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Effects of Low-Dose Hydrocortisone Therapy on Immune Function in Neonatal Horses

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ABSTRACT: Low-dose hydrocortisone (LDHC) therapy modulates inflammatory responses in adults and improves outcomes in some septic adults and neonates, but its immunologic effects have not been evaluated in neonates. The objective of this study was to evaluate effects of LDHC therapy on *ex vivo* immune function in neonatal horses (foals). We hypothesized that LDHC treatment would dampen proinflammatory responses without impairing neutrophil function. Hydrocortisone (1.3 mg/kg/d i.v.) was administered to foals in a tapering 3.5 d course. Peripheral blood leukocytes were collected from foals before, during, and after hydrocortisone treatment. A separate group of age-matched untreated foals served as controls. Endotoxin-induced peripheral blood mononuclear cell gene expression of inflammatory cytokines was measured by real-time quantitative RT-PCR. Neutrophils were incubated with labeled, killed *Staphylococcus aureus* or *Escherichia coli* for assessment of phagocytosis, and with phorbol myristate acetate, zymosan, or endotoxin for measurement of reactive oxygen species (ROS) production. Neutrophil phagocytosis and ROS production were similar in both groups. Foals receiving hydrocortisone had significantly decreased endotoxin-induced expression of TNF-α, IL-6, IL-8, and IL-1β. These data suggest that this LDHC treatment regimen ameliorates endotoxin-induced proinflammatory cytokine expression in neonatal foals without impairing innate immune responses needed to combat bacterial infection. **(*Pediatr Res* 70: 72–77, 2011)**

Cortisol is vital for the stress response to critical illness and plays an essential role in regulation of the inflammatory response (1). In some patients, though, the cortisol response to illness is inadequate, and a condition called relative adrenal insufficiency (RAI) or critical illness-related corticosteroid insufficiency (CIRCI) (2–4) arises. The physiologic consequences of RAI/CIRCI can include cardiovascular collapse and an excessive, unregulated systemic inflammatory response (3,4). A number of studies have documented a significantly higher incidence of multiple organ failure and death in septic patients with RAI/CIRCI compared with patients with intact hypothalamic-pituitary-adrenal (HPA) axis function (4–6). An exaggerated systemic inflammatory response characterized by imbalance in pro- and anti-inflammatory cytokine production is also documented in septic people and animals and is also predictive of increased disease severity and death (1,7–12). Cortisol insufficiency during sepsis is theorized to contribute to such an overwhelming inflammatory response (1,10,12).

Thus, glucocorticoids have been administered to septic patients in varying doses in attempts to quell inflammation. Although high-dose corticosteroid therapy is associated with adverse effects and decreased survival in septic patients (13–15), a number of studies have described improved shock reversal and survival in septic patients with RAI/CIRCI when supplemented with low-dose hydrocortisone (LDHC) (4,13–17).

As fetal HPA axis function does not mature until the peripartum period, cortisol insufficiency may be of particular significance in the neonate (3,18). A number of studies have documented cortisol insufficiency in septic adult and pediatric patients and in critically ill full-term and preterm infants (4,6,19–22). However, to the authors' knowledge, the effects of LDHC therapy on immune function in neonates of any species are not described. Given the numerous differences in endocrine and immune function and in hydrocortisone pharmacokinetics between adults and neonates (3,18,23), specific evaluation of LDHC therapy in the neonate is critical to optimize therapeutic recommendations for this population.

Like infants, neonatal horses (foals) exhibit substantial HPA axis immaturity in the perinatal period (24–27) and are highly susceptible to the development of bacterial sepsis (28). In addition, naturally occurring RAI/CIRCI is described in ~40% of septic neonatal foals and is correlated with increased incidence of shock, multiple organ failure, and death (29), as documented in septic adults, children, and infants (4,6,19–22). Furthermore, septic foals also exhibit similar inflammatory dysregulation to septic infants, characterized by increased expression of proinflammatory cytokine genes such as TNF-α and IL-6 (7,8,30).

Thus, the primary objective of this study was to examine the effects of LDHC therapy on measures of immune function in neonatal foals in an *ex vivo* model. In addition, effects of LDHC therapy on HPA axis function were examined. We hypothesized that LDHC treatment 1) would not significantly impair the ability of peripheral blood granulocytes to produce

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Abbreviations: CIRCI, critical illness-related corticosteroid insufficiency; **HPA,** hypothalamic-pituitary-adrenal; **LDHC,** low-dose hydrocortisone; **PBMC,** peripheral blood mononuclear cell; **PMA,** phorbol myristate acetate; **RAI,** relative adrenal insufficiency; **ROS,** reactive oxygen species reactive oxygen species (ROS) or phagocytose bacteria; 2) would significantly decrease endotoxin-induced expression of proinflammatory cytokine genes and significantly increase endotoxin-induced expression of anti-inflammatory cytokine genes in peripheral blood mononuclear cells (PBMCs); and 3) would not significantly suppress HPA axis function *in vivo* after discontinuation of hydrocortisone administration.

MATERIALS AND METHODS

Animals. Thirty-nine healthy 2- to 7-d-old full-term foals (GA ≥330 d, weight 40–60 kg) from university research herds were used. Foals were housed with the dam and were determined to be healthy during the study period by daily physical examination. Eleven foals (four females and seven males) were treated with LDHC (hydro group), and 28 age-matched foals (15 females and 13 males) were untreated (control group). Seventeen control foals were used to provide age-matched comparisons with hydro foals for immune function testing, and 11 control foals provided age-matched HPA axis function comparisons with hydro foals. Group assignments were randomized by order of foaling and were known to the investigators.

Study methods were approved by the University of Georgia's and Clemson University's Institutional Animal Care and Use Committee, and mare/foal pairs were cared for according to the guidelines stated in an Animal Use Protocol determined and approved by each university's Department of Animal Resources.

LDHC treatment and sampling protocol. Sampling protocols for hydro and control foals are shown in Figure 1. Between 36 and 60 h of age, all hydro foals had an i.v. jugular catheter placed under brief standing restraint. Beginning 12 to 18 h after catheter placement, hydro foals received a tapering 3.5 d course of hydrocortisone sodium succinate (Pfizer, NY, NY) as follows: 1.3 mg/kg/d for 48 h, 0.65 mg/kg/d for 24 h, and 0.33 mg/kg/d for 12 h. This total daily dose was determined by multiplying the daily endogenous cortisol production rate in healthy neonatal foals (31) by a factor of 2 to approximate an appropriate cortisol response to stress as described in other species (2,32,33). This total daily dose was divided into six doses administered as an i.v. bolus every 4 h.

Sixty-five milliliters of blood was collected just before the first dose of hydrocortisone (pretreatment), after 48 h of therapy before the initial dose was halved (during-treatment), and 12 h after cessation of hydrocortisone administration (posttreatment). Five milliliters of blood was allowed to clot, centrifuged, and serum stored at −80°C until analysis for cortisol/hydrocortisone concentrations. The remaining 60 mL was anticoagulated with 2 mL 100 μM EDTA for isolation of peripheral blood leukocytes. At the during-treatment sample, blood was also collected 5 min after hydrocortisone administration for measurement of peak serum hydrocortisone concentration.

In the 17 control foals used for immune function comparisons, blood was collected similarly on d 2, d 4, and d 6 of life to permit age-matched comparisons with the hydro group. Twenty-four hours after discontinuation of hydrocortisone therapy, posttreatment HPA axis function and responsiveness was assessed in 8 of 11 hydro foals. Blood was collected for measurement of basal cortisol concentrations and a paired low-dose (10 μg)/high-dose (100 μg) cosyntropin (synthetic ACTH, α 1–24 corticotropin; Amphastar Pharmaceuticals, Rancho Cucamonga, CA) stimulation test (24) was performed (Fig. 2). Ten micrograms cosyntropin, rather than the standard 1 μg dose used in people, was used for the low-dose cosyntropin stimulation test because lack of a measurable cortisol response to 1 μg cosyntropin in neonatal foals has been previously demonstrated (34).

In the 11 control foals used for HPA axis function comparisons, blood was collected on d 2 and between d 5 and 7 of age for measurement of basal cortisol concentrations for comparisons with hydro foals. A paired cosyntropin stimulation test was also performed between d 5 and 7 of age in these control foals for comparison with hydro foals' posttreatment cosyntropin stimulation responses. Some findings in this group have been published previously (24).

Cortisol and hydrocortisone assays. Serum total cortisol and hydrocortisone concentrations were determined on an automated analyzer using a chemiluminescent immunoassay (Immulite, Diagnostics Product Corporation, Los Angeles, CA) validated for use in the horse (35,36).

Ex vivo immune function testing. Peripheral blood granulocytes and PBMCs were isolated within 120 min of collection by density-gradient centrifugation over Histopaque-1077 (Sigma Chemical Co.-Aldrich, St. Louis, MO) as previously described (37,38). Viability of both cell types was greater than 95% as assessed by trypan blue exclusion.

Neutrophil ROS production in response to endotoxin (100 ng/mL; *Escherichia coli* 055:B5 LPS; List Biological Inc., Campbell, CA), zymosan (1000 ng/mL; Molecular Probes, Eugene, OR), and phorbol myristate acetate (PMA, 10^{-7} M; Molecular Probes, Eugene, OR) was measured using a fluorometric assay as previously described (37). Assessment of ROS production in response to these three stimulants permitted assessment of the cells' toll-like receptor 4 (TLR-4)-mediated ROS production (endotoxin), dectin-1-mediated ROS production (zymosan), and overall capacity to produce ROS in response to protein kinase C activation (PMA).

To determine phagocytic function, isolated neutrophils were resuspended to a final concentration of 3×10^6 cells/mL in RPMI 1640 without phenol red supplemented with 50 μg gentamicin sulfate, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Bodipy-labeled, inactivated *E. coli* and *Staphylococcus aureus* (Invitrogen, Carlsbad, CA) were diluted to a final concentration of 2×10^6/mL. Two hundred microliters of resuspended cells were incubated in duplicate with 50 μL *E. coli* or *S. aureus* or with media only for 60 min at 37°C and 5% CO_2. Cells were then washed with FACS buffer and fixed in 1% formalin. Flow cytometric analysis was conducted within 7 d of fixation (Accuri C6 Cytometer; Accuri Cytometers Inc., Ann Arbor, MI). Extracellular bacterial fluorescence was quenched with 0.4% trypan blue (Sigma Chemical Co. Chemical, St. Louis, MO), and samples were then assessed for the percent of fluorescent-positive cells using commercial software (CFlow-Plus; Accuri Cytometers Inc., Ann Arbor, MI).

After isolation, PBMCs were resuspended in RPMI 1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% equine serum (Hyclone, Logan, UT). The cells were equally divided among six sterile 60 × 15 mm petri dishes and incubated for 30 min at 37°C in 5% CO_2. Plates were then treated with 1 ng/mL endotoxin (*E. coli* 011:B4 LPS; List Biological Inc., Campbell, CA) or an equivalent volume of media and incubated at 37°C

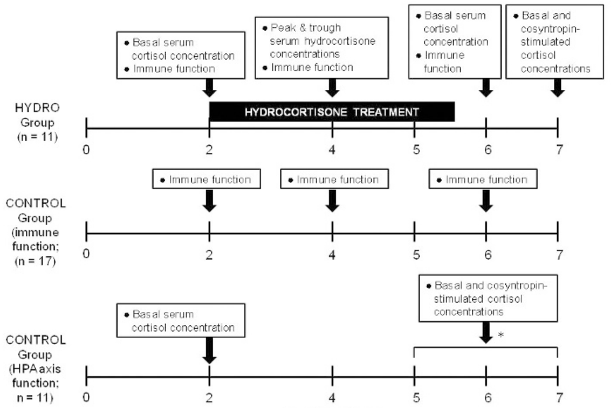

Figure 1. Foal groups and sampling protocols. *Control foals used for HPA axis function comparisons with hydro foals were sampled on d 2 of age and once between d 5 and 7 of age.

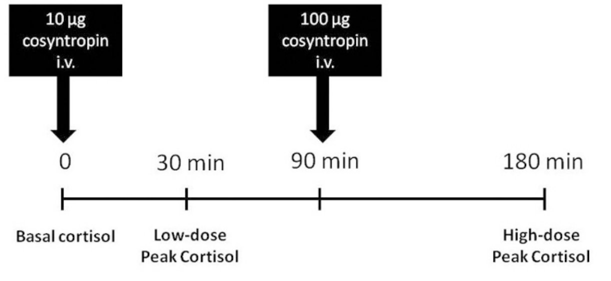

Figure 2. Paired low-dose/high-dose cosyntropin stimulation test design. At time 0, blood was collected for measurement of basal cortisol concentration, followed by administration of 10 μg cosyntropin i.v. Blood was collected 30 min later to assess the cortisol response to 10 μg cosyntropin (low-dose peak cortisol). At 90 min, 100 μg cosyntropin was administered i.v., and blood was collected 90 min later to assess the cortisol response to 100 μg cosyntropin (high-dose peak cortisol).

and 5% CO_2 for 1, 4, and 20 h. Cells were scraped from the plates in cold PBS, pelleted *via* brief centrifugation at 14,000 × g, and lysed with RNA cell lysis solution (Qiagen, Valencia, CA) in combination with 10 μL/mL 2-mercaptoethanol (Sigma Chemical Co. Chemical, St. Louis, MO) and stored at −80°C until RNA extraction. Total RNA was extracted from cell lysates using the RNeasy mini RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and treated with DNase I at 25°C for 30 min. Only samples having 260:280 nm absorbance ratios between 2.0 and 2.2 as measured on a NanoDrop spectrophotometer (ThermoFisher Scientific, Wilmington, DE) were processed for cDNA synthesis with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using 500 ng RNA as template.

Expression of TLR-4, TNF-α, IL-1β, IL-6, IL-10, IL-4, IL-8, and TGF-β were quantified using validated two-step real-time quantitative RT-PCR assays with SYBR Green detection in an Applied Biosystems 7900HT sequence detection system (Foster City, CA), with 18S ribosomal RNA used as an endogenous housekeeping control as previously reported (39,40). Changes in gene expression were calculated by relative quantification against 18s rRNA using the $\Delta\Delta C_T$ method with plain media controls used as the calibrator. Fold changes in gene expression between endotoxin-stimulated and unstimulated cells from the same foal for each incubation period (1, 4, and 20 h) at each sampling time point were calculated as $2^{-\Delta\Delta CT}$.

Data analysis. Between-group comparisons between age-matched hydro and control foals were conducted using *t* tests for parametrically distributed data and Mann Whitney *U* tests for nonparametric data, after normality was assessed with the Kolmogorov-Smirnov test. For inflammatory mediator gene expression, lipopolysaccharide-induced fold changes in gene expression ≤3-fold were not considered relevant gene induction. Thus, if both control and hydro foals failed to exhibit an endotoxin-induced change in expression of >3-fold for a specific gene, further between-group comparisons for that gene at that day and incubation time point were not conducted. Statistical analysis was performed using commercial statistical software (Prism Version 4; GraphPad Software, Inc., San Diego, CA) and statistical significance was set at $p < 0.05$.

Table 1. *Neutrophil ROS production in hydro foals before, during, and after hydrocortisone treatment and in age-matched control foals after* ex vivo *stimulation with endotoxin (100 ng/mL), zymosan (100 ng/mL), and PMA (10^{-7} M)*

	Pretreatment	During treatment	Posttreatment
Response to endotoxin (corrected AFUs)			
Hydro foals (n = 11)	6.4 ± 4.6 (0.4 to 15.1)	4.2 ± 5.9 (−1.7 to 18.2)	4.3 ± 5.7 (−1.9 to 16.6)
Control foals (n = 13)	2.9 ± 4.1 (−2.7 to 10.6)	0.7 ± 5.0 (−6.4 to 7.4)	0.4 ± 3.3 (−4.7 to 4.9)
Response to zymosan (corrected AFUs)			
Hydro foals (n = 11)	16.2 ± 3.0 (9.1 to 19.4)	15.8 ± 9.8 (−3.3 to 33.7)	19.5 ± 17.8 (−3.7 to 56.3)
Control foals (n = 13)	13.7 ± 7.2 (2.3 to 25.8)	9.3 ± 5.9 (0.4 to 18.9)	11.3 ± 2.9 (8.3 to 18.0)
Response to PMA (corrected AFUs)			
Hydro foals (n = 11)	51.6 ± 16.2 (29.8 to 75.3)	71.2 ± 22.8 (43.7 to 16.8)	100.6 ± 57.9 (41.5–227.4)
Control foals (n = 13)	65.2 ± 17.4 (41.2 to 93.1)	71.6 ± 21.9 (40.0–110.6)	89.6 ± 34.7 (45.5–176.6)

ROS production is expressed as corrected arbitrary fluorescence units (AFUs), which were calculated by subtracting background fluorescence in unstimulated control cells from fluorescence in stimulated cells. Data are expressed as mean ± SD (range). No significant differences ($p < 0.05$) were found between hydro and control foals.

RESULTS

No severe adverse effects were noted in any hydro foals. Two foals developed a partial thrombus at the jugular catheter site during treatment and one foal developed mild diarrhea 72 h into hydrocortisone treatment, all of which resolved without specific therapy.

ROS production. Neutrophil ROS production in response to endotoxin, zymosan, and PMA in hydro and control foals is shown in Table 1. No significant differences in ROS production in response to any stimulants were found between hydro and control foals before, during, or after hydrocortisone treatment.

Phagocytic function. Neutrophil phagocytosis of *E. coli* and *S. aureus* in hydro and control foals is shown in Table 2. No significant differences in phagocytic function were found between hydro and control foals before, during, or after hydrocortisone treatment.

Inflammatory molecule gene expression. Both hydro and control foals exhibited a >3-fold endotoxin-induced change in gene expression for TNF-α, IL-6, IL-1β, IL-8, and IL-10 for at least one incubation time on each sampling day, permitting between-group comparisons. Endotoxin-induced changes in gene expression for TLR-4, IL-4, and TGF-β were <3-fold for both groups and at all incubation times on all sampling days, so between-group analysis was not conducted for these genes.

No significant differences in endotoxin-induced gene expression were found between hydro and control foals at the pretreatment sample for any genes except IL-8, for which hydro foals had significantly lower expression than control foals ($p = 0.027$) after 4 h of incubation with endotoxin.

Endotoxin-induced changes in gene expression for TNF-α, IL-6, IL-1β, IL-8, and IL-10 in hydro foals during and after hydrocortisone treatment and in age-matched CONTROL foals are shown in Figure 3. At the during-treatment sample, hydro foals exhibited significantly lower endotoxin-induced expression of IL-6 ($p < 0.001$), IL-1β ($p < 0.036$), and IL-8 ($p = 0.004$ to $p = 0.019$) than control foals. In addition, hydro foals also exhibited lower expression of IL-1β and IL-10 after 4 h of endotoxin incubation that approached statistical significance ($p = 0.053$ and $p = 0.050$, respectively).

At the posttreatment sample, endotoxin-induced expression of IL-6 ($p = 0.023$), IL-1β ($p < 0.001$ to $p = 0.021$), and IL-8

Table 2. *Phagocytosis of killed, bodipy-labeled* E. coli *and* S. aureus *by isolated neutrophils from hydro foals before, during, and after hydrocortisone treatment and from age-matched control foals*

	Phagocytosis of *E. coli* (% positive cells)			Phagocytosis of *S. aureus* (% positive cells)		
	Pretreatment	During treatment	Posttreatment	Pretreatment	During treatment	Posttreatment
Hydro foals (n = 11)	16.7 ± 13.2 (2.0–44.6)	19.4 ± 12.7 (3.3–38.2)	16.7 ± 10.6 (0.0–33.5)	22.0 ± 9.2 (5.0–34.8)	17.6 ± 8.9 (6.3–34.0)	19.2 ± 11.1 (5.2–46.8)
Control foals (n = 9)	11.9 ± 9.9 (1.0–28.2)	14.3 ± 13.9 (2.7–45.1)	11.8 ± 8.3 (1.5–22.3)	19.6 ± 14.0 (1.3–41.3)	14.9 ± 10.4 (6.2–39.8)	11.1 ± 6.8 (2.2–22.3)

Phagocytosis is expressed as % positive cells detected by flow cytometry after quenching of extracellular fluorescence with 0.4% trypan blue. Data are expressed as mean ± SD (range). No significant differences ($p < 0.05$) were found between hydro and control foals.

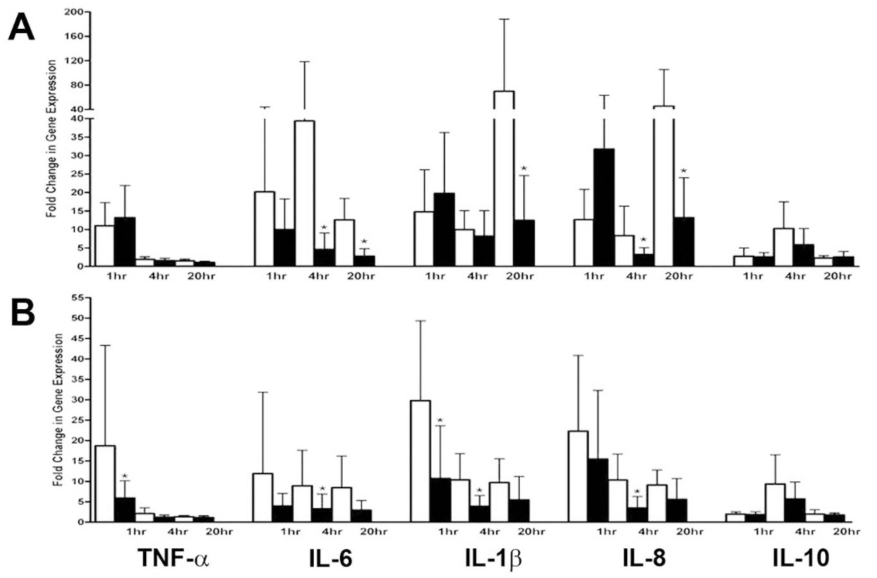

Figure 3. Mean fold change in mRNA expression of TNF-α, IL-6, IL-1β, IL-8, and IL-10 in PBMCs from hydro foals (n = 11; ■) and age-matched control foals (n = 15; □) incubated with endotoxin (1 ng/mL) for 1, 4, and 20 h. Fold change in mRNA expression is relative to unstimulated PBMCs from the same animal. Expression during and after hydrocortisone treatment is shown in (*A*) and (*B*), respectively. *Significant ($p < 0.05$) difference between hydro and control foals.

Table 3. *Serum cortisol/hydrocortisone concentrations in hydro and age-matched control foals before, during, and after low-dose hydrocortisone treatment*

	Serum cortisol/hydrocortisone* concentration (μg/dL) pretreatment (day 2 of age)	Serum cortisol/hydrocortisone* concentration (μg/dL) during treatment† (day 4 of age)		Serum cortisol/hydrocortisone* concentration (μg/dL) posttreatment (day 6 of age)
		Trough	Peak	
Hydro foals (n = 11)	2.6 ± 1.0 (1.6–4.7)	2.1 ± 1.4 (0.4–4.9)	22.3 ± 9.5 (15.0–44.9)	2.5 ± 1.0 (1.2–4.6)
Control foals (n = 11)	2.4 ± 1.0 (1.3–4.4)	n/a	n/a	2.0 ± 0.8 (1.0–3.6)

Data were expressed as mean ± SD (range). No significant differences ($p < 0.05$) were found between hydro and control foals.
* Cortisol and hydrocortisone are chemically indistinguishable.
† Samples for trough and peak cortisol/hydrocortisone concentrations during treatment were collected immediately before and 5 min after i.v. bolus administration of hydrocortisone, respectively.

Table 4. *Basal serum cortisol concentrations and serum cortisol responses to a paired low-dose (10 μg)/high-dose (100 μg) cosyntropin stimulation test in hydro and age-matched control foals*

	Basal cortisol (μg/dL)	Low-dose peak* cortisol (μg/dL)	High-dose peak† cortisol (μg/dL)	Low-dose delta‡ cortisol (μg/dL)	High-dose delta‡ cortisol (μg/dL)
Hydro foals (n = 8)	1.8 ± 0.9 (0.7–3.6)	3.1 ± 1.0 (1.9–4.6)	5.0 ± 1.7 (3.4–7.9)	1.4 ± 0.7 (0.6–2.8)	3.2 ± 1.4 (1.8–6.1)
Control foals (n = 11)	2.0 ± 0.8 (1.0–3.6)	3.3 ± 0.8 (2.3–4.4)	5.5 ± 1.1 (3.5–7.4)	1.3 ± 0.6 (0.6–2.6)	3.5 ± 1.3 (1.7–5.9)

Data were expressed as mean ± SD (range). No significant differences ($p < 0.05$) were found between hydro and control foals.
* Low-dose peak cortisol concentrations were obtained 30 min after i.v. administration of 10 μg cosyntropin.
† High-dose peak cortisol concentrations were obtained 90 min after i.v. administration of 100 μg cosyntropin.
‡ Delta cortisol concentrations were calculated by subtracting the basal cortisol concentration from the peak cortisol concentration reached after administration of 10 μg (low-dose delta cortisol) or 100 μg cosyntropin (high-dose delta cortisol).

($p = 0.002$) remained significantly lower in hydro foals than control foals. Endotoxin-induced expression of TNF-α was also significantly lower in hydro foals ($p = 0.047$) at this sample. Endotoxin-induced expression of IL-1β and IL-8 after 20 h of incubation also was lower in hydro foals and approached statistical significance ($p = 0.054$ and $p = 0.054$, respectively).

Serum cortisol and hydrocortisone concentrations. Serum cortisol and hydrocortisone concentrations in hydro foals before, during, and after LDHC treatment are shown in Table 3. Paired cosyntropin stimulation test results in hydro and control foals are shown in Table 4. There were no significant differences between hydro and age-matched control foals in HPA axis function as assessed by basal cortisol concentration before or after hydrocortisone treatment, or by peak or delta cortisol (peak − basal cortisol) responses to low- (10 μg) or high- (100 μg) dose cosyntropin after hydrocortisone treatment.

DISCUSSION

The results herein provide support for our hypotheses and illustrate that LDHC dampens the proinflammatory cytokine response to *ex vivo* endotoxin exposure without significantly suppressing neutrophil function in neonatal foals. Furthermore, HPA axis function after discontinuation of LDHC treatment was not significantly suppressed in hydro foals. In sum, these findings suggest that a similar LDHC regimen may ameliorate a detrimental proinflammatory response in septic neonates without impairing innate immune and endocrine responses to the inciting infection.

The finding that LDHC did not impair ROS production or phagocytic function in isolated foal neutrophils is consistent with previous reports from adult animals and humans (41–43). The results herein support these previous studies and suggest that innate immune mechanisms also remain intact in neonates receiving LDHC.

The significant reduction in endotoxin-induced gene expression of the proinflammatory cytokines TNF-α, IL-6, IL-1β, and IL-8 in hydro foals was also consistent with previous reports of decreased proinflammatory mediators in septic adult humans receiving LDHC (32,42–44). However, to the authors' knowledge, this is the first study to show persistence of anti-inflammatory effects after discontinuation of hydrocortisone therapy, as evidenced by a significant decrease in TNF-α, IL-6, and IL-1β expression in hydro foals 12 h after discontinuation of hydrocortisone. As transcriptional effects of corticosteroids can involve modification of regulatory molecule production, it is not surprising that some functional genomic effects might persist after steroid administration ceases. Although further study is needed, a short course of LDHC may also result in prolonged anti-inflammatory effects in clinical patients.

Although increased expression of the anti-inflammatory cytokines TGF-β and IL-4 was expected in hydro foals, a relevant $>$3-fold endotoxin-induced change in expression was not observed for these genes, and no further between-group comparisons were possible. Expression of these cytokines may not be induced until later in the inflammatory response, and differences might have been observed if longer endotoxin exposure was conducted. Alternatively, it is possible that expression of TGF-β and IL-4 is not regulated *via* TLR-4-mediated pathways in foal PBMCs.

Expression of the anti-inflammatory cytokine IL-10 was significantly increased after endotoxin exposure in comparison with unstimulated cells in both groups of foals, but expression in hydro foals was comparable to control foals. Corticosteroid treatment has been shown to increase production of IL-10 from isolated human PBMCs (10), but other studies have documented suppression of IL-10 production with LDHC therapy in septic adult humans (42). These contradictory effects may reflect temporal changes in cytokine production related to the duration of the inflammatory response in clinical sepsis *versus* experimental models. Furthermore, as this study examined IL-10 gene expression rather than protein production, posttranscriptional changes in IL-10 production may have been missed.

It is important to note that the LDHC regime used in this study used a lower daily dose and shorter course of therapy than current recommendations in septic adult humans (16). The hydrocortisone dose and q. 4 h dosing protocol used in our study was based on daily endogenous cortisol production rates and secretion patterns in healthy foals (31) and was derived similarly to LDHC recommendations in adult humans (2,32,33). This hydrocortisone regimen did suppress proinflammatory responses in leukocytes from healthy foals to a degree comparable with that achieved with higher doses in septic and endotoxemic adult animals and people (10,32,41,42). A similar daily hydrocortisone dose of 1 to 2 mg/kg divided into intermittent boluses has been recently recommended for use in human neonates with CIRCI (3). The appropriate duration of hydrocortisone treatment in both neonates and adults, however, remains unclear and is best governed by the underlying disease process being managed (*i.e.* septic shock *versus* prematurity) and the clinical response of the individual patient. A shorter LDHC course than the standard 7-d protocol (16) was evaluated here because the authors questioned whether shorter treatment duration would provide still adequate anti-inflammatory effects and thus might have potential clinical utility in patients (such as neonates) in which adverse effects of steroid treatment are particularly undesirable. Further study to evaluate the effects of shorter-duration LDHC regimens in adults and neonates in a clinical setting is needed.

An important limitation of this study is the use of an *ex vivo* model of infection with cells obtained from a small number of healthy animals. Infection in the live animal or person stimulates complex host-pathogen interactions with important endocrine and immunological consequences that could impact the response to LDHC in clinical patients. For instance, the inflammatory response in naturally occurring sepsis is likely present for a longer duration and to a greater degree than in this experimental model, so altered steroid dosing might be required in clinical patients. The anti-inflammatory effects of the LDHC protocol used in this report must be confirmed in larger scale *in vivo* studies with experimental and naturally occurring infection.

In conclusion, the LDHC therapy protocol used in the study herein dampened the *ex vivo* proinflammatory response to endotoxin in neonatal foals without significantly impairing *ex vivo* neutrophil function or endogenous HPA axis activity. These anti-inflammatory effects occurred with a lower dose and shorter course than is currently recommended in adults with RAI/CIRCI, and some anti-inflammatory effects persisted after discontinuation of hydrocortisone. Further study is needed to evaluate the immunologic and clinical effects of a similar protocol in clinical patients.

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