Published in final edited form as:

Int Forum Allergy Rhinol. 2012 ; 2(1): 63–68. doi:10.1002/alr.20110.

Human sinonasal explant system for testing cytotoxicity of intranasal agents

Jae H. Lim, M.D., Ph.D.¹, Greg E. Davis, M.D., M.P.H.¹, Tessa C. Rue, M.S.², and Daniel R. Storm, Ph.D.^{3,*}

¹Department of Otolaryngology – Head and Neck Surgery, University of Washington, Seattle, WA.

²Department of Biostatistics, University of Washington, Seattle, WA.

³Department of Pharmacology, University of Washington, Seattle, WA.

Abstract

BACKGROUND—Intranasal agents play a critical role in the management of sinonasal disorders. There are ongoing efforts to develop new intranasal medications to combat sinonasal disease. Some intranasal agents, however, can have cytotoxic effects on human sinonasal tissue. In order to facilitate safe drug discovery, we developed a simple and reliable *in vitro* screening assay using human sinonasal explants to measure the cytotoxic profiles of intranasal agents.

METHODS—We obtained sinonasal tissues from several regions of the nasal cavity from 12 patients undergoing endoscopic sinonasal surgery. These tissues were cultured on polytetrafluoroethane membrane in serum free growth medium. We determined the biochemical properties of these explants by measuring extracellular lactate dehydrogenase (LDH) levels and performing histological analyses over a period of 1–2 weeks. We then examined the cytotoxic profiles of 13 intranasal agents by measuring extracellular LDH levels using the human sinonasal explant system.

RESULTS—Sinonasal explants exhibited a rapid reduction in extracellular LDH levels indicating stabilization in the culture environment within 2 days. Histological analysis showed maintenance of good cellular architecture for up to 2 weeks. The explants displayed intact epithelium and expressed β III-tubulin and Ki-67. Of the 13 tested intranasal agents, 1% zinc sulfate, 5% zinc sulfate and Zicam application were cytotoxic.

CONCLUSIONS—Based on the unique biochemical properties of the human nasal explant culture system, we developed a simple and reliable *in vitro* screening assay to determine the cytotoxic profiles of various intranasal agents by examining extracellular LDH levels and histopathology.

Keywords

human sinonasal explants; drug discovery; organotypic tissue culture; intranasal agents; cytotoxicity

*Corresponding author: Daniel R. Storm, Ph.D., Department of Pharmacology, University of Washington, 1959 NE Pacific St, Seattle, WA 98195, dstorm@u.washington.edu, Tel: 206-543-7028, Fax: 206-616-8621.

Financial disclosures: None

Conflicts of interests: None

INTRODUCTION

Intranasal agents play a major role in the medical management of sinonasal disorders. Both prescribed and over-the-counter intranasal agents are utilized to treat wide range of sinonasal disorders, including allergic rhinitis, nasal congestion and rhinosinusitis. The ease of use and delivery, combined with the availability of many chemical compounds to target the pathogenesis of sinonasal disease has resulted in a surge of recent intranasal drug development. As with any drug discovery, however, the safety profile of the intranasal agents must be meticulously established. At present, a simple and reliable testing of intranasal agent cytotoxicity does not exist. The testing of novel intranasal agents is limited to animal studies and phase 1 clinical trials that are often time-consuming and costly. Given the recent controversy surrounding the safety of some intranasal agents ¹⁻³, a dependable assay is needed to facilitate the development of safe and effective intranasal agents.

The organotypic tissue culture system is a powerful tool to study various biological processes, and is ideal for examining the cytotoxic profiles of intranasal agents. Because organotypic culturing of tissue explants resemble the tissue *in vivo* morphologically and functionally, it offers several advantages over the dissociated cell culture system ⁴⁻⁶. Indeed, human nasal explants have been used widely to investigate a number of topics from the ciliary function in the respiratory epithelium ^{7, 8} to neurogenesis in the olfactory epithelium ^{9, 10}. These experiments have provided important insights into the relatively unknown biology behind various human nasal disorders. Over the years, the method to culture the nasal explants have changed with the parallel advancement in general tissue culturing techniques. The resultant efforts have yielded a faster, simpler and more reliable methodology. Nonetheless, some of the fundamental issues regarding the properties of human nasal explants in culture environment remain unclear. Therefore, a thorough and systematic understanding of how these explants behave in culture environment is critical for designing meaningful experiments.

In this paper, we established a simple and reliable technique to culture human nasal explants from various regions of the nasal cavity in a serum-free environment. We further used biochemical and histological assays to determine the biochemical properties of these explants over a period of 1–2 weeks. The results led us to develop an *in vitro* assay to determine cytotoxic profiles of many intranasal agents. Our findings will play a beneficial role in developing future experimental applications that will utilize the cultured human nasal tissues, and to facilitate new intranasal agent development to combat sinonasal disorders.

METHODS

Patient recruitment and tissue collection

Human subjects were recruited as a consecutive sample from a rhinology clinic at a tertiary medical center. Written consent was obtained from all subjects donating the nasal tissue. Inclusion criteria included any patient over age 18 already scheduled for an endoscopic nasal procedure for chronic rhinosinusitis, inferior turbinate hypertrophy, and/or cerebrospinal fluid leak repair. Patients were excluded if they had a history of blood borne pathogens. The human nasal tissue was collected during each subjects' surgery while under general anesthesia. We collected various nasal tissues from the middle turbinate, inferior turbinate, uncinate process, or superior nasal septum based on the planned operation. The harvested nasal tissue was immediately processed as below for establishing an organotypic tissue culture system. The recruitment of patients and the study protocol was approved by the Institutional Review Board at the University of Washington (#35031).

Human nasal explants culture system

The biopsied tissues were removed of any tissue debris or blood, and divided into 2–4mm × 2–4mm × 1–2mm pieces with a 15-blade scalpel under a dissecting scope. It was then placed on 0.4µm polytetrafluoroethane (PTFE) membrane (Millicell-CM; Millipore, Cork, Ireland) immersed in 1.5ml of culture medium consisting of 50% Dulbecco's Modified Eagle Medium High Glucose (Invitrogen, Carlsbad, CA); 25% Hank solution (Invitrogen, Carlsbad, CA); 50U/mL penicillin G and 40µg/mL streptomycin (Invitrogen, Carlsbad, CA) in a 60×15mm tissue culture dish (Corning, Corning, NY). For 24-well culture system, 300µl of culture medium was used per well. The tissue was placed on the membrane such that epithelial side was exposed to the air, and placed in humidified incubator at 37°C with 5% CO₂. The culture media was changed every day.

Lactate Dehydrogenase (LDH) Assay

Cultured nasal explants from five human subjects underwent LDH assay. CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) was used with the following modifications. On the day of the assay, 50µl of culture medium was removed and added to 50µl of Substrate Mix. Following 30 minute incubation at room temperature, the reaction was stopped with 50µl of Stop Solution. Absorbance at 490nm was obtained with Epx Precision Microplate Reader (Molecular Devices, Sunnyvale, CA). For measuring LDH levels in the human nasal explants following intranasal agent administration, we stabilized the nasal tissue in the cultured for approximately 48 hours. We then measured the baseline LDH levels, and applied 2 µl of either dH₂O, saline, 0.01% ZnSO₄, 0.1% ZnSO₄, 1% ZnSO₄, 5% ZnSO₄, Zicam (Matrixx, Inc; Scottsdale, AZ), Nasalcrom (Prestige Brands, Inc; Irvington, NY), Atrovent Nasal 0.03% Aqueous (Boehringer Ingelheim; Auckland, NZ), Afrin (Schering-Plough HealthCare Products, Inc., Kenilworth, NJ), Flonase (GlaxoSmithKline, Middlesex, UK), Omnaris (Sunovion Pharmaceuticals, Inc; Marlborough, MA), Nasonex (Schering-Plough HealthCare Products, Inc., Kenilworth, NJ), Astelin (Meda Pharmaceuticals Inc; Somerset, NJ), or Pantanase (Alcon Laboratories Inc; Fort Worth, TX) directly onto the explant. The levels of LDH were again measured 24 hours later. We repeated the addition of the intranasal agent and LDH measurement for five days.

Tissue processing and Immunocytochemistry

The nasal explants were immersed in 4% paraformaldehyde overnight at 4°C, followed by immersion in 20% sucrose overnight at 4°C. The tissue was then embedded in OCT (Sakura Finetek USA, Inc., Torrance, CA), frozen in –20°C and cryosectioned at 30µM. Immunostaining was performed as described before with the following modifications¹. β-tubulin and Ki-67 antibodies were used at 1:2,000, and 1:200 dilutions, respectively. Images were taken with Zeiss confocal microscope.

Hematoxylin and Eosin staining

Hematoxylin and Eosin (H+E) staining was performed as previously described¹. Briefly, human nasal tissues were processed as above and stained in hematoxylin and eosin (Surgipath Medical Industries, Inc, Richmond, IL) for 1 min each. Tissues were then dehydrated in ethanol, cleared in xylene and coverslipped with DPX (Fluka, Milwaukee, WI).

Statistical analysis

A linear mixed model of relative LDH was fit to determine significant differences between two agents on a given day. The linear mixed model included a random effect for each of the five tissues used in the explant systems and a random effect for each individual experiment to account for correlation between repeated measures. The model included fixed effects for

drug by day that allowed tests for differences between each agent and saline as well as tests for differences between 1% ZnSO₄ and 5% ZnSO₄ and between 5% ZnSO₄ and Zicam. For all tests, $p < 0.05$ was considered significant.

RESULTS

Morphology and histology of human nasal explants in culture system

The harvested human nasal explants were cultured in either single-well (Figure 1A) or multi-well system (Figure 1B). Grossly, the nasal explants appeared to be viable without significant discolorations and/or tissue shrinkage over ten days in the cultured environment (Figure 2). Subsequent histological evaluation with hematoxylin and eosin (H&E) staining demonstrated that the overall cellular architecture was generally well-maintained even after two weeks in culture. The epithelium remained largely intact and was resilient to cell loss or damage over 1–2 weeks in culture (Figure 3A–C). In addition, both the mucous and serous submucosal glands were present. However, some loss of submucosal glands and cells in the basal layer began to emerge around days 3 to 5 in culture (Figure 3B–C). These features were consistent for all tissues obtained from various nasal regions. In the absence of growth medium, the nasal explant exhibited rapid loss of cells throughout the tissue (Figure 3D).

Extracellular lactate dehydrogenase (LDH) release by human nasal explants in culture system

We measured the levels of extracellular lactate dehydrogenase (LDH), an enzyme released by dying cells over a period of 1 week in culture conditions. The explants showed a rapid reduction in LDH levels within 2 days, followed by stable levels (Figure 4). Again, H&E staining of the nasal tissue showed maintenance of good cellular and tissue architecture.

Presence of biological markers in human nasal explants

We performed immunostaining with β -III-tubulin (neural specific marker) and Ki-67 (cellular proliferation marker) to demonstrate the biochemical viability of the cultured human nasal explants. The tissues expressed both of these proteins, even after 12 days in culture environment. β -III-tubulin staining was apparent in neuronal cells in the epithelium and in the submucosa (Figure 5a). Ki-67 positive cells were observed in the basal layer (Figure 5b).

Extracellular LDH levels in human nasal explants following application of intranasal agents

We tested 13 intranasal agents. These included various concentrations of zinc sulfate (0.01%, 0.1%, 1% and 5%), a substance known to be toxic to olfactory epithelium in mice and human^{11, 12}. We also used Zicam, an intranasal agent previously shown to be cytotoxic to murine and human nasal tissue¹. 0.9% saline and dH₂O were used as negative controls. Of the 13 intranasal agents, only 1% zinc sulfate, 5% zinc sulfate, and Zicam displayed significant elevation of extracellular LDH levels compared to saline (Figure 6). On day 2, Zicam and 5% ZnSO₄ showed nearly identical LDH levels. However, on days 3, 4, and 5, LDH levels were significantly higher for Zicam-treated tissues than 5% ZnSO₄ ($p < 0.001$).

Cellular histopathology of human nasal explants following applications of intranasal agents

The epithelium was intact in all human nasal explants except those treated with 1% ZnSO₄, 5% ZnSO₄ and Zicam (Figure 7). The damage to epithelium was concentration dependent for ZnSO₄, with higher concentration resulting in more cellular disruption. The subepithelial layer also showed evidence of necrosis in 1% ZnSO₄, 5% ZnSO₄ and Zicam. All other

intranasal agent treated explants showed expected tissue degeneration comparable to saline and dH₂O treated samples.

DISCUSSION

The development of organotypic culture techniques for human nasal mucosal explants has led to important insights into the biology and physiology of human nasal function and dysfunction. It has provided the researchers with the ability to study human nasal tissue *ex vivo*, and to perform experiments that are deemed unethical with human subjects^{7–10}. In this paper, we have characterized the biochemical properties of human nasal explants in culture system. The results were used to develop an *in vitro* assay to examine cytotoxic profiles of intranasal agents. We have previously demonstrated a similar assay, but it was limited to a single-well system¹. Here, we have expanded it to a multi-well system to test a wide range of intranasal agents.

We undertook a systematic evaluation of the viability of human nasal explants in serum-free culture medium for a period of 1–2 weeks. Our results demonstrate a high degree of viability for at least 7–10 days in culture. The epithelium remained most resilient to damage. We detected both β -III-tubulin and Ki-67 positive cells by immunocytochemistry even after 12 days in culture. Nonetheless, gradual loss of submucosal glands and subepithelial cells began after 3–4 days in culture. We expected these changes because the role of any organotypic tissue culture system is to slow the cellular degeneration and to prolong the survival of the tissue. In fact, there is no tissue culture system that can prevent cell death – the gradual decline in tissue viability is inevitable. Therefore, it is critical to consider the timing of the culture environment when designing an experiment with human nasal explants.

Based on our organotypic tissue culture condition, the optimal time to perform experiments with human nasal explants is after 2 days in the culture system. As evidenced by the LDH assay (Figure 5), it takes approximately 2 days for the tissue to stabilize, and to adapt to the new environment. For instance, if a study examines the biological effect of a particular compound on the nasal explants, it is critical to perform the experiments only after the explants are stabilized in the culture environment. Such strategy is necessary to prevent the potential masking of meaningful and/or misleading results as the tissue stabilizes in the first few days in the culture system.

In our examination of intranasal agents, we found 1% and 5% zinc sulfate as well as Zicam to be highly cytotoxic. The findings were expected as both zinc sulfate and Zicam are known to be cytotoxic to human and murine nasal epithelium¹. In fact, Zicam, a former over-the-counter nasal spray to combat common cold has been linked to anosmia^{1, 3, 13}. It has recently been removed from the market after Federal Drug Administration (FDA) investigation. For all other intranasal agents except 1% and 5% zinc sulfate, we found no demonstrable cytotoxicity as measured by extracellular LDH levels and histology.

The *in vitro* cytotoxic assay using the human nasal explants described here offers a simple way to screen intranasal agents. The system is expandable to 24-wells to test dose-dependent response of a single agent or to screen multiple chemical compounds. The extracellular level of LDH reliably predicts the degree of cytotoxicity and correlates well with histology. The assay also has other advantages. First, it places no harm to human subjects as the tissue harvested is routinely discarded following surgery, and the testing is performed *ex vivo*. Second, it may potentially reduce testing in animal model systems. However, we emphasize that concurrent study in a murine system is necessary as it is not possible to study the long-term effects (i.e., beyond 2 weeks) of an intranasal agents using our culture system. Furthermore, electrophysiological studies to examine activities of olfactory sensory neurons

are not feasible with human nasal explants at this time. Despite these limitations, the assay described in this paper promises to enhance the discovery and safety testing of new intranasal agents to treat various sinonasal diseases.

CONCLUSIONS

In this study, we demonstrated biochemical properties of human nasal explants in culture environment. We utilized the findings to develop a simple *in vitro* assay to test the cytotoxic profiles of number of intranasal agents. The assay promises to facilitate the discovery and safety testing of novel intranasal agents.

Acknowledgments

We thank Carolyn Bea for technical assistance.

Funding sources: Supported by grants from National Institutes of Health to: J.H.L (NIH 2T32DC000018-26); G.E.D (NCRR Grant KL2 RR025015); D.R.S (DC04156)

References

1. Lim JH, Davis GE, Wang Z, et al. Zicam-induced damage to mouse and human nasal tissue. *PLoS One*. 2009; 4:e7647. [PubMed: 19876403]
2. Davidson TM, Smith WM. The Bradford Hill criteria and zinc-induced anosmia: a causality analysis. *Arch Otolaryngol Head Neck Surg*. 2010; 136:673–676. [PubMed: 20644061]
3. Alexander TH, Davidson TM. Intranasal zinc and anosmia: the zinc-induced anosmia syndrome. *Laryngoscope*. 2006; 116:217–220. [PubMed: 16467707]
4. Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. Organotypic slice cultures: a technique has come of age. *Trends Neurosci*. 1997; 20:471–477. [PubMed: 9347615]
5. Bhadriraju K, Chen CS. Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discov Today*. 2002; 7:612–620. [PubMed: 12047872]
6. Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci*. 2003; 116:2377–2388. [PubMed: 12766184]
7. Albery J, Stoll W, Rudack C. The effect of endogenous nitric oxide on mechanical ciliostimulation of human nasal mucosa. *Clin Exp Allergy*. 2006; 36:1254–1259. [PubMed: 17014433]
8. Wang D, Chen P, Quan W, Halpern M. Suprasternal gland secretion of male short-tailed opossum induces IP3 generation in the vomeronasal organ. *Biochim Biophys Acta*. 2007; 1770:725–732. [PubMed: 17306463]
9. Feron F, Perry C, McGrath JJ, Mackay-Sim A. New techniques for biopsy and culture of human olfactory epithelial neurons. *Arch Otolaryngol Head Neck Surg*. 1998; 124:861–866. [PubMed: 9708710]
10. Hahn CG, Han LY, Rawson NE, et al. In vivo and in vitro neurogenesis in human olfactory epithelium. *J Comp Neurol*. 2005; 483:154–163. [PubMed: 15678478]
11. Harding JW, Getchell TV, Margolis FL. Denervation of the primary olfactory pathway in mice V. Long-term effect of intranasal ZnSO₄ irrigation on behavior, biochemistry and morphology. *Brain Res*. 1978; 140:271–285. [PubMed: 626892]
12. McBride K, Slotnick B, Margolis FL. Does intranasal application of zinc sulfate produce anosmia in the mouse? An olfactometric and anatomical study. *Chem Senses*. 2003; 28:659–670. [PubMed: 14627534]
13. Jafek BW, Linschoten MR, Murrow BW. Anosmia after intranasal zinc gluconate use. *Am J Rhinol*. 2004; 18:137–141. [PubMed: 15283486]

Figure 1.

Organotypic tissue culture setup for human nasal explants. A) Single well setup. B) 24-well setup. The nasal tissue (black arrowhead) is placed on a 0.4 μ m PTFE membrane (red arrowhead) with the epithelial side exposed to the air. Growth medium surrounds the membrane and provides the tissue with nutrients.

Figure 2.

Human nasal explants in organotypic tissue culture system. A) Gross appearance of nasal explants from several different regions of the nasal cavity over 10 days in culture.

Figure 3. Hematoxylin and eosin staining of human nasal explants in culture. (A) Day 0 (B) Day 7 (C) Day 12. (D) In the absence of growth medium, significant degeneration of the epithelium and submucosal glands are also observed.

Figure 4. Lactate dehydrogenase (LDH) released by human nasal explants over 7 days in culture. LDH level is rapidly reduced and stabilized by day 2. LDH levels for each sample tissue is normalized to control growth medium.

Figure 5.
Cultured human nasal explants (middle turbinate) express various cellular proteins at day 12.
(A) Epithelial cells and neural bundles that traverse the submucosal layer express neural-specific β -tubulin. (B) Ki-67, a marker of cell proliferation, is visible in the basal layer.

Figure 6.

Average LDH levels for each intranasal agent by day. The LDH levels are normalized to day 1. Saline and dH₂O were used as controls. Four different concentrations of ZnSO₄ (0.01%, 0.1%, 1%, and 5%) solutions were tested. Nasalcrom, Atrovent, Afrin, Astelin, Pantanase, Flonase, Omnaris, Rhinocort, Nasonex and Zicam were also examined. Asterisks indicate significant changes in LDH levels as compared to saline (*p<0.05; **p<0.01; ***p<0.001). The error bars indicate standard error of the mean.

Figure 7.

Hematoxylin and eosin (H&E) stained human nasal explants following treatment with either saline, dH₂O, 0.01% ZnSO₄, 0.1% ZnSO₄, 1% ZnSO₄, 5% ZnSO₄, Nasalcrom, Atrovent, Afrin, Astelin, Pantanase, Flonase, Omnicort, Rhinocort, Nasonex and Zicam. Note the significant cellular necrosis with epithelium sloughing in tissues treated with 1% ZnSO₄, 5% ZnSO₄ and Zicam. Black arrow indicates basal layer of the remaining epithelium.