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Investigation of pepsin in tears of children with laryngopharyngeal reflux disease

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ABSTRACT

Objectives: Numerous investigations postulated that laryngopharyngeal reflux (LPR) is implicated in the pathogenesis of various upper airway inflammatory diseases as sinusitis or dacryostenosis.

The presence of pepsin in tears might be confirmed the presuntive hypothesis of the arrival in the nasolacrimal ducts and precorneal tears film through the laryngopharyngeal reflux of either gastric acid or stomach secretions (pepsin) with inflammatory potentialities.

The aim of this preliminary study was to identify the presence or absence of pepsin in the tears collected from children with a high suspicion of LPR who underwent 24-h pH (MII-pH) monitoring to confirm the disease.

Methods: This study enrolled 20 patients suffering from symptoms of laryngopharyngeal reflux that underwent 24-h multichannel intraluminal impedance (MII)-pH monitoring to confirm the disease. The findings of the study group were compared with those of a control group of patients with negative pH monitoring. The quantitative analysis of human pepsin concentration in the tear samples was performed by ELISA method in both groups.

Results: Four children (20%) of the study group showed pepsin in the tears. All of the subjects belonging to the control group were negative for its presence. No difference differences in the total number of reflux episodes and the number of weakly basic reflux in the pepsin positive patients vs. pepsin negative children were present.

Conclusions: 20% of the children with diagnosed LPR showed pepsin in the tears. Our specific investigation might provide information regarding sinusitis or dacryostenosis.

1. Introduction

Laryngopharyngeal reflux disease (LPRD) is defined as the reflux of gastric and/or duodenal juices (refluxate) beyond the esophagus into larynx, oropharynx, and/or nasopharynx. Although it has been initially considered an extension of gastroesophageal reflux disease (GERD), recently pediatric laryngopharyngeal reflux (LPR) tends to be identified as a unique and distinct disease process [1–4].

Several studies have demonstrated the presence of pepsin and other noxius reflux products, such as bile acids, in middle ear effusion, supporting the existence of a relationship between gastroesophageal reflux (GER) or LPR and otitis media with effusion (OME) [4–15]. This finding implies that these substances are able to reach the middle ear via the Eustachian tube, passing through several anatomical structures (larynx, pharynx and rhinopharynx) after their exit from the stomach [4,10,15–19].

Magliulo et al. [20] in 2013 hypothesized that GERD contributes to dacryostenosis and subsequent primary acquired nasolacrimal ducts obstruction as a "prime mover" and so that pepsin could be found in tears. Ascending gastric acid and stomach products might be result in initial edema of the nasolacrimal ducts mucosa which could progress toward chronic inflammation, fibrosis, and, ultimately in a complete nasolacrimal duct obstruction.

The aim of this preliminary study was to identify the presence or absence of pepsin in the tears collected from children with

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symptoms of laryngopharyngeal reflux disease who underwent 24-h pH (MII-pH) monitoring to confirm the disease.

2. Materials and methods

This prospective study enrolled 20 children (9 males, 11 females; 1–15 years of age, average age 6.6) with a diagnosis of laryngopharyngeal reflux disease between October 2013 and January 2015 at the Pediatrics Department of University "Sapienza" of Rome. The findings of the study group were compared with those of a control group of patients consisting of 20 normal subjects (10 males and 10 females, age range of 1–15 years) who underwent the same diagnostic protocol of the study group (Table 1).

Usually children with symptoms of LPRD, arriving to our Department of Pediatrics, underwent an initial screening by the reflux symptom index (RSI) as developed and validated by Belafsky et al. [21]. In too young children RSI evaluation was made with their parents help.

RSI is a self-conducted survey that includes nine questions with a maximum of 5 points for each question, giving a total of 45 points [22,23]. As suggested by Belafsky et al. [21] any RSI scores above 13 were considered as abnormal.

Children with abnormal RSI underwent multichannel intraluminal impedance (MII) and pH monitoring to confirm the LPRD.

24-h MII-pH monitoring was performed using an ambulatory system (Sleuth; Sandhill Scientific, Inc; Highland Ranch, CO, USA). The system included a portable data logger with impedance-pH amplifiers and a MII-pH catheter with an outer diameter of 2.1 mm (6.4-French), containing one pH-measuring electrode and seven impedance sensors, in the form of 4-mm cylindrical ring electrodes. The MII-pH catheter was introduced through the nose and fluoroscopically positioned so that the pH-measuring electrode overlay the third vertebral body above the diaphragm throughout the respiratory cycle. Each participant ate a regular diet and at least 4 h elapsed between each meal.

As no method has been clearly defined to calculate baseline impedance level throughout a 24 h tracing, baseline impedance values were assessed in the most distal channel over the first stable 60-s time period every 4 h. A stable period was identified when no swallowing or bolus or gas reflux was present. Baseline impedance levels during each selected time period were automatically calculated by a specific function (electronic ruler) of the software. Thereafter, the 4-hourly impedance baseline values obtained from the complete tracing were averaged to obtain the mean distal baseline values for the entire recording.

The acid gastroesophageal reflux index (RI), which represents the proportion of the total time of the recording for which the esophageal pH was less than 4.0, was calculated and expressed as a percentage value. RI $>$ 7% was the cut-off value considered for the diagnosis of acid gastroesophageal reflux, according to Ref. [24].

All 20 patients enrolled in the study group reported positive detection to the 24-h pH (MII-pH) with an RI $>$ 7, while, all the patients of the children of the study group had values of RI $<$ 7.

Besides the following MII-pH variables were analyzed: (1) total number of reflux episodes; (2) number of acid reflux (AR) episodes; (3) number of weakly alkaline episodes (Wal).

All of the patients underwent the withdrawal of the tear sample using a micropipette of clear silicone tube (diameter 0.3 cm, length 2 cm and cut 45° cut) connected to a small silicone tank (diameter 0.5 cm, length 2 cm) equipped with a 3.5 cm suction tube curved at 0.5 cm with a 30° angle. This works by aspiring of tear fluid from the lacrimal lake at inner canthus of the eyelid. All of the samples were stored at −20 °C until being analyzed.

The quantitative analysis of human pepsin concentration in the tear samples was performed by ELISA method (commercial pepsin ELISA kit – DRG Inc., Germany). The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of pepsin in mouse serum, plasma and other biological fluids as tears.

Several studies have confirmed as this test is effective to the pepsin evaluation in middle ear effusion or middle ear lavage fluid, however, no study reported the evaluation of human pepsin in tears by this method [1–5,11–13].

The manufacturer claimed as positive for pepsin an ELISA test detection ranged between 1.56 and 100 ng/ml. However in our case ELISA determination of the human pepsin concentration at 0.0, 2.5, 5, 10 and, 50 ng/ml was performed 10 times according to the manufacturer's instructions to determine the consistency and the lower limit sensitivity of the assay. The standard curve from the average value of the 10 runs had an R^2 = 0.97; pepsin (human) at 2.5 ng/ml had a net spectrometer unit increase of 33.5 ± 9.5% (mean ± SD) over the blank, which was significantly higher than that of the negative control (0.0 ng/ml pepsin consisting only of buffer and reagents) with 9.3 ± 3.7% net spectrometer unit increase ($P < 0.05$). Pepsin at 1.5 ng/ml or less had a similar net spectrometer unit increase over the blank as the negative control (0.0 ng/ml pepsin). Therefore, the empirical pepsin level differentiating positive from negative for pepsin in a tear sample was set at the lower limit of the sensitivity of the assay at 2.5 ng/ml. A patient was defined as pepsin-positive if one of the eye samples had pepsin above 2.5 ng/ml.

All patients guardians gave their written informed consent for the above mentioned tests and to enrolled these patients in the study. This research was performed in accordance with the principles of the Declaration of Helsinki and approved by the local ethics committee of the University "La Sapienza", Rome.

The only descriptive statistical analysis of data was performed due to the limited number of patients in both the study and control group.

3. Results

The percentage of human pepsin in the tears of the study group was estimated in 20% of cases (six children) all belonging to the group of patients with diagnosis of LPRD. Pepsin was detected in two patients younger than <5 years and in one 6 and one 7 year-old patients. Concentration levels of pepsin equal to 3.5, 5.4, 4.0 and 4.2 ng/ml were respectively calculated.

None of the subjects belonging to the control group (negative negative MII-pH monitoring) reported presence of pepsin in the tears.

Despite the limited number of relevant cases a different pepsin detection about the two groups was evident (Fig. 1). Table 2 summarizes the total number of reflux episodes vs. the presence of pepsin in the tears. No difference emerged in two groups because

Table 1
Patients' characteristics and ELISA pepsin evaluation in tears.

	No. of patients	Sex	Average age (years)	Positive pepsin in tears
LPRD group (positive MII-PH monitoring)	20	11 Male 9 Female	6.6	4 (20%)
Control group (negative MII-pH monitoring)	20	10 Male 10 Female	6.9	0

Laryngopharyngeal reflux disease (LPRD); multichannel intraluminal impedance (MII).