EFFECTS OF MODERATE HYPERBILIRUBINEMIA ON NUTRITIVE SWALLOWING AND SWALLOWING-BREATHING COORDINATION IN PRETERM LAMBS

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ABSTRACT

Background - Hyperbilirubinemia (HB) occurs in 90% of preterm newborns. HB induces acute neurological disorders (somnolence, abnormal tone, feeding difficulties, auditory dysfunction) and alterations in respiratory control. These findings suggest brainstem neurotoxicity that could also affect swallowing centers.

Objective - To test the hypothesis that HB impairs nutritive swallowing and swallowing-breathing coordination.

Methods - Two groups of preterm lambs (born 14 days prior to term), namely control (n = 6) and HB (n = 5), were studied. At day 5 of life (D0), moderate HB (150-250 µmol/L) was induced during 17h in the HB group. Swallowing was assessed *via* recording of pharyngeal pressure and respiration by respiratory inductance plethysmography and pulse oximetry. Effect of HB on nutritive swallowing was assessed during standardized bottle-feeding. A second recording was performed 48h after recovery from HB (D3).

Results - Swallows were less frequent ($p = 0.003$) and of smaller volume ($p = 0.01$) in HB lambs while swallowing frequency was decreased ($p = 0.004$). These differences disappeared after HB normalization. Swallowing-breathing coordination was impaired in HB lambs, with a decrease in % time with NS burst-related apneas/hypopneas at D0 and D3. Simultaneously, HB lambs tended to experience more severe desaturations (<80%) during bottle-feeding. Finally, following bottle-feeding, respiratory rate was significantly lower, along with increased apnea duration in HB lambs.

Conclusions - Swallowing and swallowing-breathing coordination are altered by acute moderate HB in preterm lambs. Decreased efficiency at bottle-feeding is accompanied by continuation of breathing during swallow bursts, which may promote lung aspiration.

INTRODUCTION

Jaundice is the most common clinical sign in the neonatal period [1] and the leading cause of hospital readmission in the first week of life [2]. While kernicterus has become very rare, recent studies highlight more subtle consequences of hyperbilirubinemia (HB) [3], including increased apneas of prematurity [4], suggesting alteration of respiratory centers. Such alteration is supported by data in rat pups showing an abnormal response to hypoxia and hypercapnia following a short HB, together with bilirubin deposits close to the respiratory centers [5].

Given that respiratory and swallowing control centers are co-located in the medulla, nutritive swallowing (NS) and NS-breathing coordination may be affected during HB, thereby increasing the risk of aspiration. Interestingly, feeding difficulties are also described with HB [3].

The present study aimed at testing the hypothesis that a moderate HB alters NS and NS-breathing coordination in preterm lambs.

MATERIAL AND METHODS

The protocol of the study was approved by the Ethics Committee for Animal Care and Experimentation of the Université de Sherbrooke (protocol # 260-10).

Preterm lamb model of moderate hyperbilirubinemia

Sixteen preterm lambs were born vaginally on gestational day 133 (term = 147 days) as described previously [7]. Premature labor was induced by mifepristone (8 mg/kg), after preparation by betamethasone (12 mg x 2). A unique preterm lamb model of moderate HB was designed. At birth, lambs were randomly assigned to either the HB or control group. On postnatal day 5, HB was induced by an intravenous infusion of bilirubin (Sigma Chemical, St. Louis, MO) dissolved in 0.1 M NaOH, stabilized with albumin (molar ratio bilirubin:albumin 14:1) and diluted in Lactated Ringer's solution to a concentration of 3.75 mg/mL bilirubin and 30 mg/mL albumin (final pH 8) [5,8]. HB lambs received a continuous infusion of 20 mg/kg/h of the bilirubin solution until reaching the bilirubinemia target of 150 - 250 µmol/L. Thereafter, bilirubinemia was maintained at 150 - 250 µmol/L for 17 hours by IV boluses of 10 mg/kg of the bilirubin solution as needed. Control lambs were infused with a solution of 30 mg/ml of albumin diluted in Lactated Ringer's solution (pH 8).

In addition to bilirubinemia levels, our ovine model of moderate HB was characterized based on an ad hoc neurological examination developed from published data in adult sheep [9] and human newborns [10]. A semi-quantitative neurological score (apathy, hypotonia or episodes of opisthotonos, hearing deficit) was calculated every 2 hours during HB and twice daily thereafter.

Daily monitoring also included vital signs (heart rate, respiratory rate, arterial oxygen hemoglobin saturation, body temperature), weight and blood samples for albumin, glucose, bilirubin and arterial blood gases.

Chronic instrumentation and recording equipment

Chronic instrumentation on postnatal day 5 included: i) two needle-electrodes inserted subcutaneously on the thorax (ECG); ii) two intravenous catheters into the jugular veins; iii) a 5Fr naso-pharyngeal catheter with its tip positioned 0.5 cm above the posterior border of the soft palate and attached to a pressure sensor (RX104A, Biopac, Goleta, CA) to monitor pharyngeal pressure (= swallowing function); iv) elastic bands for respiratory inductance plethysmography (Respitrace, NIMS, Miami Beach, FL, USA) around the thorax and the abdomen (respiratory movements); and v) a pulse oximeter sensor (Masimo, Irvine, CA, USA) at the tail base [arterial oxygen hemoglobin saturation (SpO_2)]. Leads from all electrodes were connected to our custom-built radio telemetry transmitters [11] to study non-sedated lambs under the least possible restraints. All signals were continuously recorded using AcqKnowledge 4.1 software (Biopac Systems) and the lambs were filmed using a web-cam.

Design of the study

All preterm lambs were housed and cared for with their mother in our animal quarters until experimental day. Recordings were first performed on postnatal day 5, designated as experimental day 0 (D0), to assess the immediate effects of moderate HB on NS-breathing coordination. Seventy-two hours after HB induction (D3), recordings were repeated to assess the delayed effects of moderate HB.

Following a 5-min baseline recording, the lambs, comfortably positioned in a sling, were offered two bottles of 50 ml of ewe's milk heated to 38.5^{o}C at 3h intervals. The bottle was inclined at 45^{o} to mimic lambs' head position when suckling from their mother. The bottle

was offered to the lambs a maximum of three times. Recordings were continued for 10 min after feeding.

Data analysis

All signals were carefully observed and analyzed in relation with the time period (before, during, or after feeding) as well as with the experimental day (D0 or D3).

Cardiorespiratory variables

Heart and respiratory rates (respectively HR and RR), as well as SpO_2, were averaged for each time period and experimental day. For baseline recordings, the 30-sec period closest to the feeding episode during which the lambs were calm was chosen. During feeding, values were averaged during the entire period. After feeding, calculations were performed immediately during 30 seconds as well as five minutes and 10 minutes after feeding.

Moreover, analyses of cardiorespiratory events before, during and after feeding were performed as previously described for laryngeal chemoreflexes [12]. Briefly, the number of HR slowings (decrease in HR ≥ 33%) and bradycardias (HR slowing lasting > 5s) were noted, and the % of time spent in bradycardia was tabulated. The number of apneas (defined as at least 2 missed breaths relative to baseline breathing) and the % of time spent in apnea were also noted. Finally, the number of desaturations < 90% and < 80% and the % of time spent with SpO_2 < 90% and < 80% were calculated, as well as the minimum saturation value and the maximum saturation decrease.

Nutritive swallowing activity and coordination with breathing

Nutritive swallows were recognized by brief, high-amplitude increases in pharyngeal pressure. Rhythmic stability of feeding was quantified using the coefficient of variation of NS-NS interval duration (COV: standard variation of the mean NS-NS duration, divided by the mean NS-NS duration). Only NS and breaths occurring during NS runs were used for

rhythm analysis. A NS run was defined as ≥ 3NS with inter-swallow intervals of ≤ 2 seconds [13]. Assessment of feeding efficiency included volume of milk intake per minute (mL/min) or per NS (mL/NS) and NS frequency, along with its coefficient of variation. Finally, NS-breathing coordination was assessed using the "multiple-swallow deglutition apnea" analysis [21] from the sum signal of respiratory inductance plethysmography. The total time duration spent with all NS-related hypopneas and apneas (respectively ≥ 50% and 90% decrease in signal amplitude for at least two respiratory cycles) was calculated.

Statistical analysis

Values are expressed as means (SD). Normality was tested using the Kolmogorov-Smirnov test. Variables with normal distribution (heart rate, respiratory rate, NS frequency, number of NS, NS-NS interval duration, bottle-feeding duration, volume of milk per swallow, volume of milk per minute and apnea/hypopnea total time duration relative to the duration of NS bursts) were analyzed using generalized estimating equations with repeated measures. The independent variables were group (HB or control) and experimental day (D0 or D3). The average value of the two bottle-feeding sessions was used. The non-parametric Mann-Whitney U-test was used for all other quantitative variables. Finally, count data were analyzed using the generalized estimating equation with repeated measures with a Poisson distribution. A value of $p < 0.05$ was considered statistically significant. Furthermore, given the relatively small number of studied animals, it was decided to give full consideration in the discussion to a significant trend, defined as $p < 0.1$.

RESULTS

Hyperbilirubinemia preterm lamb model

Animals

Four ewes gave birth to twins, two to triplets and two to a single lamb. Five of the 16 lambs died from dystocia or neonatal respiratory distress (survival rate 69%). The study was completed in six lambs (three males) in the control group and five lambs (three males) in the HB group. Mean birth weight was 2.38 (0.3) kg in the control group and 2.48 (0.5) kg in the HB group (p=1).

Neurological consequences of moderate hyperbilirubinemia in preterm lambs

A moderate HB was obtained for 17 hours (Fig 1) in all five lambs of the HB group. A cutaneomucous icterus was apparent in all lambs. Neurological anomalies were observed at D0 from the first hour of infusion, including hearing impairment and hypotonia in all lambs; with progression of HB, hypertonia was observed in two lambs, as well as spontaneous opisthotonos in one lamb. No such findings were found in control lambs.

At D3, while icterus had disappeared in all HB lambs (bilirubinemia = 33 ± 21 µmol/L), two lambs still had hearing impairment and one had persistent hypotonia.

Feeding efficiency

Nutritive swallowing was significantly less efficient in the HB group compared to the control group at D0 (Table 1). At D3, the only remaining significant difference was a decrease in NS frequency (p=0.02) in HB lambs.

Nutritive swallowing-breathing coordination

During bottle-feeding, NS-breathing coordination in control lambs relied on the presence of respiratory inhibition (apnea/hypopnea) to accommodate bursts of NS (Fig 2). Such

187 coordination was altered in HB lambs. Indeed, apnea/hypopnea duration relative to the
188 duration of NS bursts was 16% (13) in the HB group vs. 41% (27) in the control group
189 (p=0.02) at D0 and 38% (17) vs. 61% (15) (p=0.02) respectively at D3.
190 In addition, HB lambs tended to experience more severe desaturations (<80%) during
191 bottle-feeding than control lambs, as well as a significantly lower minimum saturation at D3
192 (Table 2).
193
194 **Cardiorespiratory variables**
195 Respiratory rate tended to be lower in HB lambs prior to feeding at D0. Following bottle-
196 feeding, RR was significantly lower and apneas were longer in HB lambs, both at D0 and
197 D3 (Table 3). No significant differences were observed for HR and saturation before and
198 after feeding.
199

DISCUSSION

The present results in preterm lambs show that moderate HB led to decreased NS efficiency and altered NS-breathing coordination. These alterations were present during HB and, though attenuated, were still apparent three days after the induction period.

Ovine model of neonatal hyperbilirubinemia

Our study is the first to assess the effects of sustained moderate HB in a preterm animal. We and others have shown that the preterm lamb is a relevant model of moderate prematurity [14], particularly for studying neurological and cardiopulmonary function [7,12,15].

The few animal models of HB in the literature widely differ from our model. Two rodent models with genetic mutations were used to study Crigler-Najjar syndrome [16,17] while two other teams studied the control of breathing in rat pups [5] or brain cell function in piglets [18]. Both represented models of severe HB, i.e. 425 µmoles/L vs. 150-250 µmoles/L in the present study, with maximal HB duration lasting only five hours. In contrast, moderate HB in our model was induced for 17h in order to better approximate HB in preterm human newborns. Relevance of our model is supported by the observation of tonus anomalies and hearing deficit, which were reversible after bilirubin normalization, as reported during acute bilirubin-induced neurological dysfunction [19].

Feeding efficiency and nutritive swallowing-breathing coordination

Our study clearly shows decreased feeding efficiency during HB (Table 1), a finding consistent with feeding difficulties described in human preterm newborns during bilirubin-induced neurological dysfunction [3]. To our knowledge, however, no previous study has investigated the effect of HB on swallowing or swallowing-breathing coordination.

In a previous study, we reported that term lambs have less than one apnea during bottle-feeding [22]. This is in sharp contrast with our present observations of prolonged apneas/hypopneas during NS bursts in control preterm lambs. A similar pattern of respiratory inhibition during NS bursts has been reported in preterm infants [21,23,24]. The attenuation of NS burst-related respiratory inhibition in HB preterm lambs is intriguing and raises the possibility of an increased maturation of NS-breathing coordination by HB. However, mature NS-breathing coordination in term lambs implies regular occurrence of swallows and respirations without apneas [22] whereas, in contrast, NS and respiratory rhythms were chaotic in HB preterm lambs. We therefore propose that prolonged apneas accompanying NS bursts in control preterm lambs, although reflecting an immature NS-breathing coordination, represent an « alternative » coordination to prevent aspirations within the context of prematurity. Loss of this « alternative » coordination with HB could promote tracheal aspirations during bottle-feeding. Of note, despite shorter time spent in apneas/hypopneas, more severe desaturations were observed with HB, both at D0 and D3 (Table 2). However, it is unknown whether these occurrences were related to aspirations.
Our results further complement recent studies in human preterm newborns and young rats suggesting that *severe* HB impairs respiratory control [4,5]. Interestingly, bilirubin deposits were shown in the medulla [5], where respiratory and swallowing centers are co-located.

Respiratory inhibition after bottle-feeding

In contrast to the decreased occurrence of NS burst-related apneas during bottle-feeding, decreased RR and longer apneas were observed within 10 minutes after bottle-feeding in HB lambs at D0 and D3. These data are consistent with the observations of more frequent apneas during HB in premature neonates [4].

CONCLUSION

The present results suggest that moderate HB is responsible for diminished feeding efficiency and impaired NS-breathing coordination, as well as increased apneas and desaturations after bottle-feeding, with some of these abnormalities persisting three days after HB. Overall, our results support the hypothesis of brainstem toxicity by HB, affecting both respiratory and swallowing centers. The present data should be kept in mind when planning hospital discharge of newborn infants after HB.

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FIGURE LEGENDS

Figure 1: Evolution of total bilirubinemia levels over time in control (n = 6) and hyperbilirubinemia (HB) (n = 5) lambs. The dotted lines define the induction period of moderate hyperbilirubinemia. Values are expressed as mean and standard deviation. ** $p < 0.05$ and * $p < 0.1$.

Figure 2: Swallowing-breathing coordination during bottle-feeding at D0 in one control preterm lamb (top panel) and one hyperbilirubinemia lamb (bottom panel). During bottle-feeding, HB lambs tended to experience more severe desaturations (<80%) than control lambs. Nomenclature from top to bottom: pharyngeal pressure, swallowing function; respiratory movements, given by the sum signal of the respiratory inductance plethysmography (inspiration upwards); SpO_2, arterial oxygen hemoglobin saturation; i, inspiration; e, expiration.

TABLES

Table 1: Effect of hyperbilirubinemia on nutritive swallowing

	D0		D3	
	Control	HB	Control	HB
Bottle-feeding duration (s)	35 (13)	70 (22)**	52 (37)	91 (59)
Number of NS / bottle	74 (23)	110 (22)**	89 (39)	88 (44)
NS frequency (min^{-1})	146 (28)	107 (21)**	131 (52)	74 (35)*
Coefficient of variation	0.8 (0.1)	1.4 (0.5)**	0.95 (0.2)	1.1 (0.2)
NS volume (mL)	0.7 (0.2)	0.46(0.1)**	0.6 (0.2)	0.59 (0.3)
Overall efficiency ($mL.min^{-1}$)	106 (39)	49 (16)**	82 (55)	48 (36)

Values are expressed as mean (standard deviation). D0, experimental day 0 (immediate effects of moderate hyperbilirubinemia); D3, experimental day 3 (delayed effects of moderate hyperbilirubinemia); HB, hyperbilirubinemia; NS, nutritive swallows; ** $p < 0.05$ vs. Control on D0; * $p < 0.05$ vs. Control on D3.

354 **Table 2: Arterial oxygen hemoglobin saturation during bottle-feeding**

	D0		D3	
	Control	HB	Control	HB
Nb of SpO_2 < 90%	1.1 (0.6)	0.9 (0.4)	0.6 (0.8)	1.1 (0.4)
Nb of SpO_2 < 80%	0.2 (0.3)	0.6 (1.1)	0.1 (0.2)	0.5 (0.5)
Total duration SpO_2< 90% (s)	17 (18)	27 (33)	8.2 (15)	16 (15)
Total duration SpO_2 < 80% (s)	2.6 (5)	10 (16)	0.3 (0.8)	9 (15)*
% time with SpO_2< 90%	44 (37)	35 (39)	19 (28)	23 (26)
% time with SpO_2 < 80%	4.3 (8)	15 (21)	0.4 (1)	14 (27)*
Minimum SpO_2 (%)	86 (5)	81 (9)	89 (9)	81 (7)*
Maximum SpO_2 decrease (%)	7.8 (6)	12.3 (6.4)	5.8 (7.5)	14.6(9.5)*

355 Values are expressed as mean (standard deviation). Nb: number; SpO_2: arterial oxygen
356 hemoglobin saturation. See table 1 for other abbreviations. Underlined exponent: $p < 0.05$;
357 normal font exponent: $p < 0.1$. * vs. Control on D3.

358

Table 3: Respiratory inhibition after bottle-feeding

	D0		D3	
	Control	HB	Control	HB
RR, min^{-1}				
Before bottle-feeding	74 (10)	53 (26)**	62 (9)	51 (16)
1 min after bottle-feeding	86 (24)	59 (14)**	71 (10)	56 (11)*
5 min after bottle-feeding	74 (17)	50 (18)**	66 (14)	48 (10)*
10 min after bottle-feeding	67 (10)	55 (21)	61 (7)	46 (13)*
% decrease in RR				
1 min after bottle-feeding	15 (21)	22 (33)	15 (19)	18 (38)
5 min after bottle-feeding	0 (14)	0 (17)	7 (12)	0 (18)
10 min after bottle-feeding	0 (11)	9 (14)	2 (15)	0 (14)
Time spent in apnea (% total time)				
Before bottle-feeding	0.6 (1.2)	0.5 (0.6)	0.9 (1)	0.5 (0.7)
After bottle-feeding	0.5 (0.5)	1.1 (0.6)**	0.9 (0.6)	1.2 (0.9)

Values are expressed as mean (standard deviation). RR: respiratory rate. See table 1 for other abbreviations. Underlined exponent: $p < 0.05$; normal font exponent: $p < 0.1$. ** vs. Control on D0; * vs. Control on D3.

FIGURES

Figure 1:

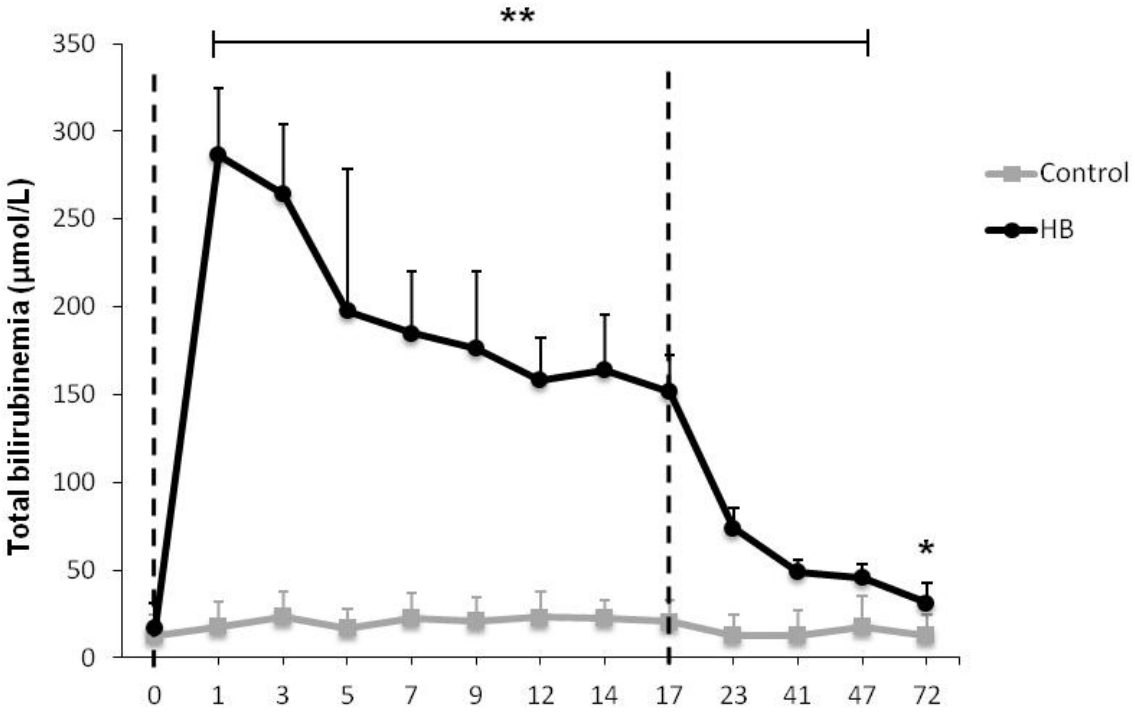
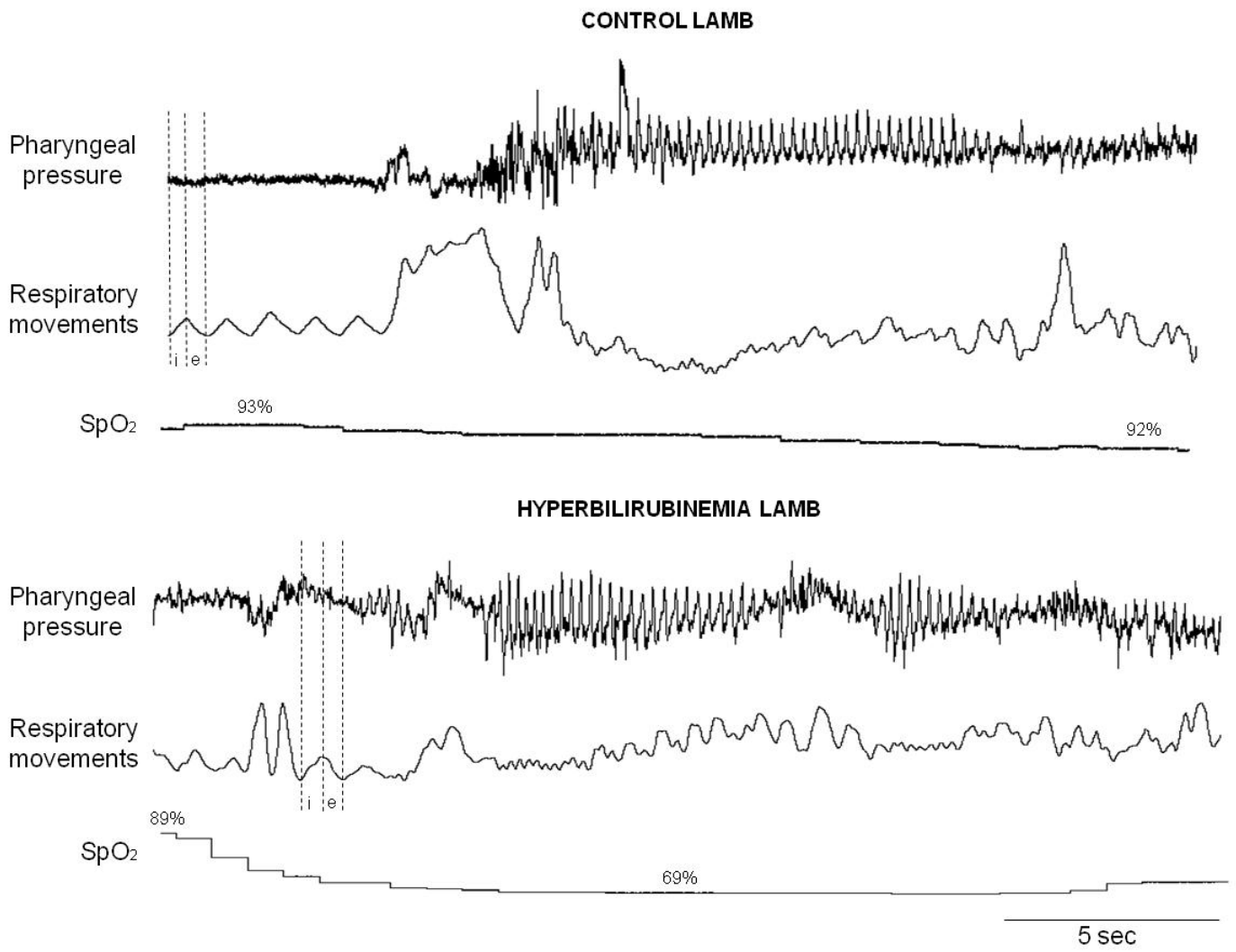


Figure 2:


CONTROL LAMB
Pharyngeal pressure
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SpO2
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HYPERBILIRUBINEMIA LAMB
Pharyngeal pressure
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