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Herbal dry extract BNO 1011 improves clinical and mucociliary parameters in a rabbit model of chronic rhinosinusitis

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Abstract

Introduction: Enhancing chloride (Cl^-) secretion in sinus epithelia represents a novel therapeutic approach to chronic rhinosinusitis (CRS). Herbal dry extract BNO 1011 enhances mucociliary clearance (MCC) via upregulation of Cl^- secretion in sinonasal cultures *in vitro* and murine epithelium *in vivo*. The objective of this study is to evaluate whether the BNO 1011 improves MCC and clinical parameters in a rabbit model of CRS.

Methods: After the development of CRS in 30 New Zealand white rabbits, animals were randomly assigned to receive oral placebo (n=10), BNO 1011 (low dose (LD), 25mg/kg/daily) (n=10), or BNO1011 (high dose (HD), 125mg/kg/daily) (n=10) for 4 weeks. Outcomes included sinus opacification (Kerschner's rabbit sinus CT grade), maxillary epithelial Cl^- secretion (sinus potential difference (PD) assay), airway surface liquid (ASL) depth using (micro optical coherence tomography (μOCT), and submucosal gland density (SMD) on histopathology. Outcome parameters were analyzed by 2 blinded investigators.

Results: BNO 1011 significantly cleared sinus opacification (HD=1.21 $\pm$ 0.63, LD=1.26 $\pm$ 0.37,) compared to placebo (4.02 $\pm$ 0.92) (p=0.009). BNO 1011 resulted in markedly greater mean sinus PD polarization (HD=-12.23 $\pm$ 1.4mV, LD=-12.0 $\pm$ 3.0mV) when compared to rabbits treated with placebo (-4.1 $\pm$ 1.1mV) (p=0.03). ASL depth was significantly improved when treated with HD (4.08 $\pm$ 0.06 μm) and LD (4.05 $\pm$ 0.06 μm) compared to placebo (3.5 $\pm$

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−0.05μm) (post-hoc analysis, $p<0.0001$). Histologically, epithelial thickness (HD=10.0±0.7μm; LD=13.7±0.9μm; placebo=21.1 ± 2.3μm; $p<0.005$), subepithelial thickness (HD=63.1±6.6μm; LD=103.2±6.7μm; placebo=113.3±6.0 μm; $p<0.001$) and SMD (HD=22.2±2.9%; LD=31.8±1.1%; placebo=43.8±1.7%; $p<0.0001$) were noticeably better with the HD.

Conclusion: Herbal dry extract BNO 1011 improves radiographic, histologic and MCC parameters in a rabbit model of CRS.

Keywords

Herbal dry extract; mucociliary clearance; rabbit model of sinusitis; chronic rhinosinusitis

INTRODUCTION

Mucociliary clearance (MCC) is the primary defense mechanism in airway epithelium that clears inhaled pathogens.^{1,2} Dysregulation of this critical function is characterized by depleted airway surface liquid (ASL) and abnormally viscous and adhesive mucus, which result in a profound decrease in mucus transport precipitating chronic infection and inflammation.^{1,3} ASL and the viscoelastic properties of the mucus depend upon the coordinated action of a number of ion transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR) and calcium activated chloride channels (CaCC).^{4–6} Mounting evidence suggests decreased chloride (Cl[−]) transport is associated with chronic rhinosinusitis (CRS).^{7–10} CRS is a hallmark feature in cystic fibrosis (CF) patients who lack functional CFTR. Healthy patients heterozygous for a CFTR mutation also have an increased likelihood of developing CRS with and without nasal polyposis.^{11,12} Furthermore, Wang et al¹³ demonstrated mild dysfunction of the CFTR is associated with the development of CRS in the general population.

Because MCC is influenced by the transepithelial movement of Cl[−], there is growing interest in the use of Cl[−] secretagogues for treatment of multiple chronic airway diseases caused by inadequate MCC.^{14–18} Enhancing Cl[−] secretion in sinus epithelia represents a unique therapeutic approach to CRS. Sinupret® (Bionorica SE, Neumarkt, Germany) is a phytopharmaceutical that has been available for therapy of rhinosinusitis in Europe for decades, and more recently in the United States.^{19,20} The product is an extract of 5 herbs: gentian root (*Gentianae radix*), primrose flowers (*Primulae flos*), elder flowers (*Sambuci flos*), common sorrel herb (*Rumicis herba*) and vervain herb (*Verbenae herba*). In the United States, it is considered a supplement and is not regulated by the Food and Drug Administration (FDA), but is a regulated drug in Europe. Herbal dry extract BNO 1011, an extract from the same plants, was recently developed and in the European market since 2012, but not currently available in the US. Using *in vitro* murine and human sinonasal epithelial cell culture models, we have previously demonstrated that herbal dry extract (Sinpuret®) stimulates ciliary beat frequency (CBF), promotes transepithelial Cl[−] secretion, and increases ASL depth in a manner likely to enhance MCC, potentially explaining its beneficial effects in sinus disease.^{2,21} These studies provide further rationale for using robust Cl[−] secretagogue based therapies as an emerging treatment modality for CRS using our preclinical rabbit model of CRS.²² Given the similarities between immunologic features of rabbit and human sinus epithelium,²³ and noting that the *in vivo* rabbit acute sinusitis

disease model has been established and is well-suited for studies of therapeutic intervention,²⁴ we used the rabbit to develop an animal model of CRS by blocking MCC (sterile obstruction of the sinus outflow).²² The objective of this study is to evaluate whether the oral intake of herbal dry extract BNO 1011 improves MCC and clinical parameters in the rabbit model of CRS.

METHODS

Test compound

For this study a genuine dry extract (BNO 1011; batch no.: 750184) derived from ethanolic extraction (extractant: ethanol 51% m/m; drug extract ratio: 3–6:1) of gentian root (*Gentianae radix*), primrose flowers (*Primulae flos*), elder flowers (*Sambuci flos*), common sorrel herb (*Rumicis herba*) and vervain herb (*Verbenae herba*) (drug ratio: 1:3:3:3:3) was used. It was provided by Bionorica SE (Neumarkt, Germany).

Animal Model (Source, Housing, Anesthesia)

Pasteurella-free, female New Zealand white rabbits (source: Charles River, Hartford, CT) weighing 2.6 to 4 kg at the time of induction of sinusitis were used. Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* in an Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. All procedures in the study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham (UAB). Rabbits were housed in stainless steel, mobile 6-cage units (4.17 ft² in each cage; Lock Solutions, Laurence Harbor, NJ) containing removable plastic (GE Noryl, Selkirk, NY) sleeves with perforated floors and removable excreta pans. Pans were lined with cage board (Techboard; Shepherd Specialty Products, Somerville, NJ) and were changed 4 times each week, whereas cage sleeves and the rack were changed every two weeks. The rabbits received a commercial high-fiber pelleted laboratory diet (Hi-Fiber Rabbit Diet, Purina Mills, St Louis, MO) ad libitum and were maintained on municipal water in glass bottles with rubber stoppers and stainless-steel sippers. Rabbits were housed at 68 ± 2 °F (20 ± 1 °C), with 45% to 65% relative humidity and a 12:12-h light:dark cycle (0600 to 1800). Before initiation of sinusitis, rabbits were housed under a social housing policy at UAB and acclimatized to the animal facility for at least 1 week. After induction of sinusitis, rabbits were housed adjacently in enclosures separated by a partition that permits social contact. For any procedure, rabbits were anesthetized with [ketamine (20 mg/kg) (MWI, Boise, ID), dexdomitor (0.15 mg/kg) (Zoetis Inc., Kalamazoo, MI), buprenorphine (0.03 mg/kg) (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA), and carprofen (5 mg/kg) (Zoetis Inc., Kalamazoo, MI)] in a warm room for comfort. Rabbits did not receive any antibiotics before or during this study.

Rabbit Model of Sinusitis

A total of 30 rabbits were used to create a rabbit model of CRS without providing exogenous bacteria or pathogens as we described previously.²² Briefly, a sterile synthetic sponge (Merocel®, Medtronic, Minneapolis, MN) was placed in the left (unilateral) middle meatus (natural outflow tract of maxillary sinus) of New Zealand white rabbits for 2 weeks. This

method (transnasal endoscopic approach) causes no damage to the sinus mucosa and is reversible by removing the sponge transnasally on week 2. On week 2, synthetic sponges were removed from the middle meatus in all 30 rabbits. After the removal, rabbits were simply observed for the next 10 weeks and assessed for the presence of CRS features at week 12. At day 0, week 2 (acute sinusitis) and week 12 (chronic sinusitis), all rabbits were examined with micro-computed tomography (microCT, X-SPECT system, Gamma Medica, Northridge, CA) scanning and nasal endoscopy (1.7 mm 30-degree endoscope (Karl Storz, Tuttlingen, Germany)), to confirm the development of sinus opacification and drainage from middle meatus.²⁵

Ingestion of Herbal dry extract BNO 1011

At Week 12, rabbits were randomly assigned into 3 groups to receive 1) oral placebo (n = 10), 2) BNO 1011 dose A (low dose, 25mg/kg, 1-fold equivalent of the human daily dose) (n = 10) and 3) BNO 1011 dose B (high dose 125mg/kg, 5-fold equivalent of the recommended human daily dose) (n = 10) for 4 weeks. We used a sealed envelope system for randomization. Once the rabbit developed CRS on week 12, animals were allocated into 1 of the 3 treatment regimens according to sequential opening of the randomized envelopes. Herbal dry extract BNO 1011 (both low and high doses) were mixed with 2ml of apple sauce and fed daily with a syringe (n = 20). For those rabbits in the placebo groups (n = 10), 2 ml of apple sauce was fed daily without BNO 1011. The study protocol is described in Figure 1.

Outcome Measurements

- 1) Micro-Computed Tomography (CT) scanning - All rabbits were scanned at week 16. Sinus opacification grading was performed using Kerschner's rabbit Sinus CT grading system (scoring each imaging study based on estimated percent (%) opacification of the maxillary sinus: 1 for <10%, 2 for 10%–19%, 3 for 20%–29%, 4 for 30%–39%, 5 for 40%–49%, 6 for 50%–59%, 7 for 60%–69%, 8 for 70%–79%, 9 for 80%–89%, and 10 for 90%).²⁶ Opacification % was measured using the ImageJ version 1.50i (National Institutes of Health, Bethesda, MD) by two blinded judges.
- 2) Sinus potential difference (PD) measurement – The measurement of sinus PD provides a direct and sensitive evaluation of Na⁺ and Cl[−] ion transport in airway epithelial cells via assessment of transepithelial bioelectric properties in *in vivo* subjects. Although PD measurements have not been validated to diagnose sinusitis, *in vivo* direct measurement of epithelial ion transport in sinus epithelium would help to understand the role of ion transport in the pathogenesis of sinusitis and to assess the efficacy of new drug trials. Sinus PD measurement was performed prior to euthanasia on the first 5 rabbits from each group (total, n = 15) to assess the *in vivo* epithelial ion transport (Figure 2). Rabbits were anesthetized using above regimen in a warm room. After eye care was given, sterile saline was used to clean the skin around the dorsum of nose (planned incision site) first, followed by betadine. Following disinfection, the skin overlying the dorsum of the nose was incised in a vertical line (1cm), next to the

maxillary sinus, and the sinus cavity was entered using a small trocar. Then, polyethylene 90 tubing was inserted into the sinus cavity to measure the PD. The solutions perfused include Ringer's lactate plus amiloride (100 μ M), a low chloride solution containing [K₂HPO₄ (2.4 mM), KH₂PO₄ (0.4 mM), Na Gluconate (115 mM), NaHCO₃ (25 mM), Ca Gluconate₂ (1.24 mM)], CFTR agonist (forskoin 20 μ M), and CFTR blocker (Inh-172 10 μ M + Glyh-101 10 μ M). Each perfused condition was maintained for at least 5 mins. After the procedure, rabbits were euthanized to harvest the sinus for histology.

- 3) Micro-optical coherence tomography (μ OCT) image acquisition and analysis – Rabbits were euthanized, and sinus cavities were harvested. Measurements of functional microanatomic parameters in *ex vivo* sinus tissue were performed on the other 5 rabbits from each group (total, n = 15, those rabbits without sinus PD) using μ OCT, a high-speed, high-resolution microscopic reflectance imaging modality, according to the protocol outlined by Liu et al.²⁷ In brief, the μ OCT instrument provides cross-sectional images of the airway epithelium at a resolution of ~ 1 μ m. For maxillary sinus tissue, images were acquired on the relatively flat mucosal surface of the medial and lateral wall with the optical beam scanner. This resolution is sufficient to directly visualize and quantify microanatomic parameters, including ASL depth, and ciliary beat frequency (CBF). Typical acquisition speed is 20,480 Hz line rate, resulting in 40 frames/s at 512 lines/frame. ASL was quantitatively evaluated by directly measuring the visible thickness of the respective layers in the image. CBF was evaluated using a time series of images and quantitatively measured by identifying peak amplitude frequency in the temporal Fourier transform of areas demonstrating oscillatory behavior.²⁷ All parameters were assessed at 5 uniformly distributed areas of the image. All images were analyzed using ImageJ version 1.50i (National Institutes of Health, Bethesda, MD) and MATLAB® R2016a (The MathWorks, Natick, MA).
- 4) Histologic evaluation – Only rabbits undergoing Sinus PD measurements were selected for histologic sectioning (5 rabbits per group). Animals selected for μ OCT analysis had to be excluded due to removal of mucosal explants. After Sinus PD measurements, rabbits were euthanized and heads harvested. Epithelial and submucosal thickness, as well as submucosal gland density for negative control were derived from the contralateral maxillary sinus in the untreated placebo rabbits. Specimens were provided to the UAB Comparative Pathology Laboratory (CPL) for sectioning and evaluation. Ten representative sections of the maxillary sinus were selected and stained with hematoxylin and eosin (H&E). Periodic Acid–Schiff (PAS) staining was performed to assess the submucosal glands. After selecting 3 slides of the maxillary sinus lateral wall, the thickness of the epithelium and submucosal layer and the density of submucosal glands were measured by 2 blinded judges. To measure the density of SMGs in the submucosal layer, the area of the SMG structures was quantified using the area tool of ImageJ version 1.50i (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Statistical analyses were conducted with Excel 2016 and GraphPad Prism version 6.0 software (GraphPad Software, La Jolla, CA) using unpaired t-test or subgroup one-way ANOVA with post-hoc Tukey's test with significance set at $P < 0.05$ based on the characteristics of comparison.

RESULTS

No adverse effects (e.g., weight loss, orbital cellulitis, decreased appetite or death) were noted during this study period. On week 12, all rabbits exhibited opacification of the left sinuses on micro-CT scans and had mucus drainage in the middle meatus on nasal endoscopy, consistent with our previous description.²²

Micro-Computed Tomography Scanning

On week 12, the average CT scores (based on Kerschner's rabbit Sinus CT grading system) from sinusitis rabbits were 4.21 ± 0.33 ($n = 30$). After 4 weeks of ingesting herbal dry extract BNO 1011, those rabbits who received either dose A (low) or dose B (high) exhibited significantly improved sinus opacification in their sinuses compared to those rabbits administered placebo (Figure 3A). Based on Kerschner's rabbit Sinus CT grading system, scores were significantly higher in those rabbits treated with placebo ($n = 10$, 4.02 ± 0.92) compared to those rabbits who received either dose A (low) ($n = 10$, 1.26 ± 0.37) or dose B (high) ($n = 10$, 1.21 ± 0.63) ($p = 0.009$, ANOVA, Figure 3B). There was no statistical difference between dose A and dose B. When compared to scores on week 12, significant improvements were noticed in rabbits treated with dose A (low) ($n = 10$, week 12: 4.19 ± 0.5 vs week 16: 1.26 ± 0.37 , $p < 0.001$, paired t-test) or dose B (high) ($n = 10$, week 12: 4.28 ± 0.46 vs week 16: 1.21 ± 0.63 , $p < 0.0001$, paired t-test). However, there was no statistical improvement in rabbits that received placebo ($n = 10$, week 12: 4.17 ± 0.76 vs week 16: 4.02 ± 0.92 , $p = 0.44$, paired t-test).

Sinus Potential Difference Measurement

After 4 weeks of treatment with herbal dry extract BNO 1011, the ion transport characteristics were significantly different from those rabbits who received placebo (Figure 4A). Sinus PD measurements revealed significantly increased response to Cl^- free ringers + forskolin in CRS rabbits treated with either low and high dose of BNO 1011 compared to those that received placebo (Cl^- free + isoproterenol PD (mv): Placebo = -4.1 ± 1.1 vs dose B (low) = -12.0 ± 3.0 , dose A (high) = -12.2 ± 1.4 , $p = 0.03$, ANOVA), suggesting that herbal dry extract BNO 1011 promoted transepithelial Cl^- secretion in a cyclic AMP dependent fashion, indicative of improved CFTR activity. There was no meaningful difference in the PD between the low and high dose of BNO 1011. Robust baseline potential difference in all three groups (Figure 4A) indicated the presence of epithelial integrity and preserved tight junctions.

Micro-optical coherence tomography (μOCT) image

It is well-established that stimulation of transepithelial Cl^- secretion leads to airway surface hydration and promotes ASL depth. To evaluate effect of the BNO 1011 on ASL, maxillary

lateral sinus walls were harvested immediately after euthanasia and μ OCT was used to assess ASL depth and CBF immediately ($n = 5$ each group; Figure 5). Mean ASL was significantly greater in the treatment group (dose A and dose B) when compared to the placebo group (placebo = $3.5 \pm 0.05 \mu\text{m}$ vs dose A (Low) = $4.05 \pm 0.06 \mu\text{m}$; dose B (High) = $4.08 \pm 0.06 \mu\text{m}$, $p < 0.0001$, ANOVA) (Figure 5B). There was no difference between dose A and dose B. The mean CBF was also significantly faster in maxillary sinus tissue treated with BNO 1011 compared to those with placebo (Placebo = $6.8 \pm 0.25 \text{ Hz}$ vs Dose A (Low) = $8.0 \pm 0.32 \text{ Hz}$; Dose B (High) = $7.9 \pm 0.29 \text{ Hz}$, $p = 0.02$, ANOVA) (Figure 5B). Again, no statistical difference was noticed between the 2 drug doses.

Histology

The maxillary sinus tissues were harvested and stained with H&E and PAS on 5 rabbits from each group (total, $n = 15$). As mucus secretion in CRS reflects the epithelial tissue remodeling and is manifested as SMG hyperplasia,²⁸ thickness of the epithelial and submucosal layer and density of SMGs within the submucosal layer were analyzed. In those rabbits who received placebo ($n = 5$), significant infiltration of inflammatory cells was noted with significant thickening of the epithelial layer and the loss of differentiated epithelial cells ranging from denuded epithelium to basal cell hyperplasia. The lamina propria (just underneath the epithelium) was edematous with dilated capillaries, plasma cells, lymphocytes, and hyperplastic seromucous glands (Figure 6A). Contralateral maxillary sinuses from placebo rabbits served as internal control measurements ($n = 5$). When measuring the height of the epithelial cell layer, sinus epithelia from those rabbits treated with placebo were the thickest ($21.1 \pm 2.3 \mu\text{m}$), followed by dose A (low) ($13.7 \pm 0.9 \mu\text{m}$) and dose B (high) ($10.0 \pm 0.7 \mu\text{m}$) (post-hoc analysis: $p < 0.005$) (Figure 6B). Those rabbits treated with high dose BNO 1011 (dose B) demonstrated the thinnest epithelia, which was not statistically different from the contralateral control sinus ($10.9 \pm 0.8 \mu\text{m}$) ($p = 0.42$) (Figure 6B).

PAS staining revealed an increased SMG density in rabbits treated with placebo compared to other treatment groups (Figure 7A). When the SMG densities were calculated at a magnification of $\times 100$ ($n = 5$, each group), there was a significant decrease in rabbits treated with dose B (high) ($22.2 \pm 2.9 \%$), followed by dose A (low) ($31.8 \pm 1.1 \%$), when compared to placebo ($43.8 \pm 1.7 \%$) (post-hoc analysis, $p < 0.0001$) (Figure 7B). Again, there was no statistical difference between the dose B and the contralateral negative control sinus from placebo rabbits ($24.9 \pm 2.0 \%$) ($p = 0.45$). We also measured the thickness of the submucosal layer along with SMG density. The submucosal layer was also thinnest in rabbits treated with dose B (high) ($63.1 \pm 6.6 \mu\text{m}$), followed by dose A (low) ($103.2 \pm 6.7 \mu\text{m}$) compared to placebo ($113.3 \pm 6.0 \mu\text{m}$) (post-hoc analysis, $p < 0.001$) (Figure 7B).

DISCUSSION

Herbal dry extract BNO 1011 has been utilized throughout Europe since 2012 as treatment for upper airway diseases and has an outstanding safety profile, yet its mechanism is not fully understood.² Using an *in vitro* epithelial cell model, we demonstrated previously that a dimethyl sulfoxide (DMSO) extract of the same drug mixture increases CBF and ASL depth

via activating Cl^- secretion (through CFTR and CaCC).^{2,21} In the current study, the impact of herbal dry extract BNO 1011 on MCC and clinical parameters in a preclinical model of CRS were evaluated. The one-fold dose level was established allometrically based on the clinically active dosage by a well-recognized conversion factor (FDA, 2005: <https://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf%23search=%27guidekines+for+industry+sfe+starting%27>: 480 mg/kg/d in humans (60 kg) corresponds to 25 mg/kg/d in the rabbit). It is therefore reasonable to use this dose level in the study to draw conclusions on the pharmacodynamic effects at the clinical dose. In addition, we would like to understand whether a further increase of the dose would elicit an even stronger effect. Therefore, a factor of five appears to be reasonable as it significantly differs from the low dose level without studying a markedly higher dose where clinical relevance would be totally lost.

After ingestion of BNO 1011 for 4 weeks, radiographic opacification, electrophysiologic findings, and parameters of MCC clearance function (CBF and ASL) were significantly improved compared to rabbits treated with placebo. No differences in these parameters were noted between dose A (low) and dose B (high). Histologically, significant decreases in SMG density and epithelial and submucosal thickness were identified in both low and high dose of BNO 1011, although rabbits that ingested the high dose demonstrated near-normal improvement compared to the other groups. These properties may explain the clinical efficacy of BNO 1011 in chronic airway diseases characterized by increased mucus production and decreased MCC.

The measurement of PD in the sinus provides a direct and sensitive evaluation of Na^+ and Cl^- ion transport in airway epithelial cells via assessment of transepithelial bioelectric properties.^{29–31} The PD has served as a diagnostic aid in difficult cases where abnormal CFTR function is suspected.^{32,33} As the only direct *in vivo* measure capable of separating Na^+ and Cl^- transport, the nasal PD assay has been used as an important endpoint in clinical trials evaluating therapeutic agents intended to replace dysfunctional CFTR, restore mutant CFTR function, or address other abnormalities in ion transport such as activators of alternate Cl^- ion channels.^{31,34–36} The markedly diminished Cl^- secretion (and Na^+ absorption) across *in vivo* sinus epithelium in human subjects with CRS with significant inflammation as measured by the sinus PD suggested acquired CFTR dysfunction in the mucosal site of inflammation in CRS. Compared to the nasal cavity, the sinuses provide an attractive model in PD measurement as they are lined with an epithelium, continuous with and qualitatively similar to the lower respiratory epithelium, including similar ion transport mechanisms and microbiology.^{37–39}

The *in vivo* sinus PD measurements (oral application) were consistent with previous *in vitro* data using primary murine and human sinonasal epithelia in Ussing chamber assays.^{2,21} An application of this formulation to the surface of primary sinonasal epithelial cells stimulated Cl^- secretion in a sustained fashion via CFTR and CaCC (Transmembrane member (TMEM) 16A channels). However, exclusively basolateral application of the formulation, which can be more consistent with cellular exposure *in vivo*, was not tested previously. In this study, the formulation was administered orally and intended physiologic effects were observed in a systemic fashion without topical application. We were unable to compare the

sinus PDs of the 3 groups with normal controls. Once multiple solutions were perfused sequentially on one side of maxillary sinus, the contra-lateral maxillary sinus was not reliable due to perfusions crossing to the contralateral side.

Clinical trials using this formulation have shown promising results in treating rhinosinusitis. A randomized, placebo-controlled trial demonstrated that Sinupret® either alone or in combination with antibiotics for CRS demonstrated significant improvement in radiologic outcomes and headache.⁴⁰ Based on 2 randomized placebo-controlled clinical trials with acute viral rhinosinusitis, BNO 1016 provided a faster and clinically relevant remission of sinusitis symptoms and improved quality of life compared to placebo.^{19,41} Palm et al. published a randomized placebo-controlled trial using BNO 1016 in a treatment period of 12 weeks in 929 CRS patients in 2017.²⁰ While the formulation was not superior to the placebo regarding the primary endpoint (sinusitis symptom scores in week 8 and week 12), secondary endpoints showed a clear trend toward superior efficacy. BNO 1016 and BNO 1011 are the same herbal dry extracts from Bionorica, SE (Neumarkt, Germany), but BNO 1016 contains excipients on top of the genuine herbal extract (BNO 1011).

There are several limitations to the current study. This is an *in vivo* preclinical investigation and this rabbit model may not represent human CRS. CRS is a heterogeneous disease and dysfunctional MCC is considered a unifying mechanism leading to chronic disease.⁴² There is no animal model that reproduces all aspects of a human disease. This model focused on micro-anatomical dysfunction (i.e. impairment of MCC) present in chronic inflammation and although immunologic reactions in rabbits are similar to those of human responses, this model may not represent subsets of human CRS that develop from primary immune dysfunction. In this preclinical model, obstructive respiratory pathologies associated with mucous hypersecretion reflect the histologic changes exemplified by thickness of epithelium, glandular (SMG) hyperplasia, and hypertrophy.²² SMG hyperplasia or hypertrophy is prevalent in the sinus mucosa of adult⁴³ and pediatric⁴⁴ patients with CRS, and in the lower respiratory tracts of patients with chronic obstructive pulmonary diseases.⁴⁵ However, our rabbit model provides an opportunity to study the impact of new interventions with a level of experimental control that is not achievable in human studies. It is also important to note that the sinuses of BNO 1011 did not completely return to normal according to microCT scans and the contralateral sinus was unable to serve as a negative control for sinus PD and μ OCT assays. Furthermore, the placebo rabbit contralateral sinus was used as the negative control for histology, which may be impacted from local or systemic crossover effects from the diseased sinus.

CONCLUSION

Herbal dry extract BNO 1011 improves radiographic, electrophysiologic, micro-functional and histologic parameters in a rabbit model of CRS. Targeting Cl^- transport may represent a novel treatment strategy that may benefit a patient population afflicted with respiratory diseases.

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Figure 1. Study Protocol

After the development of CRS in 30 New Zealand white rabbits, rabbits were randomly assigned to receive oral placebo (n=10), dose A (low, 25mg/kg/daily) (n=10), or dose B (high, 125mg/kg/daily) (n=10) for four weeks. Outcomes included sinus opacification (Kerschner's rabbit sinus CT grade), airway surface liquid (ASL) depth using (micro optical coherence tomography (μOCT)), maxillary epithelial Cl⁻ secretion (sinus potential difference (PD) assay), and submucosal gland density (SMD) on histopathology.

Figure 2. Maxillary Sinus Potential Difference (PD) tracing

After the rabbit was sedated, tubing was surgically inserted into the maxillary sinus via dorsal maxillary antrostomy site (arrow: catheter).

A.

B.

Figure 3. Micro-Computed Tomography (microCT) Scanning

A. Representative microCT images of three groups (Placebo, Dose A, Dose B). (Asterisk * = opacification) B. Kerschner's rabbit Sinus CT grading scores: CT scores were significantly higher in those rabbits who had placebo (n = 10, 4.02 ± 0.92) compared to those rabbits who received either dose A (low) (n = 10, 1.26 ± 0.37) or dose B (high) (n = 10, 1.21 ± 0.63) (p = 0.009, ANOVA, Figure 3B). There was no statistical difference between dose A and dose B (p > 0.05).

Figure 4. Sinus Potential Difference (PD) Measurement

A. Representative Sinus PD tracings B. Sinus PD assay revealed significantly increased response to Cl^- free ringers + forskolin in CRS rabbits treated with either low and high dose of BNO 1011 compared to those received placebo (Cl^- free+Isoproterenol PD (mv): Placebo = -4.1 ± 1.1 vs Dose B (Low) = -12.0 ± 3.0 , Dose A (High) = -12.23 ± 1.4 , $p = 0.03$, ANOVA). There was no statistically different change in the PD measurement between the low and high dose of BNO 1011.

Figure 5. Measurement of ASL depth using μOCT

A. μOCT images demonstrating ASL thickness in placebo, dose A (low) and dose B (high) of BNO 1011. ASL = airway surface liquid; μOCT = microoptical coherence tomography. Yellow bar = ASL depth. Scale bar = $5\mu\text{m}$. B. Mean ASL was significantly greater in the treatment group (dose A and dose B) when compared to the placebo group (placebo = $3.5 \pm 0.05 \mu\text{m}$ vs dose A (low) = $4.05 \pm 0.06 \mu\text{m}$; dose B (high) = $4.08 \pm 0.06 \mu\text{m}$, $p < 0.0001$, ANOVA). The mean CBF was also significantly faster in maxillary sinus tissue treated with BNO 1011 compared to those with placebo (placebo = $6.8 \pm 0.25 \text{ Hz}$ vs dose A (low) = $8.0 \pm 0.32 \text{ Hz}$; dose B (high) = $7.9 \pm 0.29 \text{ Hz}$, $p = 0.02$, ANOVA). ASL = airway surface liquid, CBF = ciliary beat frequency.

Figure 6. Histology – Thickness of Epithelium

A. Hematoxylin and Eosin (H&E) staining - Significant infiltration of inflammatory cells was noted with significant thickening of the epithelial layer and the loss of differentiated epithelial cells in the placebo group (top right). The lamina propria (just underneath the epithelium) was edematous with dilated capillaries, plasma cells, lymphocytes, and hyperplastic seromucous glands. Black vertical line = thickness of epithelium, Scale bar = 10µm, Magnification x 400. B. Thickness of epithelium - Sinus epithelia from those rabbits treated with placebo was the thickest (21.1 ± 2.3 µm), followed by dose A (low) (13.7 ± 2.3 µm), followed by dose B (high) (10.0 ± 1.0 µm), and control (11.0 ± 1.0 µm).

0.9 μm) and dose B (high) (10.0 $\pm$ 0.7 μm) (post-hoc analysis: $p < 0.005$). No statistical difference was noticed between the high dose of BNO 1011 (dose B) and contralateral controls (from placebo group) ($p = 0.42$).

Figure 7. Histology – Thickness of Submucosal Layer and Submucosal Gland Density

A. Periodic Acid–Schiff (PAS) staining demonstrated increased submucous glandular density with hypertrophy in the placebo group (top right) compared to the other 3 groups. Black vertical line = thickness of epithelium, Scale bar = 50µm, Magnification x 100. B. Thickness of Submucosal layer and SMG Density – Thickness of submucosal layer was also lowest in rabbits treated with dose B (high) (63.1 ± 6.6 µm), followed by dose A (low) (103.2 ± 6.7 µm) compared to placebo (113.3 ± 6.0 µm) (post-hoc analysis, $p < 0.001$). SMG density was significantly decreased in rabbits treated with dose B (high) (22.2 ± 2.9 %), followed by dose A (low) (31.8 ± 1.1 %) compared to placebo (43.8 ± 2.9 %).

– 1.7 %) (post-hoc analysis, $p < 0.0001$). There was no statistical difference between the dose B and control (24.9 $\pm$ 2.0 %) ($p = 0.45$).