Clearance of bile and trypsin in rat lungs following aspiration of human gastric fluid

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ABSTRACT

Purpose: In the clinical setting, there is no reliable tool for diagnosing gastric aspiration. A potential way of diagnosing gastric fluid aspiration entails bronchoalveolar lavage (BAL) with subsequent examination of the BAL fluid for gastric fluid components that are exogenous to the lungs. The objective of this study was to determine the longevity of the gastric fluid components bile and trypsin in the lung, in order to provide an estimate of the time frame in which assessment of these components in the BAL might effectively be used as a measure of aspiration. Materials and Methods: Human gastric fluid (0.5 mg/kg) was infused in the right lung of intubated male Fischer 344 rats (n = 30). Animals were sacrificed at specified times following the experimentally induced aspiration, and bronchoalveolar lavage fluid (BALF) was collected. Bile concentrations were analyzed by an enzyme-linked chromatogenic method, and the concentration of trypsin was quantified using an ELISA. Data were analyzed using non-linear regression and a one-phase decay equation. Results: In this experimental model, the half-life of bile was 9.3 hours ($r^2$ = 0.81), and the half-life of trypsin was 9.0 hours ($r^2$ = 0.68). Conclusions: The half-lives of bile and trypsin in the rodent aspiration model suggest that the ability to detect aspiration may be limited to a few days post-aspiration. If studies using rats are any indication, it may be most effective to collect BAL samples within the first 24 hours of suspected aspiration events in order to detect aspiration.

Introduction

Forty-four percent of adults in the US experience monthly symptoms of gastroesophageal reflux disease (GERD).[1] GERD refers to symptoms and esophageal mucosal damage caused by stomach contents, such as gastric acid and food particles, flowing back into the esophagus.[2, 3] Patients who reflux gastric content into the pharynx are at increased risk for aspiration, which can result in pulmonary complications such as chronic cough, asthma, pneumonia, fibrosis, and allograft rejection.[4–6] It is estimated that almost 10% of patients with GERD experience pulmonary manifestations,[4] although it is often difficult to effectively gauge the pulmonary consequences of GERD.[7]

While pH monitoring is the gold standard for diagnosing GERD clinically, it is not an effective tool for predicting or identifying aspiration. D’Ovidio et al examined bile salts in the bronchoalveolar lavage fluid of 120 lung allograft recipients in a cross-sectional study, and found that 25% of patients with the highest quantity of aspiration had normal proximal esophageal pH measurements.[7, 8] Bronchoalveolar lavage (BAL) is a common tool used in diagnosing and monitoring pulmonary disease, as it allows for sampling of respiratory secretions with its cellular and acellular components.[9] In the research setting, analysis of gastric fluid components in BAL fluid (BALF) has been used as a tool to evaluate gastric aspiration.[7] However, conclusions drawn from this method are limited by unknowns such as the initial concentration of components in the gastric fluid, the volume of the aspirated gastric fluid, the length of time that has passed since aspiration, and the lifetime of the gastric fluid components in the lung. The present study investigated the half-lives of two common components found in gastric fluid, bile and trypsin, which can be readily assayed...
using currently available techniques. An experimental model was utilized in which human gastric fluid was placed into the right lung of rats, and the concentration of gastric fluid components in BALF collected at different time periods following the aspiration were assessed.

**Materials and methods**

**Human gastric fluid samples**

Human gastric fluid was collected from anonymous patients immediately prior to undergoing cardiothoracic surgery at Duke University Medical Center. Collection of the gastric fluid was performed as a routine part of the standard pre-operative procedure, and that practice was not altered for the purposes of collecting the gastric fluid. Patients who had been on antibiotics prior to the perioperative period were excluded, and any prescriptions for acid-blockade (e.g. proton pump inhibitors) were noted. The pH of the samples was assessed, and the concentrations of bile and of trypsin were determined as described below. The samples were flash frozen until analysis. The collection and analyses of these human samples was declared by the Duke Institutional Review Board to be research not involving human subjects.

**Assessment of bile concentrations**

The bile concentration was analyzed by an enzyme-linked method using the Total Bile Acids Assay Kit (BioQuant; San Diego, CA, USA). The assay was run on an automated platform, Cobra Integra 400 plus Analyzer from Roche (Indianapolis, IN, USA), according to the manufacturer’s protocols.

**Assessment of trypsin concentrations**

The concentration of trypsin was quantified using a DuoSet ELISA Development Kit for Human Trypsin (R&D Systems, Minneapolis, MN, USA). ELISA assays were completed according to the manufacturer’s protocols, using the reagents provided, which included sheep anti-human trypsin as the capture antibody, biotinylated sheep anti-human trypsin as the detection antibody, and tetramethylbenzidine mixed with stabilized hydrogen peroxide as the substrate solution.

| Initial bile acid concentration in gastric fluid aspirate | BALF collection times | Other characteristics of gastric fluid aspirate |
|---------------------------------------------------------|----------------------|-----------------------------------------------|
| 0.12 μmol/L (n = 9)                                      | 3 h (n = 3)          |                                               |
|                                                         | 24 h (n = 3)         |                                               |
|                                                         | 48 h (n = 3)         |                                               |
| 165 μmol/L (n = 9)                                      | 3 h (n = 3)          |                                               |
|                                                         | 24 h (n = 3)         |                                               |
|                                                         | 48 h (n = 3)         |                                               |
| 4866 μmol/L (n = 12)                                    | 0 h (n = 3)          | Initial trypsin concentration: 52.4 μg/mL |
|                                                         | 6 h (n = 3)          |                                               |
|                                                         | 12 h (n = 3)         |                                               |
|                                                         | 18 h (n = 3)         |                                               |

**Animals**

Male (n = 30) Fischer 344 (F334; RT1Iv1) rats from Harlan Laboratories (Indianapolis, IN, USA) that were 10–12 weeks old and ~300 g were used. All experiments were approved by the Duke University Institutional Animal Care and Use Committee.

**Study design**

Rats receiving aspiration with gastric fluid were assigned into groups according to the bile acid concentration in the gastric fluid they received: 0.12 μmol/L bile acid (n = 9), 165 μmol/L bile acid (n = 9), and 4866 μmol/L bile acid (n = 12). Each group received gastric fluid samples from a unique human donor, and the individual samples were selected from a large cohort in order to obtain optimal concentrations of bile and/or trypsin prior to the initiation of the experiment. Rats received 0.5 mL/kg of gastric fluid aspirate into the right lung. Rats were sacrificed at designated time points for collection of BALF (Table 1). Rats assigned to receive gastric fluid aspirate containing 0.12 μmol/L bile acid were considered negative controls and sacrificed at 3 h (n = 3), 24 h (n = 3), and 48 h (n = 3). Rats assigned to receive gastric fluid aspirate containing 165 μmol/L bile acid were sacrificed in a similar manner to the negative control. Rats assigned to receive gastric fluid aspirate containing 4866 μmol/L bile acid were sacrificed at 0 h (n = 3), 6 h (n = 3), 12 h (n = 3) and 18 h (n = 3) in order to obtain a more accurate calculation of half-life clearance of bile acid from rat lungs. Gastric fluid aspirate containing 4866 μmol/L bile acid and BALF collected from rats assigned to this group (n = 12) were also used to calculate the half-life clearance of trypsin from rat lungs.
Laboratory rats were selected for the study because the animals are a convenient size for reliable aspiration and because aspiration procedures have been well established in the animals. Human gastric fluid was used because the antigen-specific assay for analysis of trypsin is specific for human trypsin. In addition, due to the wide range of analyte concentrations found in human gastric fluid, the concentration of both bile and trypsin could be varied by appropriate selection of individual samples of human gastric fluid.

Aspiration and BALF collection procedures

Aspiration of gastric fluid into the right lung was performed as follows: rats were sedated with isoflurane and orotracheally intubated with a 14-gauge catheter. Rats were then placed in right lateral decubitus position and reverse Trendelenburg position at a 35°–40° angle. A small silastic catheter was inserted into the distal trachea, through which 0.5 mL/kg of gastric fluid was injected into the right lung. As described above, rats were sacrificed at designated time points for the collection of bronchoalveolar lavage fluid (BALF).

Collection of BALF was performed as follows: the bronchus was dissected free and 3 mL of saline was flushed into the lungs via a second 14-gauge angiocatheter fastened to the bronchus. After removing approximately 2 mL of BALF, another 3 mL of saline was infused into the lungs. Following removal of another 2 mL of BALF, a final 1 mL of saline was added and removed. Ten milligram per kilogram of BALF was collected from both lungs. The BALF from all three washes was then pooled and stored on ice prior to use. Pooled washes of BALF were centrifuged at 600 g for 8 min at 4°C to remove all cells and debris. The resulting supernatant was flash frozen in liquid nitrogen and stored at −80°C until needed for the assays.

Half-life clearance determination

Non-linear regression was performed for the set of results associated with the BALF collected from rats aspirated with gastric fluid containing appreciable initial bile concentrations (165 μmol/L; n = 9 and 4866 μmol/L; n = 12). A one phase decay equation, \( Y = (Y_0 - Y_{\text{plateau}}) \times e^{-kX} + Y_{\text{plateau}} \), was used in which \( Y_{\text{plateau}} \) (Y at \( X = \infty \)) was set to zero, \( Y_0 \) (Y at \( X = 0 \)) is the initial bile concentration in the BALF, \( X \) is the time in hours, and \( k \) is the rate constant.

To combine results from different initial bile concentrations, the bile concentrations of the BALF collected were normalized by their initial bile concentrations (by setting \( Y_0 \) to be a constant) such that the percent remaining based on an initial concentration could be plotted together and non-linear regression performed as described above.

Statistical analysis

Non-linear regression was performed to calculate the half-life clearances of bile and trypsin using Prism 5.1 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Bile concentrations

To determine if bile acid was a specific marker for gastric fluid in the lungs, a group of rats (n = 9) received human gastric fluid aspirate containing very low levels (0.12 μmol/L) of bile and were sacrificed at 3 hours (n = 3), 24 hours (n = 3), and 48 hours (n = 3) following aspiration. As shown in Figure 1, minimal bile was recovered in the BALF of these animals, even at 3 hours, consistent with the hypothesis that any bile...
recovered in the BALF originated from aspirated gastric fluid and was not produced in the lung. To assess the half-time clearance of bile in rat lungs, a group of rats \((n = 9)\) received human gastric fluid aspirate containing 165 \(\mu\)mol/L of bile and were sacrificed at 3 hours \((n = 3)\), 24 hours \((n = 3)\), and 48 hours \((n = 3)\) following aspiration. From the decay graph, the half-life for aspirated bile in these rats was calculated to be 8.3 hours (Figure 1).

To obtain a more accurate calculation of the half-life of bile in the lung following aspiration, an additional group of rats \((n = 12)\) received human gastric fluid aspirate containing 4866 \(\mu\)mol/L of bile and were sacrificed at 0 hours \((n = 3)\), 6 hours \((n = 3)\), 12 hours \((n = 3)\), and 18 hours \((n = 3)\) following aspiration. Results from gastric fluid aspirations using appreciable initial bile concentrations (165.0 \(\mu\)mol/L; \(n = 9\) and 4866 \(\mu\)mol/L; \(n = 12\)) were combined as described in the Methods. As shown in Figure 2, the resulting half-life for aspirated bile in rats was calculated to be 9.3 hours.

**Trypsin concentrations**

The half-time clearance of trypsin was calculated using the BALF collected from a group of rats \((n = 12)\) assigned to gastric fluid aspirate containing 4866 \(\mu\)mol/L bile acid. The initial trypsin content was 52.4 \(\mu\)g/mL (Table 1). To calculate the half-life of trypsin in the rat lung, the BALF collected in this group at 0 hours \((n = 3)\), 6 hours \((n = 3)\), 12 hours \((n = 3)\), and 18 hours \((n = 3)\) after aspiration were analyzed for trypsin content. From the decay graph (Figure 3), the half-life for aspirated human trypsin in this group of rats was determined to be 9.0 hours.

As described above, the data for clearance of both bile and trypsin were fit to first order kinetics, consistent with previous studies in similar models (See Discussion). However, it is notable that the data could be fit to a zero order (linear) decay equation. For bile, the zero order decay rate (data from Figure 2 excluding data after the zero order decay reaches \(x = 0\)) had a half-life \((y \text{ at } x = \frac{1}{2} x_{\text{max}}\) of 11.0 hours \((r^2 = 0.72)\). However, the fit was not as good as for the first order equation. For trypsin, the zero order decay rate had a half-life of 6.2 hours \((r^2 = 0.67)\), with a fit similar to that of the first order decay equation. Thus, regardless of the decay model used, the clearance of gastric fluid
associated solutes was rapid, occurring within the first 12 hours of aspiration.

**Discussion**

Nearly 1 in 10 persons with GERD experience pulmonary symptoms associated with gastric fluid aspiration.\(^4\) Currently, there is no gold standard in the clinic for the diagnosis of gastric fluid aspiration. However, bronchoalveolar lavage with analyses of gastric fluid components in the lavage fluid has been used as a tool to evaluate gastric fluid aspiration in the research setting. Bile acid and trypsin are two common components of the gastric fluid that are readily assayed using currently available techniques. Unfortunately, bronchoalveolar lavage with gastric fluid component analyses is limited in its usefulness for the diagnosis of aspiration in the clinical setting. These limitations are imposed by unknowns such as the initial concentration of components in the gastric fluid,\(^10\) the volume of the gastric fluid aspirated, the length of time that has passed since aspiration, and the lifetime of the gastric fluid components in the lung.

While pH monitoring is the gold standard for diagnosing GERD clinically, it is not an effective tool for predicting or identifying aspiration. D’Ovidio et al examined bile salts in the bronchoalveolar lavage fluid of 120 lung allograft recipients in a cross-sectional study, and found that 25% of patients with the highest quantity of aspiration had normal proximal esophageal pH measurements.\(^7, 8\) Bronchoalveolar lavage (BAL) is a common tool used in diagnosing and monitoring pulmonary disease, as it allows for sampling of respiratory secretions with its cellular and acellular components.\(^9\) In the research setting, analysis of gastric fluid components in BAL fluid (BALF) has been used as a tool to evaluate gastric aspiration.\(^11\) However, conclusions drawn from this method are limited by unknowns such as the initial concentration of components in the gastric fluid, the volume of the aspirated gastric fluid, the length of time that has passed since aspiration, and the lifetime of the gastric fluid components in the lung. The present study investigated the half-lives of two common components found in gastric fluid, bile and trypsin, which can be readily assayed using currently available techniques. An experimental model was utilized in which human gastric fluid was placed into the right lung of rats, and the concentration of gastric fluid components in BALF collected at different time periods following the aspiration were assessed.

In this study, the observed clearance half-times of both bile acid and trypsin were approximately 9 hours in the rat lung and appeared to follow first order kinetics, similar to previous reports involving insoluble aspires into the lung.\(^11\) A potential mechanism of bile and trypsin clearance in rat lungs is receptor-mediated phagocytosis by resident pulmonary macrophages. Both human and rat lung possess resident pulmonary macrophages. Recently, it was found that human pulmonary macrophages possess a bile acid-specific G-protein receptor known as TGR5 that, to an unknown extent, sequesters bile acid.\(^12\) At present, investigations have not identified rat pulmonary macrophages as possessing either a TGR5 receptor analog\(^12, 13\) or other receptors for binding bile or trypsin. Thus, it may be that clearance of bile and trypsin from the lung is not the result of receptor-mediated processes carried out by rat pulmonary macrophages. Rather, given our findings that both bile and trypsin are eliminated from the lungs at a similar rate, a non-specific mechanism of bile and trypsin clearance in the rat lung may be more likely. The fact that bile and trypsin are both heterogeneous but yet are cleared with first order (or even zero order) kinetics adds further support to the view that clearance mechanism are non-specific.

It is reasonable to hypothesize that the clearance half-times of bile and trypsin in the rat lung observed in this study are the result of mucociliary escalator clearance. The rate of clearance of foreign particles via the mucociliary escalator is dependent upon several factors, such as an insoluble particle's size,\(^11\) the viscosity of the mucus layer in the bronchi,\(^11\) and the bulk of the foreign particles in the lung.\(^14\) The mucociliary escalatory appears to eliminate a fluid slower than an insoluble particle. In a study by Hofmann and Asgharian\(^11\) using an asymmetric, multiple-path model of the bronchial tree, it was found that mucociliary clearance and particle retention in the rat lung were significantly influenced by airway diameter rather than the size of the particle itself. They found the half-times of mucociliary clearance in rat lung of particles ranging from 0.1 to 7 μm in diameter to average approximately 1.25 h, with most of the particles being cleared from the rat lung after about 6 h.\(^11\) These clearance rates are significantly faster than our observed clearance of bile and trypsin in gastric fluid. Because bile and trypsin are soluble, their clearance may be dependent upon
how gastric fluid itself interacts with the mucociliary escalator. Change to the mucus viscosity as a result of aspirating whole gastric fluid may not fully explain our observed clearance half-times of both bile and trypsin in rat lungs. It is known that fluids of viscosities greater than 150 cP can cause the autoregulatory mechanism of mucociliary clearance to decompensate. However, given the whole gastric fluid utilized in this study had a viscosity ranging from 0.91 cP to 3.64 cP, it seems unlikely that viscosity played a role in prolonging clearance of bile and trypsin in this study.

It is likely the interaction between the mucus layer of the respiratory tract and gastric fluid lead to the observed clearance half-times of bile acid and trypsin. A recent study of the autoregulatory mechanism governing the human mucociliary tract lends support to this hypothesis. Liu et al found that when the load of the mucus layer is increased to compress the underlying periciliary liquid layer by more than 1 μm, then the cilia's beating ability is reduced. This prior study also showed that when the load of the mucus layer compressed the periciliary liquid layer by more than 2 μm, the autoregulatory mechanism was no longer able to compensate for the mucus load, leading to significant deceleration of mucociliary clearance.

If a nonspecific mechanism like the mucociliary escalator is the predominant mechanism for bile acid and trypsin clearance in the rat lung, our findings may be useful to inform interpretations of analyses of bile acid and trypsin from BALF with regards to diagnosing gastric fluid aspiration in humans. Numerous studies have focused on modeling mucociliary clearance of particles in human and rat lung, leaving a need for more investigation into mucociliary clearance of fluids and soluble factors. Our study suggests that mucociliary clearance of gastric fluid and at least some soluble factors, namely bile acid and trypsin, have prolonged clearance from the lungs compared to insoluble particles. A recent study employing multiple-path modeling of the human bronchial tree found the half-life of mucociliary clearance of particles ranging from 0.1 to 7 μm in diameter averaged approximately 8 h in human lung. If our inference as to the difference in mucociliary clearance of fluid and insoluble particle is correct, we postulate half-lives of bile acid and trypsin clearance in the human lung as a result of aspiration to be at least 16 hours or greater in a non-diseased lung. However, it is well appreciated that the physiological consequences of gastric fluid aspiration probably extend far beyond the time of clearance of the gastric fluid components. Indeed, this issue underlies some of the difficulty in using biomarkers as surrogates for aspiration.

The use of bronchoalveolar lavage with subsequent analyses of gastric fluid components in the BALF is currently an imperfect tool for diagnosing aspiration in the clinical setting. The present study suggests our ability to detect aspiration in experimental rat models might be limited with respect to time, perhaps to a few days, and that the optimal detection time is within the first 24 to 48 hours. However, clearance of particles from the human lung is slower than that in rats, so a longer time-frame may exist for detection of aspiration in humans. Nevertheless, the present study demonstrates that it is possible to “rule in” aspiration based on a biochemical test, but impossible to “rule it out” if the aspiration is episodic.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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