Pepsin, a Reliable Marker of Gastric Aspiration, Is Frequently Detected in Tracheal Aspirates From Premature Ventilated Neonates: Relationship With Feeding and Methylxanthine Therapy

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ABSTRACT

Objectives: To determine the frequency of pepsin detection in tracheal aspirate (TA) samples of mechanically ventilated premature neonates and its association with feedings and methylxanthine therapy.

Patients and Methods: Serial TA samples (days 1, 3, 5, 7, 14, 21, 28 and >28 days) were collected from premature neonates receiving ventilatory support. An enzymatic assay with a fluorescent substrate was used to detect pepsin. Pepsin was also measured in 10 serum samples collected in conjunction with the TA samples from 8 neonates.

Results: A total of 239 TA samples was collected from 45 premature neonates (mean birth weight, 762 ± 166 g; mean gestational age, 25.5 ± 1.5 wk). Pepsin was detectable in 222 of 239 TA samples (92.8%) and in none of the serum samples. Pepsin was significantly lower on day 1 (mean, 170 ± 216 ng/mL) when compared with all other time points (P < 0.05). Mean concentration of pepsin was significantly lower when infants were unfed (265 ± 209 ng/mL) compared with levels during feeding (390 ± 260 ng/mL, P = 0.02). The mean level of pepsin was significantly higher in infants during xanthine therapy (419 ± 370 ng/mL) compared with no xanthine therapy (295 ± 231 ng/mL, P = 0.037).

Conclusion: Pepsin, a marker of gastric contents, was detected in more than 92% of TA samples from premature infants on mechanical ventilation. The level of pepsin was higher in fed infants when compared with unfed infants. Xanthine therapy was also associated with increased pepsin in TA samples. Chronic aspiration of gastric contents may worsen lung disease in premature infants. JPGN 43:336–341, 2006. Key Words: Gastric aspiration—Gastroesophageal reflux—Methylxanthine—Pepsin—Premature infants. © 2006 Lippincott Williams & Wilkins

INTRODUCTION

Gastroesophageal reflux (GER) is very common in premature neonates (1–3). Multiple factors, including immature tone of the lower esophageal sphincter (LES), supine positioning, small stomach capacity, delayed gastric emptying, decreased gastrointestinal motility and the presence of a nasogastric tube, contribute to GER in preterm infants (4–7). In neonates, GER can be associated with apnea, bradycardia and aspiration of gastric contents into the lungs (8–10). Cuffed endotracheal (ET) tubes in mechanically ventilated adults and children minimize the aspiration of gastric contents (11,12). However, microaspiration may not be preventable in mechanically ventilated neonates in whom uncuffed ET tubes are used. Gastroesophageal reflux may also precipitate esophagitis, which lowers the esophageal sphincteric pressure and further augments reflux, increasing the risk of aspiration (13). However, literature to support the aspiration of gastric contents in premature neonates is sparse.

Low gastric pH along with pepsin and bile acid in gastric contents can damage lung tissue (14,15). Aspiration due to GER in premature neonates on ventilatory support can worsen lung disease and may contribute to the development of bronchopulmonary dysplasia (BPD) (16,17). The true prevalence of aspiration is difficult to determine because of vague definitions, nonspecific signs and symptoms, poor assessment methods and varying levels of clinical recognition. There is no reliable method to detect gastric contents in tracheal aspirate (TA) samples in premature neonates. Measurement of pH in TA samples is not a reliable marker of gastric contents because gastric pH is greater than 4 for 90% of the time in premature neonates (18,19). Hopper et al. (20) measured...
lactose in TA samples as a marker of aspiration of gastric contents. Lipid-laden macrophages in TA samples are increased in premature neonates receiving intravenous lipids (21). A specific marker of GER-related aspiration should originate in the stomach, not the lung. Detection of pepsin in TA samples is a new reliable marker of gastric contents and microaspiration and has been used as a marker of aspiration in adults (22,23). Meert et al. (24) and Krishnan et al. (25) used a similar technique to measure microaspiration in children. However, to our knowledge, no study has been done to detect pepsin in TA samples of premature neonates.

Methylxanthines (aminophylline and caffeine) are commonly used in premature neonates to prevent and treat apnea of prematurity. Xanthine derivatives are known to increase GER by reducing LES tone and increasing gastric acid secretion (14,26).

This study was performed to determine the frequency of pepsin detection in TA samples from mechanically ventilated premature neonates and its association with feeding and xanthine therapy. We hypothesized that pepsin, a marker of gastric contents, is detectable in TA samples from premature ventilated neonates and that its level increases with increased feedings and with xanthine therapy.

PATIENTS AND METHODS

Study Population

The study was conducted in a 39-bed, level III neonatal intensive care unit at Cooper University Hospital in Camden, NJ, between March 2003 and October 2004. The Institutional Review Committee approved the study, and parents signed a written informed consent. Infants born before 32 weeks’ gestation and requiring mechanical ventilatory support were eligible for participation. The decision to treat each infant with xanthine derivatives was made by the attending neonatologist. Aminophylline was given as a loading dose of 8 mg/kg, and the maintenance dose was adjusted to keep the serum level between 7 and 15 μg/mL. The initial loading dose of caffeine citrate was 20 mg/kg, and the maintenance dose was adjusted to keep the serum level between 5 and 25 μg/mL. Relevant clinical data including the infant’s demographics, clinical parameters and feedings were collected from the patient’s chart.

Sample Collections

Tracheal aspirate samples were collected on days 1, 3, 5, 7, 14, 21 and 28 while the infant was mechanically ventilated. Additional samples were obtained after 28 days in some infants if ventilatory support continued. Tracheal aspirate samples were obtained by instilling 0.5 mL of normal saline into the infant’s endotracheal tube and suctioning the residue with a 5F suction catheter after 2 or 3 ventilator breaths. The suction catheter was passed to a standardized length of 0.5 to 1 cm beyond the tip of the ET tube. This method of collection is used widely in neonates to collect TA samples (27–29), and it is well tolerated by even the most critically ill neonates. The procedure was repeated 3 times and replicates were pooled. The suction catheter was flushed with 0.5 mL of normal saline after each suctioning episode to collect the residual sample in the catheter. The samples were immediately transported to the laboratory on ice and processed within 30 minutes in the laboratory. The samples were centrifuged at 4°C for 10 minutes at 300g. The supernatant was collected, divided into aliquots and stored at −70°C for future use.

Serum samples (n = 10) from 8 neonates were also collected at the same time as the TA collection.

Pepsin Enzymatic Method

The enzymatic method was modified according to the assay developed by Krishnan et al. (25). Porcine pepsin (Sigma-Aldrich, St Louis, MO) standards (12.5–400 ng/mL) were prepared in 0.1 mg/mL bovine serum albumin (BSA) with saline. Gastric fluid from positive control patients was diluted in the same BSA/saline solution, and the enzymatic reactions were carried out in a 96-well microplate. Fifty microliters of standard or sample was pipetted to microplate wells. Sample blanks were prepared by incubating standard or sample on a 100°C dry block for 5 minutes to inactivate the enzymatic activity. To each well, 23 μL of 129 mM HCl was added to adjust the pH to 2.0 and left on ice for 15 minutes to inactivate lysosomal acid hydrolase (cathepsin D) and to convert pepsinogen to active pepsin (25). Next, 20 μL of 0.5% fluorescein isothiocyanate

![Graph](image-url)
casein (Sigma-Aldrich) was added to each well and incubated for 3 hours at 37°C. The plates were transferred back to the ice tray, and 90 K Lo f2 0 % trichloroacetic acid were added to each well for trichloroacetic acid precipitation. The plates were centrifuged for 90 minutes at 3500 rpm and 8°C in a Sorvall centrifuge. Thirty-eight microliters of the supernatant was transferred to a new, clean, flat-bottomed microplate, and 212 K Lo f5 0 0 m m o l / L t r i sw a s a d d e d to each well. The plate was read in a spectrofluorometer at excitation (485 nm) and emission (530 nm), and the net fluorescent intensity was subtracted from the blanks. The final pepsin concentration of the sample was determined based on the net fluorescent intensity of the known concentrations of the standards. The subjective pepsin level (defining positive from negative) of an aspirate was set at the lower limit (12.5 ng/mL) of the sensitivity of the assay.

**Statistical Analysis**

Statistics were performed using Sigma Stat 3.1 for Windows statistical package (Systat Software, Inc, Point Richmond, CA). A comparison of pepsin levels over time was done by Kruskal-Wallis 1-way ANOVA. The comparisons between groups (before and after feeding, xanthine therapy) were performed using the paired t test and Wilcoxon signed rank test. The difference was considered significant for \( P < 0.05 \).

**RESULTS**

A total of 239 TA samples were collected from 45 premature neonates (mean birth weight, 762 ± 166 g; gestational age, 25.5 ± 1.5 wk). Clinical characteristics of the study population are summarized in Table 1.

Pepsin was detectable in 222 of 239 (92.8%) TA samples. The median pepsin level of all aspirates was 283.21 ng/mL (range, 0–2441 ng/mL). Eight of 17 negative samples were from day 1. After day 1, 200 of 209 (95.7%) TA samples were positive for pepsin. The level of pepsin was also measured in 10 serum samples from 8 premature neonates; samples were collected at the same time as the TA. Pepsin was undetectable in all serum samples and detectable in all TA samples.

**Levels of Pepsin Over Time**

The levels of pepsin were compared across different time points (days 1, 3, 5, 7, 14, 21, 28 and &gt;28). The numbers of TA samples collected on days 1, 3, 5, 7, 14, 21, 28 and &gt;28 were 30, 30, 34, 35, 28, 25, 23 and 34, respectively. The concentration of pepsin in TA samples was significantly lower on day 1 (mean ± SD, 170 ± 216 ng/mL) when compared with all other time points (days 3, 5, 7, 14, 21, 28 and &gt;28, all \( P < 0.05 \)). There was no significant difference in pepsin level among other time points (Fig. 1).

**Pepsin and Gestational Age**

The levels of pepsin were compared in preterm infant with gestational age 23 to 25 weeks (GA 23–25, n = 23) and 26 to 31 weeks (GA 26–31, n = 22). The level of pepsin was significantly higher on days 5 and 7 in premature neonates GA 23–25 compared with GA 26–31 (Fig. 2).

**Feeding and Pepsin Level**

Thirty-four infants received enteral feeding during the study period. The data were available on 30 infants to compare the pepsin levels before and after feeding. If more than 1 sample was collected from an infant before or during feeding, the mean level of pepsin was used for statistical analysis. The mean concentration of pepsin was significantly lower when infants were unfed

*FIG. 2. The levels of pepsin in preterm infant with gestational age 23 to 25 weeks (GA 23–25, n = 23) and 26 to 31 weeks (GA 26–31, n = 22). The level of pepsin was significantly higher on days 5 and 7 in premature neonates GA 23–25.*

*FIG. 3. The levels of pepsin when infants were unfed (nil per os) and during the feed. Median concentration of pepsin was significantly lower when infants were unfed when compared with during feeding.*

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The level of pepsin and treatment with xanthine derivatives. Treatment with xanthine increased pepsin level.

*P = 0.036

(265 ± 209 ng/mL) when compared with levels during feeding (390 ± 260 ng/mL, P = 0.02) (Fig. 3).

Xanthine and Pepsin

Thirty-six infants received xanthine derivatives during the study period. The data were available on 23 infants to compare the pepsin levels before and during the xanthine therapy. The mean level of pepsin was used for statistical analysis if more than 1 sample was collected from an infant before or during the xanthine therapy. The mean level of pepsin was significantly higher in infants during the xanthine therapy (419 ± 370 ng/mL) when compared with no xanthine therapy (295 ± 231 ng/mL, P = 0.037).

Because the level of pepsin was significantly lower on day 1 in our cohort of population, we again analyzed the data after excluding the samples from day 1. The data from all 23 infants were available for this analysis. Again, the pepsin level was significantly higher during the xanthine therapy (no xanthine, 341 ± 252 ng/mL; xanthine, 523 ± 370 ng/mL; P = 0.036) (Fig. 4).

DISCUSSION

Premature infants are predisposed to GER and are at increased risk for pulmonary aspiration. Several factors that lead to aspiration of gastric contents in premature neonates are intubations and mechanical ventilation, use of sedation, immature swallowing mechanism, reduced muscle tone and suppression of reflexes that protect the airways (4–7). In infants, physiological functions seem to be impaired at different swallowing stages, increasing the predisposition to aspiration. At the same time, the control over the systems that protect against aspiration is lost due to a direct action of the endotracheal tube.

In the past, many tests have been done to look at aspiration of gastric contents such as dye studies, lipiddaden macrophages and glucose and lactose assays. In clinical practice, technetium scintigraphy is used to detect pulmonary aspiration. However, this study is performed during and after a single feed for 2 to 3 hours and, therefore, has limited ability to detect the aspiration of gastric contents (30). Hopper et al. (20) used the detection of lactose in TAs to diagnose GER aspiration in neonates. Conclusions from these studies usually are assumptions, which can give false-negative results. Recent studies have shown that modified pepsin assay has more sensitivity and specificity in detecting aspiration (23–25). An advantage of the tracheal pepsin test over other methods for assessing aspiration is that tracheal secretion samples can be collected easily at the bedside as a noninvasive procedure that does not interfere with the patient care.

In the current study, detection of pepsin was investigated as a marker for gastric contents in TA samples from premature ventilated infants. The results demonstrated that pepsin is detectable in >92% of all TA samples and in >95% of samples collected after day 1, suggesting significant aspiration of gastric contents in premature infants. In 30 acutely ill, tube-fed, mechanically ventilated adults, pepsin was detectable in only 14 of 136 TA samples (31). Meert et al. (24) reported positive pepsin in only 9 of 100 TA samples from 37 children. They also found that children with pepsin-positive TA are more likely to have clinical evidence of GER (24). Krishnan et al. (25) detected pepsin in 31 of 37 children with history of GER and respiratory symptoms and none in 26 children without history of GER or respiratory symptoms. Recently, using intraluminal impedance technique, Lopez et al. (32) reported that >79% of acid and nonacid refluxes reach the proximal esophagus in premature neonates. Valat et al. (33) measured radioactivity in ET after injecting solution of 99mTc-sulfur colloid in the gastric tube in neonates. Forty-two percent of neonates had positive radioactivity in the tracheal tube, suggesting aspiration of gastric contents. Our data indicate that aspiration of gastric contents into the lung is a widespread phenomenon in ventilated premature infants. Apart from factors discussed earlier, uncuffed ETs used in ventilated premature infants may also have a major contribution to the high-pepsin positive rate. Previous studies support the role of cuffed ET tubes in the prevention of aspiration (11,12). Small amounts of pepsinogen are detectable in human blood (34,35). To exclude a hematogenous source of pepsinogen in TA samples, pepsin was also measured in serum samples at the same time of TA collection. Pepsin was undetectable in all the serum samples.

The presence of pepsin in >95% of samples after day 1 suggests significant aspiration of gastric contents in premature ventilated infants. Aspiration of gastric contents may play a major role in worsening of lung disease in premature neonates. Twenty percent to 40% of ventilated premature neonates develop BPD (36). Multiple factors including high oxygen, ventilator-induced lung injury, symptomatic patent ductus arteriosus, various lytic proteinases and nutritional deficiencies are implicated in the etiopathogenesis of BPD, but the exact etiology is still...
unknown (36). More, recently, a new chronic lung disease called “new BPD” is emerging in premature infants, not related to oxygen therapy and mechanical ventilation (37). The new BPD develops in premature neonates with no or minimal initial lung disease and presents with gradual worsening of respiratory failure. We speculate that chronic aspiration of gastric contents may be contributing to the worsening of lung disease in premature infants. The role of chronic aspiration due to GER warrants more studies in the development of this new BPD.

Our study shows that pepsin levels in TA samples were low on day 1, suggesting minimal pulmonary aspiration of gastric contents. In animal models, after a single episode of aspiration of gastric juice, pepsin was detectable in all samples at 2 hours, at 4 hours, and in 90% of samples at 6 hours, suggesting that pepsin can be detectable up to 6 hours after aspiration (38). Low levels of pepsin on day 1 in our population are more likely related to decreased pepsin secretion immediately after birth, no feeding and, possibly, lack of sufficient time for aspiration of gastric contents. However, samples from day 3 onward showed consistent presence of pepsin in TA, suggesting ongoing microaspiration of gastric contents. The level of pepsin was consistently higher after 3 days and did not significantly change over time.

Our data indicate that the level of pepsin was significantly higher in TA sample in infants when they were fed when compared with when they were not receiving feeds. Feeding not only activates pepsin secretion but also increases reflux, leading to increased aspiration of gastric contents into the lungs. Increased volume of feeds may increase GER and aspiration. Additional studies are needed to see the effect of various volumes of feeds on the aspiration of gastric contents in premature infants.

Xanthine derivatives are commonly used in premature infants to prevent apnea of prematurity. Our data suggest that the aspiration of gastric contents is increased in premature infants receiving xanthine derivatives. Xanthises are known to increase GER by reducing LES pressure (39,40). We were unable to compare the difference in aspiration with caffeine and aminophylline, as all but 1 infant was treated with aminophylline.

We recognize some important limitations of this study. Pepsin was measured in TA samples and was potentially diluted. It is controversial whether TA samples should be corrected for dilution in neonates (41). We followed the recommendation of the European Respiratory Task Force on bronchoalveolar lavage in children and did not correct our result for dilution (41).

Enzymatic assays in our study measured both pepsin and pepsinogen. Pepsin is an active form and contributes to lung injury, whereas pepsinogen is an inactive form. Secretion of pepsin is inconsistent and low in infants (42,43), and measuring only pepsin in TA samples may underscore the severity of aspiration in premature infants.

Our study is an observational study, lacking a control group of premature infants who are not ventilated. Infants were not randomized to feed or to receive xanthine therapy. The decision to treat the infants with xanthine derivatives was made by the attending neonatologist on service; this decision may reflect the increased bradycardiac episodes associated with GER, and the xanthine use may function as a confounding factor.

Despite the above limitations, this is the first study demonstrating significant aspiration of gastric contents in ventilated premature infants. Chronic aspiration of gastric contents may cause lung injury and is likely to be important in the pathophysiology of BPD in premature infants. Additional studies are required to investigate the role of aspiration of gastric contents in the development of BPD, its relationship with pro-inflammatory mediators and the effect of preventing GER or microaspiration in premature infants.

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