Lower esophageal sphincter relaxation reflex kinetics: effects of peristaltic reflexes and maturation in human premature neonates

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The prevalence of symptoms in gastroesophageal reflux (GER) disease (GERD) in infants has been partly attributed to spatial or temporal characteristics of the gastroesophageal refluxate or to lower esophageal sphincter (LES) and/or esophageal motor dysfunctions (17, 21, 47). Esophageal peristalsis and the LES relaxations (LESR) regulate esophageal clearance and propel the bolus away from the aerodigestive tract. LES is a specialized region of circular smooth muscle that provides antireflux barrier augmented by the extrinsic contraction of the crural diaphragm (6, 31). Additionally during peristalsis, the esophagus and LES participate in propagation of contractile waveforms and sequential relaxation respectively (22, 34). Using esophageal manometry methods, we have previously identified esophageal peristalsis as 1) basal primary esophageal peristalsis initiated by a swallow and 2) adaptive esophageal peristalsis occurring after esophageal stimulation, in the form of esophageodeglutition response (EDR) or secondary peristalsis (SP), and that these mechanisms exist by 33 wk postmenstrual age (PMA) (14, 15, 36). Although changes in LES pressure (LESP) have been described during swallowing (35, 36), the characteristics of LESR during esophageal provocation-induced SP or EDR remain unknown. These mechanisms may minimize the ascending spread of the bolus and facilitate refluxate clearance.

The analysis of LES contractile and relaxation characteristics using sleeve sensor manometry methods has been previously defined in adults and neonates during swallowing (12, 31, 35, 43). Recently, we developed methods that permit the evaluation of mechanostimulation-induced esophageal motility changes on peristaltic and sphincteric reflexes during maturation (14, 19). Motility changes during neonatal maturation are important in understanding normal physiology as well as in the investigation of abnormality. This knowledge is of importance because the sensory motor pathways in evoking spontaneous basal primary peristalsis (PP) or adaptive EDR and SP during esophageal provocation are different (26, 30). These reflexes are governed by visceral afferent and effenter innervations via the vagus (26).

The objectives of the present study are to define the development of temporal changes in LES kinetics during basal or adaptive esophageal peristalsis (PP, EDR, and SP) in premature human neonates across maturation. We tested the hypotheses that LESR kinetics are different during maturation and that the differences are dependent on the type of peristaltic reflex and the stimulus characteristics. We defined the sensory characteristics of the LESR (stimulus threshold volume and response onset) and motor characteristics (relaxation period, relaxation magnitude, nadir duration) during maturation. We accomplished these goals by evaluating basal and adaptive esophageal peristalsis (PP, EDR, and SP) in premature neonates across maturation. We tested the hypotheses that LESR kinetics are different during maturation and that the differences are dependent on the type of peristaltic reflex and the stimulus characteristics. We defined the sensory characteristics of the LESR (stimulus threshold volume and response onset) and motor characteristics (relaxation period, relaxation magnitude, and nadir duration) during maturation. We accomplished these goals by evaluating basal and adaptive esophageal peristalsis (PP, EDR, and SP) in premature neonates across maturation.
SUBJECTS AND METHODS

Participants. Twenty enterally fed preterm neonates were studied twice, 4–6 wk apart in between time 1 and time 2, when they were physiologically stable. This time interval was chosen based on our prior experience of defining maturational changes in foregut motility across such durations (13, 20). Gestational age (GA) was determined by maternal history and obstetric data. Postmenstrual age (PMA) was determined by adding GA to chronological age. Subjects were studied when they have recovered from neonatal transition and respiratory distress at birth and were on a stable phase of feeding and growth. All subjects were evaluated by the principal investigator (S. R. Jadcherla) and the attending neonatologist and were deemed healthy at study. Because some subjects were placed on acid-suppressive medications empirically, a subset analysis was performed comparing those that were placed on such medications vs. none. Additionally, as per our protocol, subjects were required to be off acid-suppressive medications for at least 72 h prior to the study, and this criterion was complied in all these subjects. None were diagnosed with necrotizing enterocolitis or bronchopulmonary dysplasia during their hospital stay. Subjects with perinatal asphyxia, gastrointestinal abnormalities, chromosomal disorders, or neurological abnormalities were excluded, as well as those that were placed on prokinetics.

The study procedures were approved by the ethics committee at the Institutional Research Review Board (IRB) at the Nationwide Children’s Hospital Research Institute, Columbus, OH. The study protocol conforms to the guidelines of the local IRB policy and the health insurance portability and accountability acts (HIPAA). Informed consents and HIPAA authorization were obtained from parents prior to study.

Manometry methods and techniques. The neonatal manometry techniques using esophageal provocation methods have been developed and validated by us (11, 14, 15, 19). Briefly, the catheter assembly (Dentsleeve International, Mui Scientific, Mississauga, Ontario, Canada) was connected to the pneumohydraulic micromanometric water perfusion and recording systems via the resistors, pressure transducers (TNF-R disposable pressure transducers, MMS medical instruments, Dover, NH), and amplifiers (Solar modules, Solar-2, MMS medical instruments, Dover, NH). The esophageal manometry catheter assembly with dual sleeves, four port, a terminal gastric recording port, and a midesophageal infusion port was used. The outer diameter of the catheter was 2.0 mm. The water perfusion rate was 0.02 ml·min⁻¹·port⁻¹ for esophageal ports, 0.01 ml·min⁻¹·port⁻¹ for pharyngeal ports, and 0.04 ml·min⁻¹·port⁻¹ for the sleeves. The catheter was passed nasally in the supine lying infant without the aid of sedation. All the studies were done in the same manner with the transducers at the level of the subject’s esophagus (midaxillary line). Respiratory inductance plethysmography (Respitrace) and vital signs transducers at the level of the subject’s esophagus (midaxillary line).

Experimental study protocol. Continuous data acquisition and analysis were performed during manometric study (14, 15, 19). Midesophageal provocations with air, water, and apple juice were performed to test the effects of mechanostimulation, osmostimulation, and chemosensitive stimulation respectively. Graded infusion volumes were infused abruptly in triplicate (0.1, 0.5, 1.0, 2.0 ml) to test the effects of stimulus-response relationships. Each volume was given in the same order during esophageal quiescence and after an interval of at least 1 min elapses for esophageal clearance.

Data analysis. Manometry data analyses were done by two investigators who were blinded from the purpose of the study, and statistically significant concordance was reached. We have previously described the data analysis pertinent to the spontaneous PP (dry swallow), infusion-induced EDR (similar to PP waveforms), and SP (11, 14, 15, 19). Spontaneous swallows or PP were identified as a fully propagated pharyngoesophageal sequence that begins in the pharynx and is associated with UES relaxation and propagation of the peristaltic wave front across the proximal, middle, and distal esophagus and accompanied by LESR (9). SP is defined as the propagation of waveforms into the proximal, middle, and distal esophageal segments in the absence of pharyngeal waveform and UES relaxation. SP occurs in response to midesophageal infusion. The onset of proximal esophageal upstream stroke from the stimulus onset defines the response latency for SP (16). EDR was defined as the deglutition response upon esophageal stimulation, which began with onset of the pharyngeal waveform associated with UES relaxation, and propagation into the proximal, middle, and distal esophageal segments (16) is associated with LESR.

The changes in LES characteristics are analyzed as described by us and others (11, 35, 36). Specific to the present study, LES resting pressure and relaxation were evaluated as follows: 1) LES resting pressure was evaluated before the onset of swallow or the infusion, as a mean of five observations of LES pressure in relation to gastric pressure at end expiration (Fig. 2C.1). 2) LESR started when LES pressure dropped at least 5 mmHg below LES resting pressure. 3) LES response time began with the onset of pharyngeal swallow signal during spontaneous swallows or onset of midesophageal infusion to onset of LESR (Fig. 2C.2). 4) Onset of LESR to onset of nadir was defined as the onset of LESR to the point where LES drops to 5.0 mmHg or less (Fig. 2C.3). 5) Nadir is defined as the lowest pressure point reached by the LES, and nadir pressure was the lowest pressure taken during nadir duration (Fig. 2C.4). 6) Nadir duration was considered as the period during which LES pressure dropped to 5.0 mmHg or less and up to the point LES pressure recovered to 5.0 mmHg (Fig. 2C.5). 7) Changes in LES pressure during spontaneous swallows were identified as a fully propagated pharyngoesophageal sequence (PP; Fig. 1A) that begins in the pharynx and is associated with UES relaxation and propagation of the peristaltic wave front across the proximal, middle, and distal esophagus and accompanied by LESR. Temporal changes in LES pressure from the baseline were evaluated in relation to the pharyngeal swallow signals. 8) Changes in LES pressure during SP and EDR. Esophageal stimulation-induced SP (Fig. 2A) or EDR (Fig. 2) events were scored for their respective frequency occurrence, as well as the frequency occurrence of LESR and the temporal changes in the LES pressure (Fig. 2, A–C).

Statistical analysis. The reflexes were visually identified (S. R. Jadcherla) at the time of study, and the coinvestigators (E. M. Pena and V. N. Parks) were trained in the recognition and spatiotemporal analysis of the waveform sequences. LESR was marked by visual inspection of each event using the a priori criteria described. Two blinded coinvestigators analyzed the data in random order, and concordance between the investigators was statistically significant for LESR characteristics with Kendall’s W values varying between 0.4 and 0.8.
LESR characteristics were compared across the two types of peristaltic reflexes evoked upon provocation, EDR vs. SP, and across maturational stages time 1 vs. time 2. Since several observations were made within each subject, multinomial mixed models or linear mixed models were used to study the stimulus-response relationship while taking into the account the correlation within subject. Compound symmetry covariance structure was fitted, and this structure specifies that measures at all times have a constant correlation regardless of the lag time.

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Fig. 1. Esophageal manometry describing spontaneous primary peristalsis (PP) and associated lower esophageal sphincter (LES) relaxation (LESR). An example of spontaneous PP characterized by the onset of pharyngeal waveform, upper esophageal sphincter (UES) relaxation, and propagation across the proximal (PE), middle (ME), and distal (DE) esophageal segments and associated with LESR. A: completely propagated PP sequence associated with complete LESR. B: incomplete LESR relaxation during PP. C: failed LESR during PP. *True LES = LES – Stomach.

Fig. 2. Effect of esophageal stimulation on peristaltic reflex response and LESR. In A, secondary peristalsis is noted in response to esophageal provocation, and associated LESR can be observed. In B, esophagodeglutition response is seen (as distinct from spontaneous PP) during which LESR is seen. The inset describes the analytical methods as to how the LESR was analyzed during these events. Schematic analysis of LESR is described. In response to swallow or esophageal stimulus (depicted as peak), the changes in LES tone are characterized as follows: 1) Resting LES pressure measured in relation to the intragastric pressure. 2) Response latency measured as the time between the stimulus-swallow peak signal to the onset of LESR defined as a pressure decrease by ≤5 mmHg. 3) LESR time defined as the time from the onset of LESR to complete LESR. 4) LES nadir pressure, defined as lowest LES nadir pressure (≤4 mmHg). 5) Duration of LES nadir, described as the duration of the flat portion of the LESR curve.
between pairs of repeated measurements. These models were fit by use of PROC GENMOD for categorical responses and PROC MIXED for continuous responses in SAS (SAS v.9.1 Institute, Cary, NC). Generalized estimating equations methods were applied to evaluate differences between the maturational stages. Descriptive data are reported as means or least square means and SE, percentages, or range, unless stated otherwise.

RESULTS

Participant characteristics. Twenty premature infants were studied (11 male, 9 female) twice. The mean ± SE for GA at birth was 28.5 ± 0.9 wk, birth weight was 1.2 ± 0.2 kg, birth length was 38.2 ± 1.6 cm, and birth head circumference was 26.3 ± 1.0 cm. All subjects were of appropriate growth for GA at birth and at time 1 and time 2. The mean ± SE for PMA at time 1 study was 34.7 ± 0.8 wk, weight was 1.8 ± 0.2 kg, length was 41.7 ± 0.9 cm, and head circumference was 29.9 ± 0.8 cm. The mean ± SE for PMA at time 2 study was 39.1 ± 1.0 wk, weight was 2.6 ± 0.2 kg, length was 45.7 ± 1.2 cm, and head circumference was 33.2 ± 0.6 cm. PMA, weight, length, and head circumference at the longitudinal studies (time 1 vs. time 2) were all significantly different (all P < 0.0001). Subjects tolerated study procedures without concerns; at discharge, all subjects were taking full nipple feeds.

At time 1, 80% were receiving feeds via gavage, 10% by gavage and orally, and 10% all orally. At time 2, 5% were receiving feeds via gavage, 40% received gavage and oral feeding, and 55% of patients were all orally fed. Feeding methods were different between the time 1 vs. time 2 (P < 0.0001).

Four subjects were empirically placed on acid-suppressive medications before the first study in addition to another two subjects before the second study. Subgroup analysis in subjects that were exposed to acid suppressive vs. none did not show any significant difference at either time 1 or time 2 for all the variables of interest with reference to LES kinetics [all variables, P = not significant (NS), mixed statistical models].

Testing the maturational differences in LESR characteristics during spontaneous PP. A total of 200 spontaneous swallow events (10 per subject) at time 1 and 197 swallow events at time 2 (9.5 ± 0.4 per subject) were evaluated for LES resting pressure and relaxation characteristics. Three types of LES responses were recognized (Fig. 1): 1) LES relaxed completely to a nadir LES pressure below 5 mmHg (Fig. 1A), 2) incomplete LESR when LES relaxed to a nadir LES pressure > 5 mmHg (Fig. 1B), and 3) absence of LESR (Fig. 1C). The frequency occurrence of complete LESR, incomplete LESR, and absence of LESR was 53.5, 10.5, and 36%, respectively, at time 1 and 61.0, 16.2, 22.8%, respectively, at time 2 (time 1 vs. time 2, P = 0.03, χ² test). LESR kinetics during PP at time 1 and time 2 are compared in Table 1, and maturational changes were significant for the magnitude of resting LES pressure (P = 0.001) and the degree of fall in pressure during LESR (P = 0.01).

Testing the maturational and mechanistic differences to the effects of midesophageal provocation on the overall frequency occurrence of LESR. A total of 616 infusions (248 air, 181 water, 187 apple juice) at time 1, and 651 infusions (269 air, 190 water, 192 apple juice) at time 2 were analyzed to evaluate the effects of esophageal provocation on the recruitment of peristaltic reflexes and LESR. An example of LESR during EDR and SP is shown in Fig. 2. Between time 1 vs. time 2, the frequencies of peristaltic reflex were 54.3% (34.3% EDR, 65.7% SP) vs. 58.4% (26.7% EDR, 73.3% SP), respectively (P = 0.1). The frequency of LESR at time 1 was 56.1% and at time 2 was 58.6% (P = 0.4). The proportion of LESR during EDR vs. SP at time 1 (62.2 vs. 64.2%) and at time 2 (65.7 vs. 70.7%) were similar (P = 0.7 and P = 0.4, respectively).

However, significant differences with the frequency of LESR were associated when peristaltic reflexes occurred compared with when no peristaltic reflexes occurred, at time 1 and at time 2 (both P < 0.0001; Fig. 3). No maturational difference (time 1 vs. time 2) was noted for LESR (P = 0.8).

Testing the maturational differences in LESR characteristics categorized by the esophageal peristaltic mechanisms. Specific LESR response characteristics evolved upon midesophageal infusion during EDR and SP are shown in Fig. 4. We further analyzed the effect of the variables (maturational time and type of peristalsis) on the repeated measures: 1) infusion onset to LES response onset interval (Fig. 4A), 2) LESR response onset to nadir onset (Fig. 4B), 3) infusion onset to nadir onset (Fig. 4C), and 4) duration of LESR nadir (Fig. 4D). Mechanistic differences were noted in the response latency to LESR, LESR relaxation time, and infusion onset to nadir onset interval, in the way that EDR had significantly longer latency than SP on all the three variables at time 2 only (P = 0.02, P = 0.04 and P = 0.003, respectively). Maturational time variable had significant effect on LESR time, infusion onset to nadir onset, and LESR nadir duration (P = 0.03, P = 0.05 and P = 0.05, respectively).

We also tested whether the LES nadir pressure (mmHg) was different between the maturational periods and between the mechanisms by comparing deglutition responses at time 1 vs.
**Table 2.** LESP characteristics between those evoked due to spontaneous dry swallow PP vs. esophageal infusion-induced EDR

<table>
<thead>
<tr>
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<th>Time 1</th>
<th>Time 2</th>
<th>P Value</th>
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<tbody>
<tr>
<td>LESP resting pressure, mmHg</td>
<td>10.1 ± 1.7</td>
<td>10.8 ± 1.9</td>
<td>0.6</td>
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<tr>
<td>Response time to LESP, s</td>
<td>2.0 ± 0.2</td>
<td>4.6 ± 0.3</td>
<td>&lt;0.0001</td>
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<tr>
<td>Relaxation time, s</td>
<td>1.0 ± 0.3</td>
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<td>0.2</td>
</tr>
<tr>
<td>LESP nadir pressure, mmHg</td>
<td>-0.2 ± 1.4</td>
<td>-1.3 ± 1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>LESP nadir duration, s</td>
<td>4.4 ± 0.6</td>
<td>6.6 ± 0.7</td>
<td>0.009</td>
</tr>
<tr>
<td>LESP pressure drop, mmHg</td>
<td>15.7 ± 1.3</td>
<td>16.2 ± 1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LESP frequency</td>
<td>64.0%</td>
<td>61.5%</td>
<td>0.6</td>
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Values are LSmeans ± SE; significance is based on mixed model with repeated measurements. EDR, esophagodeglution response.

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**Fig. 4.** Comparison of LESP characteristics during maturation and during the type of peristaltic reflexes. In A, the response time to LESP is significantly longer for esophagodeglution response (EDR) than secondary peristalsis (SP) (*P = 0.02) at time 2 only. In B, EDR has significantly longer LESP time than SP (*P = 0.04) at time 2 only. In C, the onset to nadir latency is significantly longer for EDR than SP (*P = 0.003) at time 2 only. EDR has longer latency from infusion onset to nadir onset at time 2 vs. time 1 (P = 0.03). In D, EDR has similar LESP nadir duration as SP at either time points. Maturation changes between time 1 and time 2 are significant for LESP time, onset to nadir latency, and LESP nadir duration.

**Table 2.** LESP characteristics between those evoked due to spontaneous dry swallow PP vs. esophageal infusion-induced EDR

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media evoking LESR or peristaltic reflex responses are shown in Fig. 5. Water and apple juice were combined as the liquid category since no difference was noted between these two media (water vs. apple juice, respectively) for the response time to LESR (5.0 ± 0.4 s vs. 4.8 ± 0.4 s at time 1, P = 0.9; 3.8 ± 0.3 s vs. 4.7 ± 0.3 s at time 2, P = 0.2) or the response time to PR (4.1 ± 0.2 s vs. 4.3 ± 0.2 s at time 1, P = 0.9; 4.4 ± 0.2 s vs. 4.8 ± 0.2 s at time 2, P = 0.3). Stimulation with liquids had resulted in significantly longer response onset to LESR than air at both time 1 and time 2 (P < 0.0001 and P = 0.004, respectively; Fig. 5A). Maturation effect was significant for liquids only (P = 0.05). The response latency to peristaltic reflexes (EDR or SP) was significantly longer for liquid vs. air at both time (P = 0.05 and P < 0.0001, respectively; Fig. 5B). Time effect was not significant for any air or liquids (P = 0.5 and P = 0.06, respectively).

Testing the effects of graded stimulus volumes vs. recruitment frequency of LESR across maturation. Effect of infusion medium (air or liquids) and magnitude of stimulus volumes (graded doses, 0.1 ml to 2 ml) on the recruitment frequency of LESR at time 1 and time 2 were tested. Again, water and apple juice were combined as the liquid category since no difference was noted between these two media for the LESR frequency (53 vs. 47% at time 1, P = 0.3; 58 vs. 55% at time 2, water vs. apple juice, P = 0.6). A significant relationship was noted between the increments in the stimulus volumes vs. the frequency occurrence of LESR reflex (Table 3; Fig. 6). However, no significant dose (graded volume)-response relationships were noted with regard to the duration of LESR, or the duration of LES nadir, or the magnitude of LESR (all, P = NS).

A significantly positive correlation was noted between infusion volume and duration of infusion administration at both time 1 (r = 0.36, P < 0.0001) and time 2 (r = 0.38, P < 0.0001). Therefore, as a subaim, to test whether the average flow of infusion (infusion volume divided by duration of administration) had indeed an effect on the recruitment of LESR reflex, we analyzed the relationships of average flow rate vs. reflex response characteristics. The average flow rate (ml/s) of air infusions at time 1 and time 2 were 2.3 ± 0.1 and

Table 3. Graded stimulus volume-response relationship on LESR frequency across maturation

<table>
<thead>
<tr>
<th>% LESR</th>
<th>Time 1</th>
<th>Time 2</th>
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</thead>
<tbody>
<tr>
<td>Media</td>
<td>Odds Ratio (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>Air</td>
<td>1.3 (1.1–1.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>Liquid</td>
<td>1.6 (0.9–2.8)</td>
<td>0.1</td>
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To characterize the relationship between the independent variables (time 1 and time 2, the 2 media, and graded volumes) and the dependent variable (binary outcome of the LESR), multiple logistic regression and generalized estimating equation (GEE) methods were applied. Significant values denote a positive correlation between the volume increments and the frequency of LESR. For example, with a unit increase in dose volume of air, the occurrence of LESR at time 1 was 1.3 times. 95% CI, 95% confidence interval.
2.7 ± 0.1 respectively, and the flow rate of liquid infusions at time 1 and time 2 were 0.3 ± 0.01 and 0.4 ± 0.1, respectively. Significant differences (P < 0.0001 air vs. liquid) were noted in the magnitude of flow at both periods. A significant relationship was found between the increments in the flow rates vs. the frequency occurrence of LESR reflex (Table 4). However, no significant differences were noted with flow rate-response relationships with regard to the duration of LES, or the duration of LES nadir, or the magnitude of LESR (all, P = NS).

DISCUSSION

In this study, we define and compare the sensory-motor characteristics of LESR reflex in human premature infants during maturation. Specifically, we analyzed the changes in LES kinetics 1) during swallow-induced PP and 2) upon esophageal provocation, using novel esophageal manometry and provocation methods. The significant findings from this study (summarized in Fig. 7) are 1) resting LES pressure increases with maturation; 2) an increase in magnitude of LES pressure change is noted during LESR with spontaneous PP with maturation; 3) LESR is noted upon esophageal provocation, regardless of whether there is a peristaltic reflex or not, and the frequency of LESR was significantly greater when there is a peristaltic reflex (vs. none) at both stages of maturation; 4) with esophageal provocation, mechanistic differences in characteristics of LESR are noted when there is EDR or SP; 5) discrimination of stimuli (air vs. liquids) was noted with respect to response latency to evoke LESR or peristaltic reflex; and 6) presence of graded stimulus volume-LESR response relationship is noted with air and liquids. In contrast, similarities at both stages of maturation were noted for 1) comparable threshold volumes for evoking LESR or peristaltic reflexes, 2) frequency occurrence of LESR concomitant with SP or EDR, and 3) specific characteristics of LESR during spontaneous PP. 4) Similarities were also noted between water and apple juice for the sensory thresholds, frequency recruitment, and LESR kinetics at both maturational stages. To our knowledge this is the first study that examined the effects of maturation and esophageal provocation on the sensory-motor characteristics of LESR kinetics and peristaltic reflexes (PP, SP, and EDR) in human premature neonates.

The LES provides a tonic barrier at the gastroesophageal junction and facilitates esophageal emptying by relaxation. LES also relaxes as in transient LESR and contributes to GER. During either of these relaxation functions, bolus in the esophagus can be present such as during swallowing or during GER events, and the mechanosensitive characteristics of the bolus can be variable. Regardless of the bolus sensory properties (volume, flow, solid, liquid, gas, osmolarity, acid or nonacid), it is important to prevent the bolus volume from going upstream and facilitate clearance downstream (45). From our study, in infants born as young as 28.5 wk mean gestation, the LESR reflex is evident in healthy premature infants by 34.7 wk and undergoes further maturation by 39.1 wk mean PMA. Indeed, in this study, the frequency occurrence and relaxation characteristics of LESR differ between the maturational stages and also between the mechanisms of peristaltic reflexes. These

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Table 4. Graded stimulus flow rate-response relationship on LESR frequency across maturation

<table>
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<tr>
<th>% LESR</th>
<th>Time 1</th>
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<tr>
<td>Medium</td>
<td>Odds Ratio (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>Air</td>
<td>1.4 (1.1–1.8)</td>
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<td>Liquid</td>
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To characterize the relationship between the independent variables (time 1 and time 2, the 2 media, and graded flow rate) and the dependent variable (binary outcome of the LESR), multiple logistic regression and GEE methods were applied. Significant values denote a positive correlation between the flow rate increments and the frequency of LESR. For example, with a unit increase in flow rate of air, the occurrence of LESR at time 1 was 1.4 times.
findings suggest that LESR, a function that occurs due to modulation of excitatory and inhibitory control of LES tone, is modified during maturation in human premature infants. Indeed, the feeding capabilities of these infants also support their level of maturation; at time 1 (34.7 wk) they were predominantly fed by nasogastric tube and by time 2 (39.1 wk) they were predominantly fed via oral route. Thus brain stem maturation of oral feeding and swallowing capabilities are evident with advanced maturation. During this period, the modulation and regulation of LESR kinetics also advance.

The relationship between LESR and spontaneous swallow (solitary, multiple) as well as transient LESR was discussed before (35). However, neither the effects of esophageal provocation nor the effects of swallows on LESR kinetics were assessed. The increased frequency of swallowing either as solitary swallows or multiple swallows seen in premature neonates may be one of the mechanisms needed to facilitate complete LESR and steer esophageal clearance away from the aerodigestive tract. Omari et al. (36) discussed the relationship between LESR and swallow (solitary, multiple); however, that study did not assess the effects of esophageal provocation, or the relation of swallows on LESR kinetics. These observations are evident in the form of deglutition reflex seen upon esophageal provocation, during which LESR also happens to facilitate esophageal clearance. The significant presence of deglutition and SP reflexes during esophageal provocation are complementary, and that hypervigilant state may be protective against more proximal retrograde entry of bolus. Such phenomena are also seen as in esophagoglottal closure reflex responses in neonates (16, 18).

The stimulus volume-dependent increment in the frequency recruitment of LESR reflex supports the volume sensitivity of the neonatal esophagus to facilitate esophageal clearance of greater volumes or flow (Tables 3 and 4). This process happens by the mechanisms of downstream inhibitory neurotransmission (LESR) and upstream contractile mechanisms (deglutition reflex or secondary peristalsis). This is in accordance to the Starling’s law of intestine (1). At least in healthy asymptomatic premature infants as in this study, these reflex mechanisms were present, and quantifiable measurements of sensory-motor characteristics of these reflexes are possible. Indeed, with more proximal onset of peristalsis as in deglutition response, the onset to the LESR takes longer (compared with SP; Fig. 4, A–C). This finding may be explained by more central nature of the deglutition reflex as opposed to the peripheral nature of the SP (4, 42). For example, PP is a centrally mediated response with peristalsis originating and propagating from striated muscles in the pharynx, UES, and proximal esophagus to the distal smooth muscle segment of esophagus and LES. On the other hand, the SP may be a peripherally mediated response to direct mechanodistention of the esophageal smooth muscle and activation of local intramural nerves, which interact with the vagus, consequently inducing LESR (3, 4, 8, 10, 38). However, with maturation the duration of LESR nadir decreases, implicating faster clearance at the LES and quicker restoration of LES resting pressure. Indeed, swallowing-associated LESR has also been implicated as one of the causative mechanisms for GER; neonates swallow frequently, and that shorter duration of LESR nadir and faster restoration of LES resting pressure may be protective against GER. However, in some instances true LESR nadir pressure was negative with reference to intragastric pressure, and similar findings were reported before (35) and can be explained as follows: 1) In neonates, since the LES is mainly intrathoracic (intra-abdominal part of the LES develops during infancy) and the intrathoracic part of the esophageo-LES motility is influenced by respiration and intrapleural pressure changes, it is possible that the LESP measurement becomes more negative with relaxation during inspiration (7, 12, 23, 31–33). 2) Another possibility is that during esophageal stimulation-induced longitudinal muscle contraction (31) the sphincter may be pulled oral and may squeeze water out of the sleeve, therefore producing more negative pressure. In this study we are interested in the LES pressure decline (as the marker for LESR reflex) and therefore reported it as such.

Similarly, comparisons were made between spontaneously occurring PP (dry swallows) and midesophageal infusion-induced EDR. Upon activation of EDR at both stages of maturation (compared with spontaneous PP), whereas resting LESP remains similar, we noted an increase in LESR response time, LESR duration, and LESR nadir duration (Table 2). Indeed, with dry swallow initiated PP, the origin of sequence begins with the pharyngeal waveform. In contrast with EDR, the origin of the sequence begins with the onset of midesophageal provocation. In the latter scenario, we speculate that it takes longer time for the esophageal afferents to activate the EDR and LESR reflexes (as opposed to dry swallow-initiated LESR). In the case of dry swallow-initiated PP, there is a sequential anterograde peristalsis. However, in the case of EDR, sequential anterograde peristalsis is occurring as a result of esophageal provocation.

Data from adult human and animal models suggest that LES tone measured as pressure is modulated by a balance between excitatory and inhibitory neurotransmitter activity (3, 8, 10, 38). The inhibitory tone is modulated by vagal neurons via nitric oxide or VIP (26). Increase in cholinergic tone augments LES contractile pressure, and increase in inhibitory tone facilitates relaxation. This is largely controlled by the vagovagal reflexes (31). Thus, as noted in our study, it is possible that the recruitment of inhibitory pathways may be dependent on increased graded stimulus volumes (4).

In the present study, although the threshold volumes were similar (Table 5), the neonatal esophagus clearly discriminated between the mechanosensitive nature of the physical characteristics of the stimulus in evoking LESR, in that air resulted in a faster inhibition and therefore faster onset to LESR (vs. liquids) (Fig. 5A). Indeed, with maturation liquids also yielded a faster onset to LESR (Fig. 5A). Similarly, liquids also took a longer time to evoke peristaltic reflexes (Fig. 5B) at both stages

**Table 5. Threshold volume of infusion media vs. specific reflexes**

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>Water</th>
<th>Apple Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time 1</strong></td>
<td>PR</td>
<td>0.5 ± 0.5</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LESP</td>
<td>0.4 ± 0.5</td>
<td>0.2 ± 0.2*</td>
</tr>
<tr>
<td><strong>Time 2</strong></td>
<td>PR</td>
<td>0.5 ± 0.4</td>
<td>0.6 ± 0.5†</td>
</tr>
<tr>
<td></td>
<td>LESP</td>
<td>0.5 ± 0.4</td>
<td>0.5 ± 0.5†</td>
</tr>
</tbody>
</table>

Values are means ± SD (in ml). *P = 0.04 LESR, water vs. apple juice, time 1; †P = 0.04 water PP vs. LESR, time 2.
of maturation. These findings may be due to several reasons: 1) although the volumes of stimulus mode were identical, the average speed of infusion flow was about eightfold faster with air, and air may invoke more mechanosensitivity resulting in a hypervigilant state to protect proximal aerodigestive tract; 2) liquids may cause slow distention locally compared rapid spread of air; or 3) differences between air vs. water stimulation are more related to the viscosity of the two media. Air rapidly propagates through the lumen and is more likely to propagate proximal and distal to the site of infusion. Liquid (water) takes longer to dissipate and likely remains localized at the site of infusion until peristalsis propogates the bolus distally. Furthermore, the similarities in the onset time to peristaltic reflex vs. onset time to LESR suggest that proximal excitatory (cholinergic) input and distal inhibitory (nitricergic or VIP-ergic) input are occurring simultaneously to facilitate clearance and airway protection.

This study has important clinical implications in the understanding of developmental physiology or pathophysiology of esophageal motility in human neonates, as described below: 1) Evaluation of LES functions during peristalsis may define the regulation of cholinergic and nitricergic tone at the level of LES. For example, two important LES dysfunctions underlie in inadequate LSR as in dysphagia and achalasia or more frequent LSRs as in GERD. Investigating the sensory-motor aspects of esophageal and LSR reflexes may enhance the understanding of vagal pathobiology in neonatal dysphagia, feeding problems, aspiration syndromes, airway disease, chronic lung disease, GERD or achalasia. 2) The data analytical methods described in this study may be helpful in the evaluation of LES contractile and relaxation functions during pharmacological manipulation of LES or before and after surgeries involving manipulation of gastroesophageal junction. Neonates and young infants are sometimes treated with pharmacological agents such as caffeine or theophylline (for respiratory stimulation), metoclopramide (for antiemetic and prokinetics activity), baclofen (to suppress transient LSR), acid-suppressive medications (to decrease gastric acidity), or sildenafil (for pulmonary hypertension). Indeed, all these agents influence the function of the esophagus or the LES, either directly by their effects on the GABA-ergic, cholinergic, dopaminergic or nitricergic signaling mechanisms, or indirectly by altering the physicochemical nature of the gastric contents [2, 24, 37, 39, 41, 44, 46]. In fact, there are undesired effects of these medications that can be detrimental in conditions they are not intended. For example, caffeine, theophylline, and sildenafil have been associated with decreasing LES tone [25, 27, 28, 40, 48]. Thus these medications can potentially relax the LES more (favorable in dysphagia due to inadequate relaxation of LES), or potentially contribute to more GER events. On a different note, metoclopramide has been implicated with making oromotor coordination and dysphagia worse owing to its extrapyramidal effects. Indeed, developing infants have physiological maturational delays in the functions of aerodigestive tract and LES kinetics. This finding is supported by the fact that at time 1 study infants were predominantly gavage fed and were orally fed by time 2. As swallowing functions are developing during this period, usage of medications can be detrimental to the neuromotor functions of the developmental aerodigestive tract, unless benefits outweigh the risks. Further studies are needed to evaluate the longitudinal development of the enteric neuromotor interactions during maturation in neonatal diseases of the aerodigestive tract.

In conclusion, we analyzed the changes in LES kinetics 1) during swallow-induced PP and 2) upon esophageal provocation, using novel esophageal manometry and provocation methods. We defined the development of temporal changes in LES kinetics during basal or adaptive esophageal peristalsis in premature human neonates across maturation. We conclude that sensory-motor characteristics of LSR depend on the mechanosensitive properties of the stimulus (media, volume, flow) as well as the postnatal maturation and that postnatal maturation may modulate an increased recruitment of inhibitory pathways that favor LSR. To our knowledge this is the first study that examined the effects of maturation and esophageal provocation on the sensory-motor characteristics of LESR kinetics and peristaltic reflexes in human premature neonates. This study has important clinical implications in the study of developmental feeding disorders in infants.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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