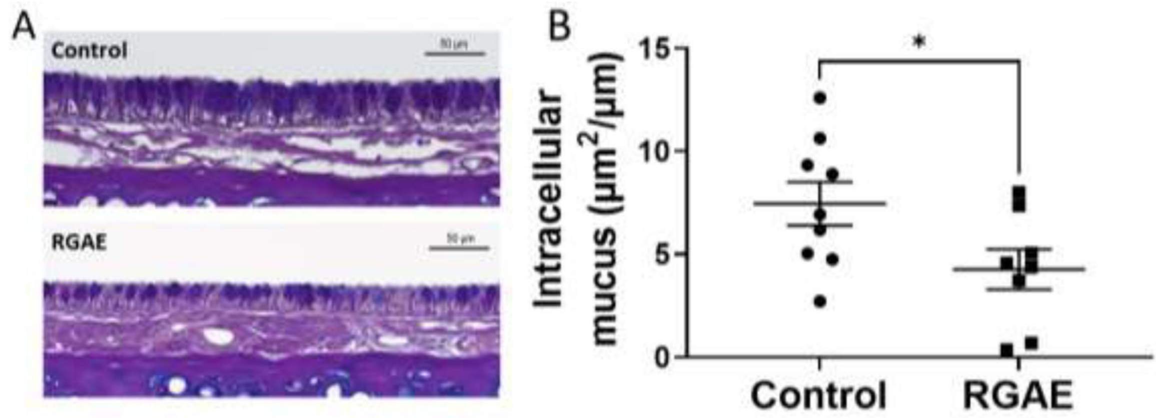

Figure 4.

Representative Alcian Blue – Periodic Acid Schiff staining of nasal septum epithelium (A) from control and ginseng-treated CF rats. Summary data demonstrates significant reduction in intracellular mucus in RGAE-treated CF rats (*P<0.05, unpaired t test).

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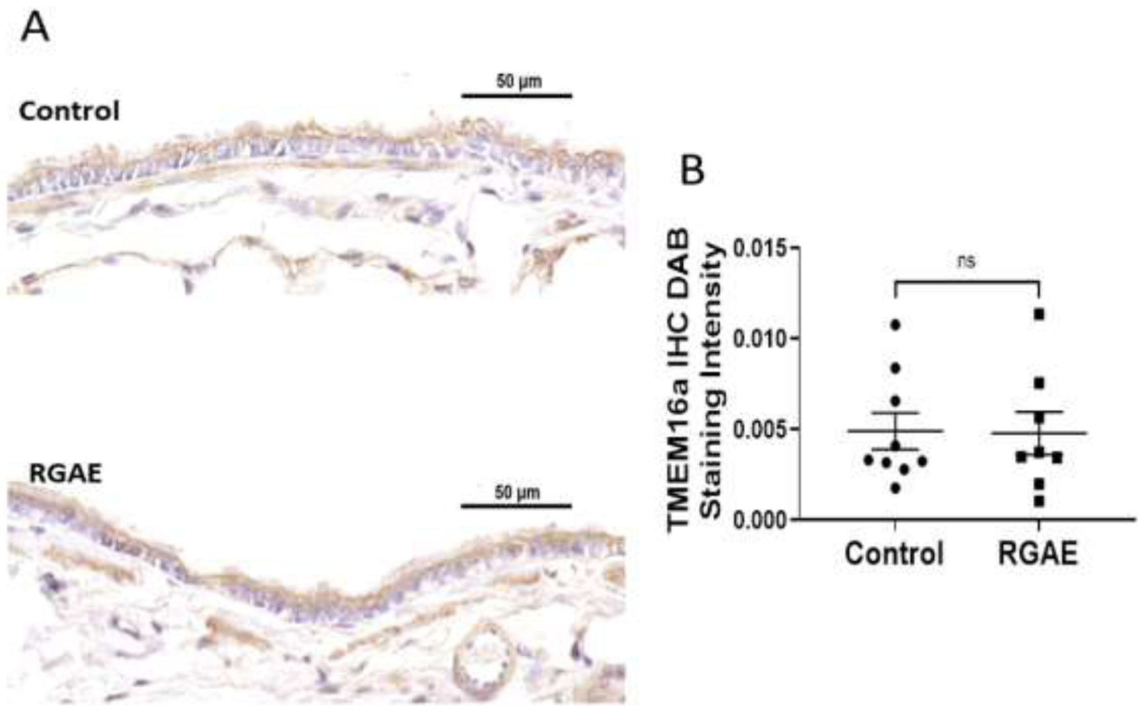

Figure 5.

Representative TMEM16A immunohistochemical staining between control and RGAE-treated CF rats (A). Summary data demonstrates no increase in TMEM16a staining intensity in RGAE treated CF rats (B).