Gestational age at time of the initial exposure to lipopolysaccharide determines the severity of diaphragmatic contractile dysfunction in preterm lambs

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The integrity of the immature diaphragm may be a critical determinant of the resilience to respiratory failure after preterm birth. Approximately 70% of extreme preterm births are associated with intra-uterine inflammation, commonly manifest as chorioamnionitis (Goldenberg, 2000). Using an ovine model of chorioamnionitis, we showed previously that acute exposure to intra-amniotic lipopolysaccharide (LPS) caused diaphragm contractile dysfunction in preterm lambs. Therefore, we hypothesized that the developing diaphragm muscle may be differentially vulnerable to an inflammatory response depending on the gestational age at initial exposure and/or single or multiple LPS exposures during gestation.

This study was approved by the Animal Ethics Committee of The University of Western Australia. Time-mated Merino ewes with singleton fetuses received LPS (10 mg/mL) or saline (control) by intra-amniotic injection at 2 d, 7 d, 21 d (single exposures) or 7+14+21 d (multiple exposures) before operative delivery at 121 d gestational age (preterm). At post-mortem, the right hemi-diaphragm muscle was dissected and placed in Krebs physiological salt solution (in mM: NaCl, 109; KCl, 5; MgCl, 1; $CaCl_2$, 4; $NaHCO_3$, 24; NaH_2PO_4, 1; $C_3H_3NaO_3$, 10). A longitudinal strip of diaphragm muscle was isolated and mounted in an *in vitro* muscle test system (model 1205, Aurora Scientific In., Canada). The organ bath was maintained at 25°C and continuously bubbled with 95% O_2/5% CO_2. The muscle strip was manually adjusted to the optimal muscle length (Lo) at which maximum isometric twitch force (P_t) and maximum tetanic force (P_0) were recorded. The fatigue resistance of the diaphragm muscle was assessed by applying 80 Hz tetanic contractions of 0.33 s duration once every second for 150 s. The fatigue index (FI) was calculated from the ratio of the force produced during the 150th contraction relative to the 1st contraction; a higher ratio representing a greater resistance to fatigue. The susceptibility of diaphragm to stretch-induced damage was evaluated using a series of 5 eccentric contractions at 2 min intervals: the percentage reduction in P_0 after the stretch protocol indicates the severity of muscle damage. Following the assessment of contractile function, the mass of the muscle strip and its length (Lo) were measured in order to determine the specific force (normalised for cross sectional area; $N \cdot cm^2$). Sigmaplot was used for statistical analysis. For normally distributed data, differences among treatment groups were determined using one-way ANOVA, followed by the HolmSidak/Dunns test as a *post hoc* analysis. Nonparametric data were analysed using ANOVA on ranks. Values are means ± SEM, unless specified otherwise.

	Saline	**LPS Exposure Groups**			
	Controls	**2 day**	**7 day**	**21 days**	**7+14+21 days**
$P_t (N \cdot cm^2)$	7.96 ± 0.86	5.66 ± 0.80*	6.07 ± 0.33	5.32 ± 0.20*	5.26 ± 0.41*
$P_0 (N \cdot cm^2)$	15.58 ± 1.45	11.2 ± 1.30*	12.14 ± 0.76	9.27 ± 0.50*	9.55 ± 0.58*
½ RT (s)	0.19 ± 0.03	0.22 ± 0.02	0.22 ± 0.02	0.31 ± 0.02*	0.33 ± 0.06*
FI	0.34 ± 0.04	0.34 ± 0.03	0.33 ± 0.04	0.51 ± 0.04*	0.54 ± 0.03*
Damage	16.55 ± 2.39	16.30 ± 2.21	14.38 ± 1.42	6.53 ± 0.92*	7.77 ± 1.78*

* significantly different to Saline Controls

Intra-amniotic LPS exposure in preterm lambs significantly reduced contractile force generation in the diaphragm (as shown in the Table). Exposure to LPS through a single dose at 21 d or multiple doses at 7+14+21 d before birth significantly reduced maximum specific force by ~40 %, relative to the control saline group. Similarly, the peak twitch force (P_t) decreased by ~30%, and was accompanied by a significant increase in ½ RT in these groups. Furthermore, the resistance to fatigue and to stretch induced muscle damage were significantly reduced in 21 d and multiple LPS treated groups compared with controls. P_0 was also significantly reduced with short term (2 d) single LPS exposure, albeit to a lesser extent than the long-term (21 d) exposures.

In conclusion, intra-amniotic LPS exposure significantly impaired muscle contractility in the preterm diaphragm. The severity of contractile dysfunction was greater when the initial exposure occurred 21 d prior to delivery compared to short term single LPS exposures at 2 d or 7 d prior to delivery. Importantly, multiple exposures to LPS did not cause additional impairment in contractile function over that seen with a single exposure 21 day prior to delivery. Therefore, the gestational age at the time of initial exposure rather than the frequency of inflammatory exposure determines the severity of contractile dysfunction in the preterm diaphragm.

Goldenberg R, Hauth JC, Andrews WW. (2000) *New England Journal of Medicine* **342:** 1500-7.