Effects of caffeine and/or nasal CPAP treatment on laryngeal chemoreflexes in preterm lambs

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Boudaa N, Samson N, Carrière V, Germim PS, Pasquier JC, Bairam A, Praud JP. Effects of caffeine and/or nasal CPAP treatment on laryngeal chemoreflexes in preterm lambs. J Appl Physiol 114: 637–646, 2013. First published January 10, 2013; doi:10.1152/japplphysiol.00599.2012.—Current knowledge suggests that laryngeal chemoreflexes (LCR) are involved in the occurrence of certain neonatal apneas/bradycardias, especially in the preterm newborn. While caffeine and/or nasal continuous positive airway pressure (nCPAP) are the most frequent options used for treating apneas in preterm newborns, their effects on LCR-related apneas/bradycardias are virtually unknown. The aim of the present study was to test the hypothesis that caffeine and/or nCPAP decreases LCR-related cardiorespiratory inhibition in a preterm ovine model. Seven preterm lambs were born vaginally on gestational day 133 (normal gestation: 147 days) after intramuscular injections of betamethasone and mifepristone. Five days after birth, a chronic surgical instrumentation was performed to record states of alertness, electrocardiogram, systemic arterial pressure, and electromyographic activity of a laryngeal constrictor muscle, as well as to insert a transtracheal supraglottal catheter. LCR were induced in quiet sleep under four conditions: 1) control (without caffeine or nCPAP); 2) nCPAP (5 cmH2O, without caffeine); 3) caffeine (10 mg/kg infused intravenously for 30 min, without nCPAP); and 4) nCPAP + caffeine. Our results showed that nCPAP consistently blunted LCR-related cardiorespiratory inhibition vs. control condition, contrary to caffeine whose overall effect was nonsignificant. In addition, nCPAP condition was characterized by a more consistent and rapid arousal after HCl injection. No significant differences were observed between all tested conditions with regard to swallowing and cough. It is concluded that nCPAP should be further assessed for its usefulness in treating neonatal apneas linked to LCR.

LARYNGEAL CHEMOREFLEXES (LCR) are a group of reflexes triggered by the contact between a liquid, such as milk or gastric juice, and the laryngeal mucosa and are primarily aimed at preventing tracheal aspiration (33, 46). In a mature mammal, including in the healthy full-term newborn, LCR include cough, deglutition, and arousal (43). However, in the immature newborn mammal, such as the preterm lamb, LCR rather encompass significant and, at times, life-threatening apneas, bradycardias, and desaturations (44), for which treatment is currently unknown. Relevance for the study of LCR stems from their likely involvement in neonatal pathologies linked to abnormalities of cardiorespiratory control, such as apneas of prematurity (AOP) (33, 46).

AOP represent a very frequent and worrisome pathology, which affects 50% of all newborns before 37 wk of gestation and virtually all extremely premature infants born before 28 wk of gestation (47). Although a number of AOP can be prevented by either avoiding triggers or prone positioning or can be halted by gentle cutaneous stimulations, further treatment is often needed (32). Caffeine and nasal continuous positive airway pressure (nCPAP) undoubtedly represent the mainstay of treatment for severe AOP. However, additional intervention in the form of nasal or even endotracheal intermittent positive pressure ventilation is sometimes necessary (50). AOP have a complex physiopathology, mainly related to immaturity of respiratory control (27). While immature respiratory reflexes such as LCR are involved in some AOP in response to triggers, such as laryngeal penetration of upper airway secretions or gastric refluxate, or during milk or vitamin ingestion (33, 46), the effects of caffeine and/or nCPAP on LCR-related cardiorespiratory events are virtually unknown. Very preliminary results on the effect of caffeine administered in a noncontrolled manner in three preterm lambs suggested that caffeine prevents severe LCR-related cardiorespiratory events (44). The aim of the present study performed in our unique ovine preterm model was hence to test the hypothesis that caffeine and/or nCPAP can prevent LCR-related cardiorespiratory events.

MATERIAL AND METHODS

Animals

Experiments were performed in seven preterm lambs with a postconceptional age of 133 days (normal gestation: 147 days) and a mean birth weight of 3.11 kg (SD 0.61) (range: 2.14–4.24 kg). The protocol of the study was approved by the Ethics Committee for Animal Care and Experimentation of the Université de Sherbrooke.

Preterm lamb model. Seven preterm lambs were born vaginally on gestational day 133. An ultrasound to confirm pregnancy, as well as the number of lambs and their viability was performed a few days before induction of premature labor in all ewes. To induce premature labor, ewes first received two intramuscular injections of 12 mg betamethasone on gestational days 131 and 132 to stimulate lung surfactant maturation (25). Second, on gestational day 132, an intramuscular injection of mifepristone (RU 486, a progesterone receptor antagonist, 8 mg/kg) was administered to all ewes (16). Thereafter, ewe behavior was carefully monitored by video surveillance, followed by one or more vaginal examinations until lambing. Lambing occurred, on average, 30 h (SD 3) (range: 26–36 h) after mifepristone injection. Delivery was only assisted in case of dystocia to prevent fetal neurological distress. During the first 48 h following birth, vital signs, including temperature, Heart rate (HR) and respiratory rate (RR), oxygen saturation, blood glucose level, and weight were meticulously monitored. When needed, lambs were placed under a
heated lamp and received reconstituted ewe’s colostrum or milk by bottle or feeding tube to maintain normal vital signs. The presence of an experimenter 24 h/day during the first 48 h was deemed necessary to perform these vital tasks, as well as for assisting the preterm lamb in its attempts at successful interactions with its mother.

**Chronic Instrumentation**

Chronic instrumentation was performed on the 5th day of life under general anesthesia (2% isoflurane, 30% N2O, balance O2), as recently described (8). Briefly, anesthesia was preceded by an intramuscular injection of ketamine (5 mg/kg), atropine sulfate (0.1 mg/kg), and morphine (0.016 mg/kg) and an intravenous bolus (10 ml/kg) of Ringer lactate solution. One dose of ketoprofen (3 mg/kg) was also injected intramuscularly for analgesia and repeated if needed the next day. Antibiotics (5 mg/kg gentamicin and 50 mg ampicillin, twice a day) were administered intramuscularly before surgery and daily thereafter.

As previously described (8), two right-angled needle-electrodes (E7-12, Grass Technologies, West Warwick, RI) were inserted into the parietal cortex directly through the skull for electrocorticogram recordings, while a third needle-electrode (F-E2M, Grass Technologies) was inserted under the scalp as a ground. Eye movements (electrooculogram) were recorded with two custom-built silver electrodes inserted subcutaneously next to the right eye socket. An electrocardiogram was recorded with two needle-electrodes (F-E2M, Grass Technologies) inserted under the peristeum of the fifth rib, on both sides of the thorax, and directly glued on the ribs. For electrical activity (electromyogram (EMG)) recording, custom-designed bipolar electrodes, built from right-angled gold connectors (Sullins Connector Solution, Digi-Key, Thief River Falls, MN), were inserted into both thyroarytenoid muscles (TA; a glottal constrictor) through the lateral aspect of the thyroid cartilage. Both electrodes were then glued on the external surface of the thyroid cartilage. A catheter was also introduced into the right carotid artery to monitor arterial blood pressure and blood gases. In addition, a supraglottal catheter was inserted transcutaneously to allow injection of liquids into the laryngeal vestibule (14). Finally, a plastic tubing (internal diameter: 1 mm) was subcutaneously tunneled to the lamb’s back to connect the external part of the supraglottal catheter to our custom-built, radio telemetry-driven injector. Leads from each electrode and catheter were subcutaneously tunneled to a common exit on the lamb’s back. Postoperative care included antibiotics and daily flushing of the arterial catheter to prevent agitation and ambulation when it was connected to nCPAP. Antibiotics (5 mg/kg gentamicin and 50 mg ampicillin, twice a day) were administered intramuscularly before surgery and daily thereafter.

**Experimental Equipment**

**Ventilatory Equipment.** Nasal CPAP was applied using the Infant Flow nCPAP system (Cardinal Health, Dublin, OH) with heated (37°C), humidified air. A custom-built nasal mask, filled with dental paste (Examix, Servident, St-Laurent, Quebec, CAN), was installed on the lamb’s muzzle to deliver nCPAP while enabling the lamb to open its mouth at will (4).

**Recording equipment.** Lamb instrumentation was completed immediately before recordings. Elastic bands for respiratory inductance plethysmography (Respirtrace, NIMS, Miami Beach, FL) were installed around the thorax and abdomen to monitor respiratory movements and to assess lung volume variations qualitatively. Nasal CPAP values were continuously monitored from the nasal mask (RX104A pressure transducer, Biopac Systems, Goleta, CA). A reflectance pulse oximeter sensor (Masimo Radical, Irvine, CA) was attached at the base of the tail for continuous monitoring of oxygen hemoglobin saturation (SpO2), as well as pulse wave. Arterial blood gases were also measured in four lambs (IL 1306, Instrumentation Laboratory, Lexington, MA) and corrected for rectal temperature of the lamb (1). Finally, the subcutaneous plastic tubing connected to the supraglottal catheter was attached to a custom-built, radio telemetry-driven injector, where liquids to be injected were warmed and maintained at the lamb’s body temperature. All recordings were performed using our custom-designed radio telemetry system (38) to obtain prolonged recordings in nonsedated lambs under the least possible restraining conditions. The raw EMG signals were rectified, integrated, and moving time averaged (100 ms). All parameters were continuously recorded on a PC using AcqKnowledge software (version 4.1; Biopac Systems, Goleta, CA).

**Design of the Study**

All preterm lambs were housed and cared for with their mother in our animal quarters until the experimental day. Polysomnographic recordings were performed on postnatal days 7 and 8 (n = 5) or postnatal days 8 and 9 (n = 2) and included simultaneous recording of respiratory movements, states of alertness, TA EMG, electrocardiogram, systemic arterial blood pressure, and SpO2. Accordingly, lambs were placed in a Plexiglas chamber (1.2 × 1.2 × 1 m, corresponding to guidelines from the Canadian Council for Animal Care) and comfortably positioned on a mattress with minimal contention (under the constant presence of an experimenter near the lamb to prevent agitation and ambulation when it was connected to nCPAP). Arterial blood gases and pH were measured in four lambs before each of the four experimental conditions to eliminate the presence of baseline hypoxia, hypercapnia, and/or a low pH, which have been shown to affect LCR (18, 19). LCR were induced by injection of 0.5 ml of hydrochloric acid (HCl, pH = 2, diluted in saline or ewe milk via the supraglottal catheter during quiet sleep (QS). Both solutions were injected twice in random order during four experimental conditions: 1) control (without caffeine or nCPAP); 2) nCPAP (5 cmH2O, without caffeine; 3) caffeine (10 mg/kg infused intravenously for 30 min, without nCPAP); and 4) nCPAP + caffeine. Due to the long half-life of caffeine in the newborn (2), the two experimental conditions involving caffeine had to be performed on the second recording day. On each day, the order of the two experimental conditions (with and without nCPAP) was randomized. The supraglottal catheter was systematically flushed with 1 ml of saline between each injection of test solutions (dead space of the catheter 0.5 ml), and each lamb was given at least 15 min of recovery time between two injections. Events such as agitation, cough, arousal, and/or full awakening were noted by an observer present throughout the recordings. Finally, plasma caffeine level was systematically measured at the end of the second recording day.

**Data Analysis**

**States of alertness.** Standard electrophysiological and behavioral criteria were used to define QS (35). Cortical arousal from QS was defined by the association of a change in electrocorticogram (decrease in amplitude + increase in frequency) for 3 s or more, with at least two of the following modifications: a 10% increase in heart rate (HR), or change in respiratory rate (RR) or movement (21). Full awakening was defined when the lamb was still awake after 1 min (17).

| Table 1. Mean baseline vital signs of the seven preterm lambs during room air breathing from birth to the last day of experimentation |
|-----------------------------------------------|---------------|
| VITAL SIGNS                                      | MEAN (SD)     |
| Heart rate, beats/min                          | 225 (7)       |
| Respiratory rate, breaths/min                  | 69 (5)        |
| SpO2, %                                       | 97 (0.5)      |
| Body temperature, °C                           | 39.9 (0.1)    |
| Glucose, mmol/l                               | 5.7 (2.4)     |
| Mean weight gain/day, g                        | 227 (72)      |
| SpO2, hemoglobin O2 saturation                |               |
LCR. Analysis of the LCR was performed as described previously within the first minute following each laryngeal injection (43). First, cardiorespiratory responses were assessed as follows. The percentage of decrease in HR [%Dec HR = (HR_{BL} - HR_{min}) / HR_{BL} \times 100] was calculated, with HR_{BL} representing the baseline HR value averaged over 30 s before challenge, and HR_{min} representing the minimal HR value observed during the first minute after the challenge. The %Dec SpO2 was calculated in the same manner. Any presence of bradycardia (defined by a %Dec HR > 33%) was noted, as well as the number of bradycardias and total summed duration of bradycardias. The presence of apneas (defined as at least two missed breaths relative to baseline breathing) was also noted, including the number of apneas and their total summed duration. Moreover, any presence of desaturation was noted, as defined by a decrease in SpO2 of at least 4% or <90%, as well as the minimal SpO2 (SpO2_{min}). The percentage of increase in mean arterial pressure (%Inc MAP) and the maximum mean arterial pressure (MAP) value (MAP_{max}) were also noted. In addition to cardiorespiratory responses, the following responses, usually considered to be protective against tracheal aspiration (33), were assessed. First, the number of swallows (defined as a brisk, high-amplitude, and short-duration TA EMG burst) (34) occurring within the first minute after laryngeal stimulation was tallied. Second, the number of coughs (including laryngeal expiratory reflexes, which could not be discerned from coughs in our study) was also inferred from visual observations, as well as analysis of TA EMG and respiratory inductance plethysmograph signals. Third, the presence of arousal or full awakening was noted. For all stimulations, the following parameters were measured: the time duration between the moment of stimulation (defined for the purpose of the present study as the moment of the first swallow observed following injection onset) and the moment of HR_{min}, the onset of the first apnea and first cough, SpO2_{min}, MAP_{max}, and arousal occurrence (respectively, HR_{min}, apnea, cough, SpO2_{min}, MAP_{max}, and arousal occurrence times). Finally, the time needed to recover from LCR induced by HCl (= LCR recovery time, defined as the first consecutive 10 s following LCR without any

Table 2. Influence of nCPAP and/or caffeine treatment on baseline cardiorespiratory values during quiet sleep in seven preterm lambs

<table>
<thead>
<tr>
<th>Baseline Values</th>
<th>Control</th>
<th>nCPAP</th>
<th>Caffeine</th>
<th>nCPAP + Caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>207 (26)</td>
<td>221 (20)</td>
<td>211 (29)</td>
<td>211 (23)</td>
</tr>
<tr>
<td>Respiratory rate,</td>
<td>60 (19)</td>
<td>52 (18)^\text{ab}</td>
<td>61 (17)</td>
<td>55 (17)^\text{d}</td>
</tr>
<tr>
<td>breaths/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean arterial</td>
<td>73 (8)</td>
<td>84 (14)^\text{ad}</td>
<td>73 (9)</td>
<td>78 (11)^\text{c}</td>
</tr>
<tr>
<td>pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SpO2, %</td>
<td>94 (2)</td>
<td>96 (1)^\text{c}</td>
<td>94 (3)</td>
<td>96 (1)^\text{d}</td>
</tr>
</tbody>
</table>

Values are means (SD). nCPAP, nasal continuous positive airway pressure. \(P < 0.05\) vs. \(\text{control}\) and \(\text{caffeine}\). \(P < 0.1\) vs. \(\text{control}\) and \(\text{caffeine}\). All other \(P\) values are >0.1.

LCR. Analysis of the LCR was performed as described previously within the first minute following each laryngeal injection (43). First, cardiorespiratory responses were assessed as follows. The percentage of decrease in HR [%Dec HR = (HR_{BL} - HR_{min}) / HR_{BL} \times 100] was calculated, with HR_{BL} representing the baseline HR value averaged over 30 s before challenge, and HR_{min} representing the minimal HR value observed during the first minute after the challenge. The %Dec SpO2 was calculated in the same manner. Any presence of bradycardia (defined by a %Dec HR > 33%) was noted, as well as the number of bradycardias and total summed duration of bradycardias. The presence of apneas (defined as at least two missed breaths relative to baseline breathing) was also noted, including the number of apneas and their total summed duration. Moreover, any presence of desaturation was noted, as defined by a decrease in SpO2 of at least 4% or <90%, as well as the minimal SpO2 (SpO2_{min}). The percentage of increase in mean arterial pressure (%Inc MAP) and the maximum mean arterial pressure (MAP) value (MAP_{max}) were also noted. In addition to cardiorespiratory responses, the following responses, usually considered to be protective against tracheal aspiration (33), were assessed. First, the number of swallows (defined as a brisk, high-amplitude, and short-duration TA EMG burst) (34) occurring within the first minute after laryngeal stimulation was tallied. Second, the number of coughs (including laryngeal expiratory reflexes, which could not be discerned from coughs in our study) was also inferred from visual observations, as well as analysis of TA EMG and respiratory inductance plethysmograph signals. Third, the presence of arousal or full awakening was noted. For all stimulations, the following parameters were measured: the time duration between the moment of stimulation (defined for the purpose of the present study as the moment of the first swallow observed following injection onset) and the moment of HR_{min}, the onset of the first apnea and first cough, SpO2_{min}, MAP_{max}, and arousal occurrence (respectively, HR_{min}, apnea, cough, SpO2_{min}, MAP_{max}, and arousal occurrence times). Finally, the time needed to recover from LCR induced by HCl (= LCR recovery time, defined as the first consecutive 10 s following LCR without any

![Fig. 1. Potentially life-threatening cardiorespiratory reflexes triggered in one preterm lamb following instillation of 0.5 ml HCl onto the laryngeal mucosa during quiet sleep in control condition [no nasal continuous positive airway pressure (nCPAP)/no caffeine]. From top to bottom: ECoG, electrocorticogram; EOG, electrooculogram; TA, electrical activity of the thyroarytenoid muscle (laryngeal constrictor); Sum, sum signal of the thoraco-abdominal movements measured by respiratory inductance plethysmograph, inspiration upward; ECG, electrocardiogram; HR, heart rate [mean and maximum (max); beats/min]; SpO2, hemoglobin O2 saturation; AP, arterial pressure [mean and maximum (max); mmHg]. Note the prolonged, potentially life-threatening responses with awakening, repetitive apneas followed by periodic breathing, severe bradycardia down to 71 beats/min, and severe SpO2 down to 69%.](http://japp.physiology.org/)
apnea and bradycardia, and with less than three swallows) was assessed for each stimulation.

Statistical Analysis

For descriptive statistics, results were first averaged in each lamb and then averaged for the seven lambs as a whole. Quantitative data are expressed as mean (SD), whereas qualitative binary data (arousal and awakening, yes/no) are expressed as relative frequency.

Statistical analyses for comparing the four treatment conditions were performed on raw data for all variables. Given that a solution effect was not part of the research query, the effect of HCl was analyzed separately from that of ewe milk. Normality was first tested using the Shapiro-Wilk test. Quantitative continuous variables with a normal distribution (cardiorespiratory components of LCR, %Inc MAP, and MAP max occurrence time) were analyzed through a general linear model one-way ANOVA for repeated measures using PROC MIXED of the SAS software (version 9.1, Cary, NC), with treatment (nCPAP and/or caffeine). Quantitative continuous variables not normally distributed (baseline cardiorespiratory variables and other arousal occurrence times) were analyzed by Friedman’s test followed by Wilcoxon’s signed rank test, using the GraphPad Prism software (version 5.0, La Jolla, CA). Binary data (arousal and awakening) were analyzed with a logistic regression model using the GENMOD procedure of the SAS software for repeated measures and adjusted by the Sidak correction, while count data (number of coughs, swallows) were analyzed by Friedman’s test followed by Wilcoxon’s signed rank test. Finally, LCR recovery time was analyzed through generalized estimating equations for multinomial responses (1 to 5 categories of 15 s), calculated with the Wald $\chi^2$ test, to account for repeated measures.

Differences were deemed statistically significant if $P < 0.05$. In addition, given the relatively small number of studied lambs (related both to the complexity of the preterm ovine model and ethical constraints), it was decided to give full consideration to the presence of a significant trend, defined as $P < 0.1$.

RESULTS

Premature Lamb Model

Seven pregnant ewes were included in the study. Lambing occurred on average 30 h (SD 3) (range 26–36 h) after mifepristone injection, on gestational day 133. The total number of births was 12, including 6 females and 6 males, with 5 ewes delivering twins and 2 ewes delivering a single lamb. Eleven of the twelve preterm lambs survived, with two lambs from the same litter needing resuscitation at birth due to severe dystocia. One of these twins was stillborn and could not be resuscitated. One lamb was found cyanotic and bradycardic 24 h after birth, possibly following a prolonged AOP, but was successfully resuscitated. The other remaining lambs did not require oxygen at birth and presented normal vital signs from birth up to the last day of experimentation (Table 1). Lamb feeding initially began by presentation of the ewe’s teat for a maximum of 5 min at approximately every 2 h and was followed, if needed, by supplemented bottle feeding (ewe colostrum or milk). Two lambs were tube fed during the first 24 h of life due to a poor sucking reflex. All lambs were

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Fig. 2. Effect of nCPAP and/or caffeine treatment on apnea number and duration (A); bradycardia number and duration (B); and the % decrease in SpO2 (C), following instillation of 0.5 ml of milk (solid bars) and HCl (shaded bars) onto the laryngeal mucosa during quiet sleep. Values are expressed as means (SD). **$P < 0.05$. *$P < 0.1$. Apnea and bradycardia duration represents the sum of duration of all laryngeal chemoreflexes (LCR)-related apneas or bradycardias observed.
self-sufficient in feeding themselves after 24–48 h of life. A total of seven preterm lambs (one lamb from each litter) were included in the study.

Effects of nCPAP and/or Caffeine Treatment

Baseline cardiorespiratory values. Baseline cardiorespiratory values obtained in all seven lambs and in each experimental condition are detailed in Table 2. Overall, nCPAP alone or combined with caffeine decreased RR and increased MAP, as well as SpO2 (see Table 2 for details on specific interactions). In addition, baseline arterial blood gases and pH measured in four of seven lambs were found normal in all experimental conditions: arterial PO2 = 80 Torr (SD 4) (range 76–88 Torr); arterial PCO2 = 41 Torr (SD 2) (range 39–45 Torr); pH = 7.36 (0.01) (range 7.34–7.37).

LCR. A total of 111 laryngeal stimulations were performed during QS; one LCR (milk) was not induced, as one caffeine-treated lamb was unable to sleep during the recording. Plasma caffeine level in the seven lambs was 58 mg/l (SD 6) (range 35–67 mg/l) after completion of the second recording session, ~4 h after caffeine infusion.

Life-threatening cardiorespiratory events were observed in three of seven lambs following HCl injection in control, caffeine, and nCPAP/caffeine conditions. These events included repetitive, prolonged central apneas or periodic breathing epochs with bradycardias (HRmin 32 beats/min) and hemoglobin desaturation (SpO2 min = 20%). The events lasted from 95 s to 9 min before spontaneous recovery (Fig. 1). No severe cardiorespiratory event was observed under nCPAP condition in any of the lambs.

LCR-related cardiorespiratory events. Cardiorespiratory events occurring during LCR are summarized in Fig. 2 and Table 3 and are illustrated by tracings obtained in one lamb in black, hence the reflectance sensor unable to analyze blood color, and one lamb with its tail severely bitten by the ewe). Overall, nCPAP significantly blunted the LCR-related cardiorespiratory components elicited by HCl compared with control and to caffeine treatment. A beneficial effect of nCPAP treatment was also observed for apnea number and/or duration, as well as for bradycardia number and/or duration and for %Dec HR. In addition, HRmin and SpO2 values were higher during nCPAP treatment (see Table 3 for details on specific interactions). Although cardiorespiratory inhibition was less pronounced following milk injection, it was similarly blunted under nCPAP treatment compared with control and caffeine treatment (Table 3). By contrast, caffeine alone or when combined with nCPAP had no significant effect, on average, on LCR-related cardiorespiratory inhibition. Further analysis revealed that, contrary to nCPAP, the effect of caffeine was notably variable from one lamb to another for apnea number and/or duration (Fig. 4A), as well as for bradycardia number and/or duration for both HCl (Fig. 4B) and milk. Overall, the %Inc MAP was lower during nCPAP and nCPAP + caffeine treatment compared with control, independently of the solution used to induce the LCR.

Table 3. Effects of nCPAP and/or caffeine treatment on the cardiorespiratory components of LCR during quiet sleep in seven preterm lambs for HCl and ewe milk individually

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>nCPAP</th>
<th>Caffeine</th>
<th>nCPAP + Caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Dec HR</td>
<td>44 (22)</td>
<td>35 (15)</td>
<td>50 (19)</td>
<td>39 (21)</td>
</tr>
<tr>
<td>HRmin, beats/min</td>
<td>121 (53)</td>
<td>145 (34)</td>
<td>105 (51)</td>
<td>128 (43)</td>
</tr>
<tr>
<td>HRmin occurrence time, s</td>
<td>20 (14)</td>
<td>12 (10)</td>
<td>20 (13)</td>
<td>18 (11)</td>
</tr>
<tr>
<td>SpO2min, %</td>
<td>87 (5)</td>
<td>92 (5)</td>
<td>87 (4)</td>
<td>91 (7)</td>
</tr>
<tr>
<td>SpO2min occurrence time, s</td>
<td>29 (15)</td>
<td>22 (8)</td>
<td>37 (10)</td>
<td>20 (9)</td>
</tr>
<tr>
<td><strong>Ewe milk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Dec HR</td>
<td>38 (18)</td>
<td>30 (14)</td>
<td>42 (16)</td>
<td>39 (21)</td>
</tr>
<tr>
<td>HRmin, beats/min</td>
<td>126 (38)</td>
<td>152 (32)</td>
<td>122 (40)</td>
<td>132 (54)</td>
</tr>
<tr>
<td>HRmin occurrence time, s</td>
<td>11 (8)</td>
<td>8 (6)</td>
<td>17 (3)</td>
<td>13 (10)</td>
</tr>
<tr>
<td>Apnea occurrence time, s</td>
<td>4 (5)</td>
<td>5 (5)</td>
<td>7 (7)</td>
<td>10 (13)</td>
</tr>
<tr>
<td>SpO2min, %</td>
<td>87 (7)</td>
<td>94 (3)</td>
<td>89 (6)</td>
<td>92 (3)</td>
</tr>
<tr>
<td>SpO2min occurrence time, s</td>
<td>27 (15)</td>
<td>19 (7)</td>
<td>19 (5)</td>
<td>18 (2)</td>
</tr>
</tbody>
</table>

Values are means (SD). LCR, laryngeal chemoreflexes; %Dec HR, percentage of decrease in heart rate; HRmin, minimal heart rate; SpO2min, minimal SpO2. P < 0.05 vs. control and/or caffeine. P < 0.1 vs. control, caffeine, and nCPAP/caffeine. All other P values are >0.1.

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DISCUSSION

Statement of Principal Findings

The present study provides new and intriguing findings on the effects of nCPAP and/or caffeine on LCR in preterm lambs. The most striking observation is the consistent blunting effect of nCPAP on LCR-related cardiorespiratory inhibition, and the absence of a similar effect with caffeine. We believe these unique results are important information to consider when discussing therapeutic options for AOP.

Methodological Considerations

Our preterm lamb model. Over the years, many improvements have been made to our preterm lamb model, which has resulted in a substantial increase in the survival rate and health outcome of our animals (3, 30, 34, 35, 44). Previously, intensive neonatal care, which included delivery by caesarean section under epidural anesthesia, immediate endotracheal intubation at birth to deliver exogenous surfactant replacement, supplemental nasal oxygen, and tube feeding, were systematically applied, with an average survival rate of roughly 50% at day 7 of life (3, 30, 34, 35, 44). In contrast, 11 of 12 preterm lambs delivered in the present study survived (one stillborn lamb due to severe dystocia) with constant care focusing on optimal ewe-lamb interactions during the first 48 h of life, while eliminating invasive procedures. This intensive, albeit noninvasive neonatal care probably explains our high survival rate (90%), which compares favorably to the rates recently reported by others for a similar preterm ovine model (11).

Clearly, our preterm lamb model is more akin to the late preterm than to the extreme premature infant. Nevertheless, it represents a unique asset enabling new insights on anomalies linked to immature cardiorespiratory control at birth, as well as the assessment of the various treatment modalities used in premature infants presenting with cardiorespiratory events.

Assessment of caffeine effect. Given the long half-life of caffeine in the newborn (2), the two experimental conditions involving caffeine had to be performed on the second recording day. The impossibility to randomize the two recording days with and without caffeine could theoretically be viewed as a limitation of the study, for it could bring into question the presence of a systematic bias responsible for the overall enhancement of LCR-related cardiorespiratory inhibition observed with caffeine on the second recording day. This is, however, very unlikely. On the contrary, habituation of the reflex, as well as its maturation (44), would have blunted...
LCR-related cardiorespiratory inhibition on the second day. In addition, the contribution of postoperative inflammation of the larynx, if any, would obviously have been more important in the first experimental day; yet previous data suggest that laryngeal inflammation would have increased LCR-related cardiorespiratory inhibition (8), which is contrary to our observations. Finally, hypoxemia or hyperthermia, which can increase LCR-related cardiorespiratory inhibition (18, 48, 49), was absent on both experimental days. Thus we believe that systematic assessment of caffeine effect on the second experimental day does not constitute a limitation in the present study.

Effects of Caffeine and/or nCPAP Treatment on LCR

Caffeine is undoubtedly the first-line treatment for severe AOP, due to its general efficiency in reducing AOP and its relative short-term and long-term safety profile, including up to cardiorespiratory inhibition (8), which is contrary to our observations. Finally, hypoxemia or hyperthermia, which can increase LCR-related cardiorespiratory inhibition (18, 48, 49), was absent on both experimental days. Thus we believe that systematic assessment of caffeine effect on the second experimental day does not constitute a limitation in the present study.

Table 4. Effects of nCPAP and/or caffeine treatment on the protective mechanisms of LCR during quiet sleep in seven preterm lambs for HCl and ewe milk individually

<table>
<thead>
<tr>
<th></th>
<th>Control nCPAP</th>
<th>Caffeine</th>
<th>nCPAP + caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCl</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Inc MAP</td>
<td>21 (6)</td>
<td>14 (4)≤b</td>
<td>22 (8)</td>
</tr>
<tr>
<td>MAP occurrence time, s</td>
<td>18 (13)</td>
<td>11 (9)≤b,c</td>
<td>19 (8)</td>
</tr>
<tr>
<td>No. swallows</td>
<td>11 (9)</td>
<td>9 (6)</td>
<td>16 (13)</td>
</tr>
<tr>
<td>No. coughs</td>
<td>2 (2)</td>
<td>1 (2)</td>
<td>5 (5)</td>
</tr>
<tr>
<td>Cough occurrence time, s</td>
<td>12 (12)</td>
<td>10 (8)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>No. arousals</td>
<td>5/14</td>
<td>10/14</td>
<td>7/14≤c</td>
</tr>
<tr>
<td>Arousal occurrence time, s</td>
<td>8 (12)</td>
<td>2 (3)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>Efficient arousal index</td>
<td>1 (2)</td>
<td>3 (2)≤a</td>
<td>1 (1)</td>
</tr>
<tr>
<td>No. awakenings</td>
<td>5/14</td>
<td>1/14</td>
<td>5/14</td>
</tr>
<tr>
<td><strong>Ewe milk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Inc MAP</td>
<td>21 (7)</td>
<td>12 (3)≤b</td>
<td>26 (20)</td>
</tr>
<tr>
<td>MAP occurrence time, s</td>
<td>7 (3)</td>
<td>10 (10)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>No. swallows</td>
<td>10 (5)</td>
<td>9 (4)</td>
<td>11 (5)</td>
</tr>
<tr>
<td>No. coughs</td>
<td>0 (0)</td>
<td>0 (1)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>No. arousals</td>
<td>8/14</td>
<td>11/14</td>
<td>8/13</td>
</tr>
<tr>
<td>Arousal occurrence time, s</td>
<td>4 (6)</td>
<td>6 (9)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>No. awakenings</td>
<td>3/14</td>
<td>1/14</td>
<td>2/13</td>
</tr>
</tbody>
</table>

Quantitative variables are means (SD), and qualitative variables (arousal and awakening) are expressed as relative frequency. MAP, mean arterial pressure; %Inc MAP, percentage of increase in MAP; no. arousals, number of LCR with arousal/total number of LCR; no. awakenings, number of LCR with awakening/total number of LCR. "P < 0.05 vs. control, "caffeine, and "nCPAP/caffeine. P < 0.1 vs. control. All other P values are >0.1. Note that statistical analysis was not possible for arousal occurrence time because of too many missing values (= absence of arousal).

Fig. 4. Individual cardiorespiratory responses following instillation of 0.5 ml HCl on apnea duration (A) and bradycardia duration (B). Note the high variability of the effect of caffeine (left) from one lamb to another, compared with the much more consistent effect of nasal CPAP (right).

Fig. 5. Time needed to recover from LCR induced by HCl (defined as the first consecutive 10 s following LCR without any apnea, bradycardia and with less than 3 swallows). The LCR recovery time is illustrated for each treatment by intervals of 15 s. Overall, preterm lambs recovered more rapidly under nCPAP treatment.
school age (2, 28, 39, 40). Caffeine is both a powerful central nervous system stimulant and respiratory stimulant. The latter is partly related to its potent A1 and A2A adenosine receptor antagonist action and includes an increase in minute ventilation and CO2 sensitivity, as well as a decrease in hypoxic depression and periodic breathing (26). However, caffeine alone can be insufficient to treat AOP (6), and nCPAP (4 – 6 cmH2O) must often be added to caffeine for an optimal effect on AOP. The beneficial effect of nCPAP is deemed related to the maintenance of optimal lung volume and chest wall fixation, as well as to upper airways splinting, resulting in lower work of breathing and higher arterial PO2 (12, 27, 50).

Blunting of LCR-related cardiorespiratory inhibition by nCPAP. While the increase in pulmonary oxygen stores under nCPAP likely contributed to preventing the late desaturations and bradycardias following LCR-related apneas (12, 27), this effect, however, cannot explain the consistent beneficial effects of nCPAP on the early LCR-related apneas and bradycardias. On the contrary, present knowledge would rather suggest an enhancing effect of nCPAP on LCR-related cardiorespiratory inhibition. Indeed, nCPAP is known to dilate the supraglottal larynx and increase glottal opening (10, 15, 22) and may also favor a greater spreading of HCl (or milk) over the laryngeal mucosa. The above could increase both the surface and exposure time of the laryngeal mucosa to HCl (or milk) and could, in turn, enhance LCR-related cardiorespiratory inhibition. A similar conclusion could be reached from the increased possibility of tracheal aspiration of HCl (or milk) with nCPAP, due to increased glottal opening (24). In fact, explanation of the blunting effect of LCR-related cardiorespiratory inhibition may come from the following two considerations. First, regarding the sequence of events taking place during HCl-induced LCR (Fig. 6), a striking observation is that arousal with nCPAP, but not caffeine, occurred more often and more rapidly than in control condition (see Table 4, “efficient arousal index”). The consequent arousal related-noradrenergic release would, in turn, promote both cardiac and respiratory activity (5). Second, nCPAP may directly alter the function of the laryngeal chemoreceptors responsible for LCR, in such a manner that their response is blunted. For example, the transient receptor potential vanilloid 1 receptors, which are located on laryngeal C-fiber endings and involved in LCR (36, 49), have been shown to be pressure sensitive (9, 41, 45).

High variability of caffeine effects on LCR-related cardiorespiratory inhibition. Our previous preliminary results in only three preterm lambs treated with caffeine for clinically significant apneas and bradycardias 3 days before LCR studies had initially led us to hypothesize that caffeine treatment was an effective means to prevent LCR-related cardiorespiratory events (44). This appeared in agreement with earlier results obtained with aminophylline in four piglets (23). Thus our present observation that caffeine blunted the LCR-related cardiorespiratory inhibition in only two of seven preterm lambs was rather unexpected, especially in conjunction with a consistent blunting effect of nCPAP. Such observation may also seem contradictory with the amplifying effect of A2 adenosine receptor stimulation on GABA release from neurons in the nucleus tractus solitarius, which, in turn, inhibits inspiratory and expiratory activity of neurons in the ventral respiratory column (49). However, such amplifying effect of adenosine via its A2 receptors has been observed in hyperthermia, but not in normothermia (48), suggesting that it is only relevant in cir-

Fig. 6. Sequence of events observed during HCl-induced LCRs for each treatment condition. Occurrence times of minimum HR (bradycardia), \( \text{SpO}_2 \text{min} \) (desaturation), first apnea, increase in mean AP (MAP), arousal, and first cough are defined as the time durations between the moment of stimulation [defined for the purpose of the present study as the moment of the first (immediate) swallow observed following onset of HCl injection] and the onset of each event. Each segment represents the average and full range of occurrence times for a given event.
cumstances in which adenosine is abundant, such as during hypoxia, inflammation, or hyperthermia. Overall, although a satisfactory explanation for the highly variable effect of caffeine on LCR-related cardiopulmonary inhibition remains elusive, a similar variability of action is well known for AOP reduction (13, 20, 29), as well as for prevention of hypoxic episodes and bradycardias (7) in preterm infants. Unfortunately, although confirming to a certain degree the beneficial effect of nCPAP, results obtained with nCPAP + caffeine do not offer any further clue for understanding the overall absence of caffeine effect.

Protective mechanisms related to LCR. Aside from cardiorespiratory events, LCR also elicit coughing, swallowing, arousal, and awakening. These components of the LCR are generally considered to be protective mechanisms aimed at both clearing liquids from the laryngeal region and preventing tracheal aspiration. The potential importance of a rapid arousal has already been highlighted in discussing the nCPAP effect. The absence of effects of nCPAP on swallowing during LCR may appear surprising, given our previous report that non nutritive swallowing is decreased by nCPAP via reflexes originating from bronchopulmonary receptors (37). However, our recent study showing that nCPAP (up to 10 cmH2O) does not decrease nutritive swallowing (4) likely indicates that the inhibiting effect of nCPAP on swallowing does not operate in instances of urgency, when repetitive swallowing is deemed crucial by the swallowing centers to clear the offending liquid. To our knowledge, there have been no previous reports of the effect of caffeine on swallowing. Finally, the absence of differences in the number of swallows and coughs between the various experimental conditions suggests that these protective mechanisms do not explain the different effects of nCPAP and caffeine.

Clinical Implications

The present results, while obtained in lambs, suggest that nCPAP is more efficient than caffeine for treating AOP related to LCR, as observed in preterm infants with laryngeal penetration of upper airway secretions or gastric reflexes. However, given that the proportion of AOP related to LCR is currently unknown (31, 42), additional experiments in infants are clearly warranted to clarify conditions where nCPAP may be preferred over caffeine as first-line treatment of AOP.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: N.B., N.S., and P.S.G. performed experiments; N.B. and V.C. analyzed data; N.B. and N.S. prepared figures; N.B. drafted manuscript; N.S., J.-C.P., and J.-P.P. conception and design of research; N.S., A.B., and J.-P.P. interpreted results of experiments; N.S., A.B., and J.-P.P. edited and revised manuscript; J.-P.P. approved final version of manuscript.

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