A clinically relevant tapering protocol for Hydrocortisone Treatment in Newborn Animals

Honors Undergraduate Thesis by Jean Lee

Neuroscience Concentration in the Department of Mollecular, Cellular and Developmental Biology (MCDB) and Psychology

University of Michigan, Ann Arbor

April 2011

Key Words: Premature Infants, Glucocorticoid (GC), Dexamethasone (DEX), Hydrocortisone (HC), Surfactant Protein A (SP-A) , 11-β-HSD1, Surfactant Protein Synthesis.

Acknowledgement

I would like to thank Dr. Delia Vazquez, my mentor and research sponsor, for her continuous and generous support. I have not only gained various laboratory techniques, but also gained a sense of research planning and community. From her instructions, I learned to think with a scientist's mind in executing the research for this project and for the future ones to come. I thank her for inviting and accepting me as a member of Vazquez lab and being my role model as a passionate researcher. Also, I want to express my appreciation to all the members of the lab, especially Shirley Huang, for having me. Shirley disciplined and took care of me since my sophomore year without failing. She made my three years in the lab rewarding and fun.

I appreciate Professor Robert Denver, my co-sponsor, for his encouragement and willingness to help. He has been advising me for two consistent years, in addition to countless number of other students from the University of Michigan. Special thanks to Professor Jacinta Beehner for serving as a reader of my thesis. This research was supported by NIH grant RO1 HDDK37431.

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Abstract

Background

Glucocorticoid (GC) steroid agents are used to enhance lung development in premature infants due to its capacity to promote the production of surfactant proteins. Surfactant proteins reduce surface tension in the lungs and in turn facilitate oxygenation of body tissues. Until recently, dexamethasone (DEX) has been the agent of choice to treat infants with respiratory distress of prematurity. However, the association of DEX with negative long term neurological consequences has led to the use of hydrocortisone (HC), a less potent GC agent. HC use in premature infants may be relatively safe but, there are limited information on the effects of HC tapering dose schedules on markers of lung maturation and on the long term neurological consequences. A clinically relevant tapering protocol for HC treatment in newborn animals would facilitate the development of an animal model to study long term neurological consequences of HC treatment. In this study compared HC with DEX following two dosing tapering protocols to determine the protocol that best compared to DEX effect on surfactant protein synthesis.

Methods

Three experiments were performed. Experiment 1 consisted of four injections of decreasing dosage of either DEX or HC on postnatal days (PD) 3, 4, 5, and 6 (Protocol A: DEX 0.5mg/kg to 0.05mg/kg; HC: 5mg/kg to 0.5mg/kg); Experiment 2 consisted of two scheduled injections of decreasing dosage of either DEX or HC on PD 5-6 (Protocol B: DEX 0.5mg/kg and 0.1mg/kg; HC 5.0mg/kg and 1.0mg/kg); Experiment 3 compared varying dose concentrations of Protocol B after conducting Experiment 1 and 2. Vehicle treated pups served as controls in all cases. Body weights were measured, plasma was collected after 6 and 24 hours, and lung and spleen tissues were collected 24 hours after the tapering protocols. Protein levels of surfactant

protein A (SP-A) were measured utilizing Western Blot; mRNA levels of SP-A, 11-β-HSD1, GR, and MR were measured by RT-PCR. Plasma corticosterone hormone levels generated by the tapering HC protocols were assessed by RIA.

Results

We found that 24 hours after the first injection, dexamethasone treated pups had significantly lower body weight than both vehicle and hydrocortisone treated animals. Irrespective of tapering protocol, DEX and HC treated animals had increased SP-A protein synthesis. For both protocols, DEX significantly increased SP-A mRNA and 11-β-HSD1 mRNA levels. HC only increased SP-A protein and failed to affect the others. Two short-term HC tapering doses that had sufficient potency at enhancing SP-A protein levels are as follows: 5 mg/kg HC given at PD 5 followed by 1mg/kg on PD6, and HC 20mg/kg given at PD5 followed by 4mg/kg on PD6.

Conclusions

We conclude that dexamethasone and hydrocortisone are potent stimulators of lung maturation. However, unlike dexamethasone, hydrocortisone does not result in adverse generalized effects on the organism. In addition, we have defined the optimal dose as 5 mg/kg on PD 5 followed by 1 mg/kg the next day to investigate other effects of HC by specific systems.

Introduction

The initiation of ventilation by the respiratory system of an infant is important for the adaptation to extra-uterine life [1]. Pulmonary surfactant protein plays a crucial role during this adjustment by reducing surface tension in the epithelial lining fluid and participating in the inhibition of inflammatory reactions in the lung [2], both of which improve oxygen and carbon dioxide exchange and respiratory function [3-5]. Synthetic glucocorticoid (GC) hormones effectively enhance the synthesis of surfactant proteins [5-7]. Dexamethasone (DEX) has been the GC agent of choice in the neonatal intensive care unit to reduce risks of chronic lung disease and injuries associated with mechanical ventilation [6, 8-12]. However, numerous follow-up studies revealed the adverse effects of DEX therapy on neuromotor function and somatic growth [13, 14]. Because the brains of premature infants are especially malleable, DEX administration has been associated with complex neuro-developmental impairments that vary on the child's age. Delayed psychomotor development and bipolar disorder may appear at pre-school age [15-17]. Increased incidences of social competence problems and attention-deficit hyperactivity disorders (ADHD) were reported at teenage [18-23]. Previous results concurred that the early treatment of DEX can lead to decreased weight gain and smaller head circumferences [24-26]. Based on the systematic review of the DEX experience in neonates, [27, 28] the American Academy of Pediatrics recommended to limit the use of DEX in infants in 2002 [29], and less potent hydrocortisone (HC) has become the preferred GC agent used in the medical management of premature infants [30].

Many studies have shown that early HC treatment in a population of extremely low birth rate infants has decreased the likelihood of chronic lung disease, and the duration of mechanical ventilation treatment [31, 32]. Some report that HC use may be relatively safe with respect to neurodevelopment since there is a lack of generalized adverse effects on motor or cognitive

functions [33, 34]. However, some reported long term consequence of HC exposure on gross neurological and electroencephalographic abnormalities [35]. In particular, reduced head circumference has been reported in treated infants at the age of 7 years [36]. This implies that exposure to HC early in life has an impact on brain development. Causality may be difficult to determine since premature infants may have multiple complex medical problems, treatment with multiple medications and multiple courses of HC during the first months of life. The development of an animal model that would be clinically relevant would facilitate the study of the effect of HC on the developing brain. Our ultimate objective would be to provide DEX and HC during postnatal ages that correspond neurodevelopmentally to time points at which human premature infants are likely to receive prolonged GC courses in neonatal intensive units, and to utilize doses that are clinically relevant to the primary condition targeted by GC treatment. Though much is known about DEX effects on lung maturation [37], to our knowledge, relatively little is known about the effects of HC tapering doses on markers of lung maturation.

We evaluated the effect DEX and HC on surfactant protein A, a glucocorticoid sensitive hydrophilic triplet molecule that is abundant in lung tissue, which plays an important role in reducing the surface tension in alveoli thereby facilitating oxygenation [38]. We also evaluated on DEX and HC tapering regiments on the corticoid receptors that mediate GC action, namely glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) [39, 40]. The endogenous glucocorticoids in human and in rats are cortisol and corticosterone, respectively. Because cortisol (hydrocortisone) and corticosterone have a high affinity for MR when compared to GR, receptor selectivity is determined by the activity of the enzymes 11-Beta-hydroxysteroid dehydrogenase (11-β-HSD) 1and 11-β-HSD 2 [41, 42]. The 11-β-HSD2 is a unidirectional dehydrogenase that converts cortisol and hydrocortisone into inactive steroids that will not occupy MR. On the other

hand, 11-β-HSD1 though a bidirectional oxidoreductase in vitro (producing cortisol in one direction and deactivating cortisol in the reverse direction) [43], it is primarily a reductase in vivo producing active cortisol that binds to MR. The occupation of MR through 11-β-HSD1 activity appears to play a role in lung maturation at early stages of development [43, 44]. Therefore, in the present study we compared two GR/MR agonist, DEX and HC, and two tapering regimen, long and short time course, in the generation of a marker of lung maturation: SP-A protein levels, and SP-A, 11-β-HSD1, GR and MR mRNA levels. We examined whether pharmacological doses of DEX and HC may lead to induction of surfactant synthesis, high level of MR and 11-β-HSD1activity in lung tissue. We found that (1) DEX reduced weight gain; (2) both long and short courses of DEX and HC administration increased SP-A protein synthesis in the lungs; (3) both long and short courses of DEX increased SP-A and11-β-HSD1 mRNA levels; (4) protein determination is more reliable to ascertain the GC –induced generation of SP-A surfactant protein. We conclude that DEX and HC are potent stimulators of lung maturation. However, unlike DEX, HC does not result in undesirable generalized effects on the organism. In addition, we have defined the optimal dose and duration of tapering schedules to investigate other effects of HC by specific systems.

Methods

Animal procedure

Pregnant Sprague-Dawley rats of known gestational age (Day 19) were purchased from Charles Rivers (Wilmington, MA) and kept in accordance with NIH guidelines for the Care and Use of Laboratory Animals under constant temperature and lighting. Eight to 16 pregnant females were used for each experiments performed. All animals were housed individually until the day of birth, postnatal day 1 (PD1). On PD2, animals in each litter were sexed and culled to 6 males and 6 females. On PD3, litters were assigned to three different treatment groups: vehicle control (VEH), dexamethasone (DEX) and hydrocortisone (HC). Animals in different treatment groups, DEX, HC, and VEH, were permanently marked by clipping the tip of the outer toe within each paw.

Experiment 1: Long Tapering Protocols (Protocol A). In this experiment, we followed dosing schedules presented in Table 1. Animals received treatments on PD 3, 4, 5 and 6 of life following Protocol A. On injection days, all animals were weighed, handled and placed in a warm pad (30-35°C). For protocol A, pups in the DEX group received intramuscular injection of 0.5mg/kg DEX on PD3, 0.25mg/kg on PD4, 0.125mg/kg on PD5 and 0.05mg/kg on PD6; HC group received 5mg/kg of HC on PD3, 2.0mg/kg on PD4, 1.0mg/kg on PD5 and 0.5mg/kg on PD6. VEH group served as controls receiving the equivalent volumes of sterile saline. Seven males and seven females were studied per treatment group, and three treatment groups were sacrificed by decapitation per two time periods: 6 hours and 24 hours after the last injection. Plasma was collected from all pups and assessed by RIA; lung tissues were collected from 24 hr cohort: right lung for Western Blot and left lung for RT-PCR. Spleens served as negative control. All samples were weighed and immediately frozen at -80°C.

Experiment 2: Short Tapering Protocols (Protocol B): In this experiment we followed dosing schedules presented in Table 1. Animals received treatments on PD 5 and 6 of life following Protocol B. DEX group received 0.5mg/kg of DEX on PD5 and 0.1mg/kg on PD6; HC group received 5.0mg/kg of HC on PD5 and 1.0mg/kg on PD6. All were weighed, handled, and placed in a warm pad (30-35°C) after receiving the injection. VEH group served as controls receiving the equivalent volumes of sterile saline. Seven males and seven females were studied per treatment group, and three treatment groups were sacrificed by decapitation per two time periods: 6hours and 24 hours after the last injection. Plasma was collected from 6 hr and 24 hr cohort and assessed by RIA; lung tissues were collected from 24 hr cohort: right lung for Western Blot and left lung for RT-PCR. Spleens were also rapidly dissected for negative control. All samples were weighed and immediately frozen at -80°C.

Experiment 3: Plasma Hormone Assay. Following the results from Experiment 1 and 2, a dose response experiment was performed following the protocol B for HC only. HC doses were injected for two days on PD5 (5mg/kg, 20mg/kg, 80mg/kg or 160mg/kg) followed by a serial of doses that were 5x less given on PD6 (1mg/kg, 4mg/kg, 16mg/kg or 32mg/kg correspondingly). Vehicle treated animals were used as controls. Sixteen to twenty pups were studied per treatment group. All treatment groups were either sacrificed 6 hours or 24 hours after the last injection, and trunk blood was obtained and stored frozen at -80°C. Plasma corticosterone hormone levels generated by the tapering protocols were assessed by RIA on the last day of the protocol (see Radioimmunoassay section).

RT-PCR for SP-A, 11-beta-HSD1, GR and MR

The total cellular RNA was extracted using Trizol RNA isolation reagent. Ambio's DNA-free™ kit was used on the RNA samples to remove the residual genomic DNA. The first-strand cDNA was synthesized by SuperScript™ II Reverse transcriptase-mediated synthesis of Oligo-dT24V. PCR reactions (50ul) contained 10ul of cDNA template (1:20 dilution) and 40ul of master mix (25 ul of 2xSyberMix, 2ul forward and reverse primer mix - 5pM each primer, 0.125 ul of SyberGreen-1, 0,055 ul of Taq polymerase and 12.82 ul of ddH2O). Reactions were carried out in a Bio-Rad iCycler. The Cycling conditions were as follows: polymerase activation 3 minutes at 95°C and 35 cycles extension for 15 sec at 95°C, 56°C for 15 sec, 72°C for 10 sec and 1 cycle elongation at 72°C for 5 minute followed by 47 cycles of melt curve analysis at 72°C for 10sec. After PCR is finished, the PCR specificity was examined by 2% agarose gel. All reactions were performed in triplicate, and each experiment contained –RT reaction for each sample and a no-template control for each primer set. Each gene specific expression was normalized in parallel reactions with the housekeeping gene, HPRT. Table 3 lists the sequences of the forward primer and reverse primer for all genes that were studied. The calculation for relative quantification was based on the mathematical model described by Pfaffl et.al [45].

Western Blot Analysis for Surfactant protein A (SP-A)

Lung and spleen tissues were homogenized using Polytron in lysis buffer (PH=7.6) consisting of 10mM Tris, 3mM MgCl2, 40mM KCl, 2mM DTT, 5% Glycerol, 0.5% Triton-X-100 and 5% Proteinase inhibitor cocktail. The homogenates were incubated on ice for 30minutes and centrifuged at 600g for 10min at 4°C. The supernatants of the homogenates were analyzed for protein assay and 20ug of each sample were loaded in 10% NuPage Bis-Tris gel (Novex, San

Diego, CA) in reduced conditions [46] for immunoblotting analysis. The SP-A (H-148, sc-13977, at 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody and goat anti-rabbit horseradish peroxidase-conjugated as secondary antibody (diluted in 1:10,000, Jackson ImmunoResearch, West Grove, PA). The detection was carried out with the ECL-Plus detection system (RPN 2135, Amersham BioSciences).

Radioimmunoassay

Plasma hormone Corticosterone (CORT) level was assessed in animals studied in experiment 3 using standard RIA kits (MP Biomedicals, LLC). The detection limit of this assay is 0.17ug/dL. The intra and inter-assay coefficients of variation are 5.3% & 7.5%, respectively. Hydrocortisone was measured by RIA using "Coat-A-Count" RIA kit (DPC; Los Angeles, CA). This assay was modified for rat samples using adrenalectomized rat serum with known amount of hydrocortisone concentrations. The detection limit is 0.2µg/dl. Intra and inter coefficient of variation are ±2% and ±5%, respectively. The assay has 1.4% cross reactivity with CORT.

Statistical analysis

In all cases, tissues collected were identified by number only, and the individuals performing the gene/protein or hormonal determinations did not have knowledge about the group assignment until the end of data acquisition. ANOVA was used, followed by Post-hoc Fisher paired least significant difference test (PLSD). Significance was set at $p<0.05$. Data is presented as (Mean±SE).

Results

Body Weight

In order to better understand the effect of prolonged postnatal GC exposure in the whole animal and its somatic growth, we followed body weight. ANOVA analyses revealed significant treatment ($p<0.0001$), time effect ($p<0.0001$) and no sex differences for both protocols. Figure 1, Panel A shows combined sex body weight progression for protocol A (PD3-6) for all treatments; Panel B shows the same for protocol B (PD5-6). For protocol A, we found that compared to VEH, DEX treated pups demonstrated significantly different weight from PD4 to PD 6 (post-hoc, $p<0.0001$); HC treated pups demonstrated indifferent weight compared to VEH (Fig 1). Similarly, for Protocol B, DEX decreased weight at PD 6 (post-hoc, $p<0.0001$); HC did not effect weight.

Surfactant Protein A levels in the Lungs

a. Western Blot for SP-A protein level

The western blot analyses revealed a 31-35KD band consistent with molecular weight of the SP-A protein. Figure 2a shows negative control of the spleen sample in lane 1; lane 2 and 3 shows male and female samples respectively; lane 4 shows positive control of the SP-A protein. For Experiment 1 and 2, multifactorial ANOVA revealed significant treatment effect ($p=0.0001$) but no protocol ($p=0.1582$) or sex ($p=0.3249$) differences. Figure 2b shows that DEX significantly increased SP-A protein levels in both Experiment 1 (protocol A: Fisher PLSD, $p=0.0009$) and Experiment 2 (protocol B: Fisher PLSD, $p=0.0098$) when compared to VEH. HC significantly increased SP-A protein levels in Experiment 1 (Protocol A: $p=0.0363$), but not in Experiment 2 (Protocol B). However, Experiment 3 (Protocol B with more animals) revealed differences in SP-A protein levels (Figure 6, Panel B).

b. RT- PCR for SP-A mRNA level

To compare transcription activity of SP-A mRNA in response to DEX and HC, we assessed the long term and short term protocols by RT- PCR. The two way ANOVA showed that there is a significant treatment effects (ANOVA with post-hoc, $p<0.0001$, $n=7$ for each group) and sex effects ($p=0.0097$, $n=7$ for each group). The levels of the SP-A mRNA were much higher in female samples. SP-A mRNA levels were significantly increased in DEX treated animals for both protocols A and B in male and female samples compared to VEH and HC groups. There was no difference in SPA mRNA levels in the HC treated animals compared to VEH groups.

Overall results for the SP-A protein and mRNA synthesis are as follows: 1) No protocol differences— DEX and HC dosing in consecutive days up-regulated SP-A protein levels no matter short (PD5-6) or long (PD3-6) course protocols; 2) DEX increased SP-A mRNA levels for both protocols, but not HC.

11-Beta-HSD1, GR and MR mRNA levels in the Lungs

The two way ANOVA showed that there is a significant treatment effects (ANOVA with post-hoc, $p<0.0001$, $n=7$ for each group) but no protocol and sex effects in 11-β-HSD1 mRNA levels. The 11-β-HSD1 mRNA levels in DEX treated animals compared to VEH and HC groups were significantly increased for both long course and short course protocols. Notably, there was no difference in 11-β-HSD1 mRNA levels in the HC treated animals compared to VEH groups. Based on the ANOVA analysis, there is no significant difference in MR and GR mRNA levels, when compared among all the groups.

CORT and SP-A protein response to varying doses of HC

Experiment 3: HC administration on PD 5-6 (Protocol B) at different doses. No sex effect was found for either SP-A lung protein levels or plasma CORT with a two-way ANOVA, so the data was collapsed across this variable. A significant dose effect was observed for plasma CORT levels (Figure 6, Panel A, ANOVA, $p=0.0001$) and for SP-A protein levels (Figure 6, Panel B, ANOVA, $p<0.0001$). The CORT levels collected at 24 hours following the HC protocols were not detectable due to the rapid metabolism of CORT in plasma. The circulating CORT from 6 hours after the last HC injection, which ranged between 1.2 ± 0.08 and 24.2 ± 1.3 μg/dl (Mean ± SE), were all significantly elevated from VEH (see Figure 6, Panel A). SP-A protein levels were significantly increased with injection of 5 mg/kg HC at PD 5 followed by 1mg/kg on PD6, and HC 20mg/kg given at PD5 followed by 4mg/kg on PD6 (Figure 6, Panel B).

Discussion

In the present study, we have compared long or short course postnatal dexamethasone (DEX) and hydrocortisone (HC) treatment effects on somatic growth, generation of Surfactant Protein A (SP-A) and steroid receptor system in the lung of the infant rat pup. The major findings are as follows: (1) DEX treatment had significant reduction in body weight in the developing animal, whereas HC had no impact; (2) both long and short DEX tapering protocols increased SP-A protein, SP-A mRNA and 11-β-HSD1 mRNA synthesis in the lungs; (3) both long and short HC tapering protocols increased SP-A protein synthesis. This suggests for the first time that DEX treatment decreases somatic growth of a developing infant, whereas HC treatment is safer due to normal somatic growth. DEX significantly increases protein and mRNA synthesis for both protocols, suggesting that PD 5 and 6 are the critical period for protein up-regulation. HC increases SP-A protein synthesis following the long and short course injection, which shows that HC is able to significantly impact lung maturation as effectively as DEX.

The tapering doses we used for postnatal DEX in rat model were based on the prolonged DEX course proposed by Cummings, D'Eugenio and Gross from the current clinical procedures in humans [47]. The individual DEX doses from 0.5 to 0.025 mg/kg have been directly applied to a rat postnatal animal model by others [48, 49] and had been proven to be effective in the enhancement of lung maturation. Our data is in agreement with published results, concurring that DEX significantly improves lung maturation. We extrapolated the DEX human dose schedule to a HC schedule, which is also the dosage utilized in the intensive care unit for the treatment of human neonates [25, 36, 50, 51]. Steroid initiation and duration in rat pups were consistent with respect to human long parameters because the general sequence of brain growth between rodents and humans are similar. The main differences are in the timing of events that lead to spurts in brain

growth [52, 53]. In humans, excluding the cerebellum and hippocampus, neuronal proliferation is essentially completed before 24 wks gestation [52]. In contrast, it is estimated that on postnatal day 10, the rodent brain is roughly equivalent to that of the full term human brain of 38 to 40 weeks post-conception [52-54]. Extrapolating from this model, the brain of a rodent pup at birth (PD1) corresponds to that of a human fetal brain at or near 19–21 weeks gestation [53, 55]. The PD2 pup approximates that of a 22-23 week human in its neurodevelopment, PD3 corresponds with that of a 24-26 wk human, and PD6 approximates a 30-32 wk human. Our objective is to provide essential DEX and HC dosing protocol during postnatal ages that correspond neuron-developmentally to time points at which human premature infants are likely to receive prolonged GC courses in neonatal intensive care units. This timing is critical, because normal development of the human LHPA axis provides a low CORT milieu during this period, an environment that favors the bulk of alveolar formation in the developing lung in the rat.

Consistent with reviews, our results confirm that DEX decreases body weight [15-17]. Figure1 suggests that nutritional issues are a concern, particularly given that weight loss and slow somatic growth result in DEX treated pups. This was observed between DEX treated versus control animals, only after 24 hours of injection, which lead us to the notion that decreased nutritional intake, particularly free water, may play an acute role in immediate weight disparity. Inability to attach to the mother's nipples and inefficient sucking is conceivable: other investigators have shown that the dam spends more time giving nutrition, stimulation and warmth to a litter that is perceived to have poor health [56, 57]. Some factors that appear to heighten maternal sensitivity to their baby are decreased weight, reduction in motor movement, and enhanced vocalizations [58, 59]. Another possible explanation for the decreased somatic growth observed in the DEX-Treated pups is the direct DEX effects on catabolism and tissue accretion as

DEX has been shown to increase protein catabolism and prevent adequate growth [60-63]. Figure1 also suggests that HC does not alter somatic growth for both short and long-term treatments. More likely, the HC doses used are sub-optimal compared to DEX. This can be due to hydrocortisone's relative potency compared to DEX (DEX: HC = 1:10 to 1:100) or because of metabolic considerations that are unique to the rodent (i.e. increased metabolic rate of degradation). Another consideration is the fact that HC is not the endogenous GC in rodents, but this should not be an issue since both steroids are of the similar chemical classification such that GR and MR activation and metabolic pathways of degradation are identical [64].

We addressed the issue of DEX versus HC pharmacotherapy in this study by evaluating specific drug levels achieved and specific immediate effects of the GC dose schedules on parameters of GC action in lung tissues. An important GC's immediate effect on lung maturation can be quantified by measuring Surfactant Protein-A synthesis [38]. SP-A reduces alveoli surface tension for increased surface area of gas exchange [3, 65-67], and also takes a part in innate immunity and inflammatory processes to act as a first line of defence against inhaled microbes and pathogens [5]. With a goal of understanding GC's action on lung development, Floros and co-workers evaluated the postnatal DEX stimulation of SP-A synthesis in lung tissue and showed that within 24h of DEX injection, SP-A expression increased [37, 68]. Recently, Broussard and co-workers described lung growth and provided a baseline for changes in respiratory function [69]. As a next step, this study shows specific SP-A levels that are met after a series of DEX or HC injections and corresponding effects on lung tissue. Our data is consistent with published reports on lung protein induction by DEX [70] and suggests that the DEX protocol is enhancing surfactant synthesis and 11-β-HSD1 levels.

Focusing on Surfactant Protein-A protein synthesis, ANOVA revealed there is a treatment effect but no protocol effect. Figure 2b shows potent effect of DEX, increasing 1.7-1.9 fold SP-A translation compared to VEH. Consecutive DEX injections effectively up-regulated the fibroblast surfactant protein synthetic capacity for both Experiment 1 (Protocol A, PD 3-6) and Experiment 2 (Protocol B, PD 5-6). No protocol effect suggests that PD 5 and 6 may be critical for up-regulation of protein synthesis, and Protocol B may be a useful for other studies. Figure 4 shows that although DEX significantly increases SP-A gene transcription level in all animals, there was a sex effect with higher mRNA levels in females than males, which was especially obvious in Protocol B. Therefore, DEX protocol is a clinically relevant dose schedule; however, HC dose schedule was less effective at affecting SP-A mRNA levels. Since SP-A protein levels are more sensitive to GC, protein may be a better marker for lung maturation than other mRNA markers. For Experiment 1, HC administration on PD 3-6 effectively up-regulated SP-A protein synthesis; however, Experiment 2 on PD 5-6 showed no effect. A failure to observe an increase SP-A protein with short course (Protocol B) HC injection in Experiment 2 suggests type I error. A small sample size may attribute to the non-significant effects of HC because Experiment 3 clearly shows significant increase in SP-A protein synthesis following the same Protocol B with increased animal numbers (14 pups in Exp. 2, 16-20 pups in Exp. 3). Of four total HC short course protocols examined in the Experiment 3, the two lower doses were significantly increasing SP-A protein levels (Figure 6, Panel B). Comparing Panel A and B of the Figure 6 suggest that increasing HC doses injected corresponds to increasing pattern of CORT plasma level. In the higher HC doses, a decrease in SP-A protein production may be perhaps due to cell toxic effects of high plasma CORT level. For Experiment 3, we examined SP-A protein production by HC injection on PD5-6 for several reasons. First, limiting the amount and the time of exposure to GC agents have become a major

goal in intensive care units (NICU) [71]. Second, the greatest survival rate of 95% observed in NICU nationwide is in premature infants born at 28 weeks gestation [72, 73], and this newborn age coincides with postnatal day 5 in the rodent. Since, HC treatment typically lasts 3 to 4 weeks [74, 75], the period of PD 5 and PD 6 selected also coincides with the treatment length extensively used in NICUs.

The enzyme 11-β-HSD1 and the interaction among SP-A, GR and MR are important for lung maturation. 11-β-HSD1 is primarily a reductase in vivo producing active cortisol that binds to MR to enhance lung maturation at early developmental stages [43, 44]. This has led many scientists to the claim: "low endogenous fetal cortisol may exert actions at the high affinity MR in vivo" [76]. Our results show that there is a significant treatment effect, but no protocol and sex effects on 11-β-HSD1 mRNA levels in lung tissue. No protocol effect reinforces the idea that favors shorter duration of the protocols. The 11-β-HSD1 mRNA levels in DEX compared to VEH were significantly higher for both protocols; on the other hand, 11-β-HSD1 mRNA levels in HC compared to VEH were not significant for both protocols. This demonstrated potent DEX dosage, but leading to a question of insufficient HC dosage. However, the insufficient HC dosage hypothesis may be eliminated since SP-A protein level showed expected difference corresponding to HC treatment after increasing animal numbers. Perhaps, a failure to observe difference in 11-β-HSD1 mRNA levels may be related to specific time points of data collection. We collected 11-β-HSD1, GR and MR mRNA after 24 hours of last GC injection, arranging about 3 to 5 days after the first GC injection. Although significant for the protein assay, it may not be an optimal time point for mRNA assay, since other studies suggest that rapid induction of mRNA transcription takes as little as 15-30 minutes [77]. Also, GCs initially up-regulate GR transcription in infants; however, prolonged GC down-regulates GR by negative feedback [78]. Therefore,

11-β-HSD1, GR and MR mRNA after prolonged 3-5 days of injection may be too late to see rapid GC effects on mRNA transcription. Finally, DEX is an agonist for glucocorticoid receptor (GR); HC is an agonist for both GR and mineralocorticoid receptor (MR) [40]. Because HC has the ability to bind to both GR and MR, a lack of GR mRNA down-regulation by early HC treatment found in our preliminary data should not be construed as a lack of GR effect by HC.

Over 540,000 premature infants are born each year, and the preterm birth rate has increased by 36 percent in the United States since the 1980s [79]. There is a long history of corticosteroid treatment in newborn intensive care in order to aid critically ill infants' survival; however, even when premature babies do survive, they encounter the serious risk of lifetime physical and behavioral problems, such as breathing troubles, feeding difficulties, insensitivities to environmental changes, attention deficit disorder, emotional instability and mental retardation [79, 80]. As mentioned above , we focused on the use of GCs in the first week of life, because it is commonly used in neonatal clinical care for treatments, such as chronic lung disease and refractory hypotension [47]. In order to explain the biochemical and neuroanatomical changes by the early DEX and HC treatments, we assessed the GC schedules in our animal model to look for lung maturating markers: SP-A, 11-β-HSD1, GR and MR. With choices of doses and two protocols investigated, we can now experiment neurodevelopmental side-effects of corresponding GC doses.

Conclusions

We conclude that dexamethasone and hydrocortisone are potent stimulators of lung maturation, increasing significant amount of SP-A protein levels. Dexamethasone is especially potent since it also increased SP-A and 11-β-HSD1 mRNA synthesis following injection on PD 5-6. However, unlike dexamethasone, hydrocortisone does not result in adverse generalized effects on the organism. In addition, we have defined the optimal dose as 5 mg/kg on PD 5 followed by 1 mg/kg the next day to investigate other effects of HC by specific systems.

Our broader future goal is to understand and prevent the mechanisms that may lead to vulnerability to mental illness in a population with early GC exposures in life. Our hope is to establish protocols that would limit the use of GCs to the essential level and to find novel uses of established pediatric agents to reverse negative psychological effects of early GC exposure.

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Figures

Figure 1 – Body weight for Protocol A and B

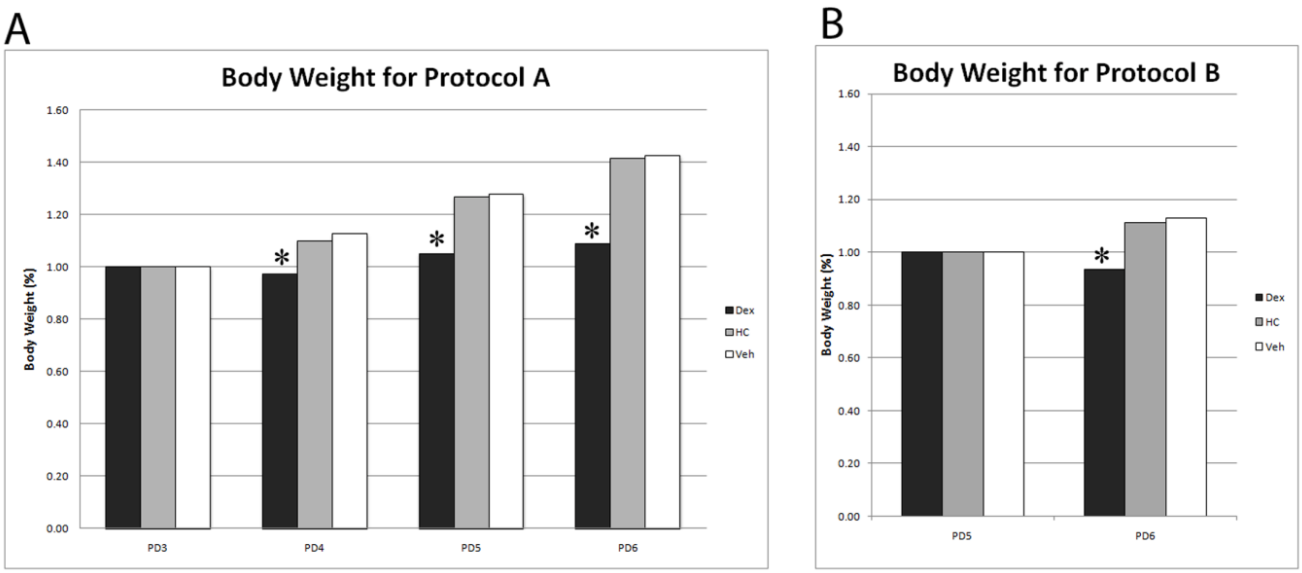


Figure 1. % Body Weight for Protocols A and B
*: DEX treated pups show significant difference in percent body weight compared to VEH, 24 hours following the last injection

Experiment 1: Panel A shows the body weight progression for pups in Protocol A (DEX group received 0.5mg/kg, 0.25mg/kg, 0.125mg/kg and 0.05mg/kg on PD 4-6; HC group received 5mg/kg, 2.0mg/kg, 1.0mg/kg and 0.5mg/kg on PD 4-6; VEH group received saline).
For Protocol A, ANOVA revealed significant treatment ($p<0.0001$), time effect ($p<0.0001$) and Post-hoc Fisher PLSD showed DEX different from VEH ($p<0.0001$) from days PD 4-PD6.

Experiment 2: Panel B includes a bar graph showing body weight progression for Protocol B (DEX group received 0.5mg/kg and 0.1mg/kg on PD 5-6; HC group received 5.0mg/kg and 1.0mg/kg on PD 5-6; VEH group received equivalent volumes of sterile saline).
For Protocol B, ANOVA revealed significant treatment effect ($p<0.0001$) and Post-hoc Fisher PLSD showed DEX different from VEH ($p<0.0001$) at PD6.

Figure 2a – Western Blot for SP-A Protein

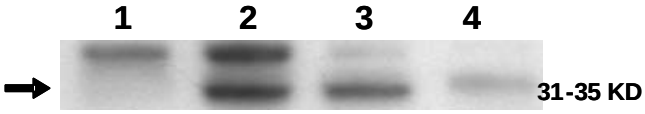


Fig 2. SP-A Western Blotting. Lane 1-3 contained 20ug of total protein. Lane1- negative control from spleen sample, Lane 2- male rats lung samlple, Lane 3- female rats lung sample, Lane 4- positive control from Santa Cruz (loaded 5ul, Cat#:sc-2390)

Figure 2b – SP-A Protein levels for Protocol A and B

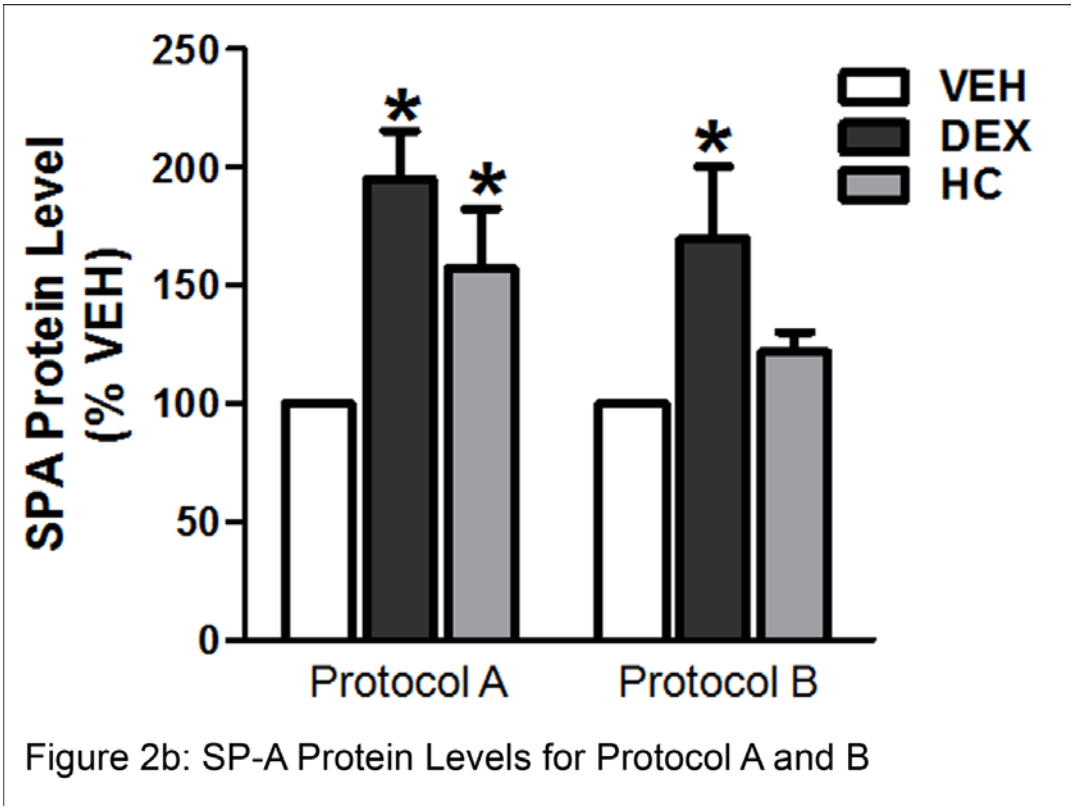
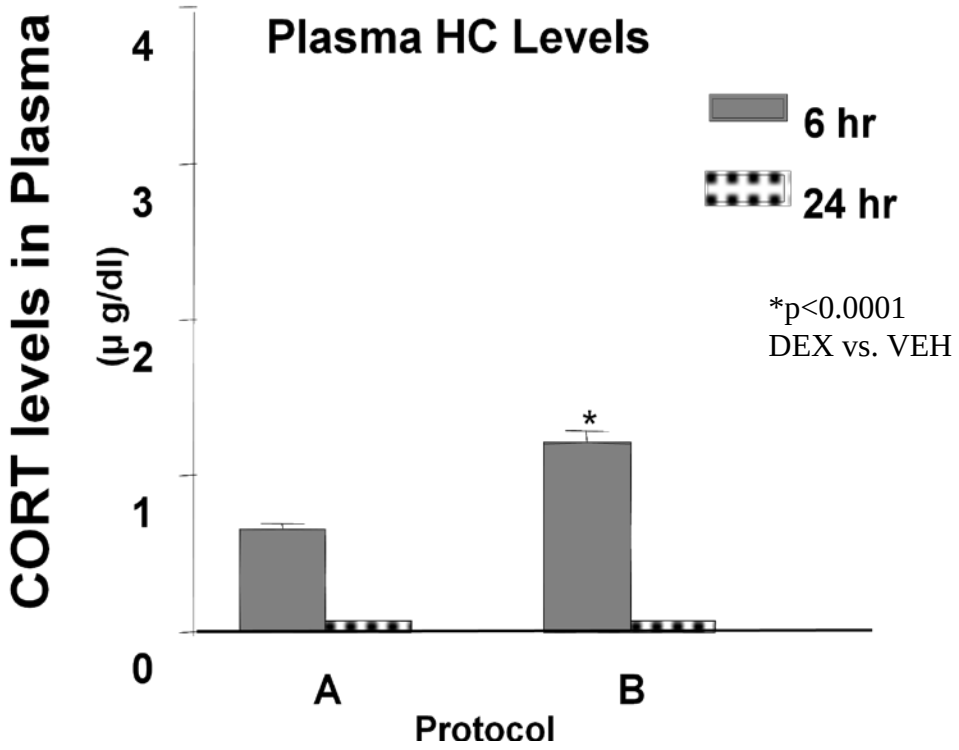


Figure 2b: SP-A Protein Levels for Protocol A and B

Protocol A (PD3-6):DEX—0.5mg/kg on PD3, 0.25mg/kg on PD4, 0.125mg/kg on PD5 and 0.05mg/kg on PD6 and HC—5mg/kg on PD3, 2.0mg/kg on PD4, 1.0mg/kg on PD5 and 0.5mg/kg on PD6; Protocol B (PD5-6): DEX—0.5mg/kg on PD5, 0.1mg/kg on PD6; HC—5.0mg/kg on PD5, 1.0mg/kg on PD6. *$p<0.05$ DEX or HC vs. VEH
ANOVA revealed treatment effect ($p=0.0001$). Post-hoc Fisher PLSD showed, DEX is significantly different from VEH in Protocol A ($p=0.0009$) and Protocol B ($p=0.0098$); HC is significantly different from VEH in protocol A only (Protocol A: $p=0.0363$).

Figure 3 – Plasma CORT levels for Experiment 1 and 2



Experiment 1. Protocol A (PD3-6):DEX—0.5mg/kg on PD3, 0.25mg/kg on PD4, 0.125mg/kg on PD5 and 0.05mg/kg on PD6 and HC—5mg/kg on PD3, 2.0mg/kg on PD4, 1.0mg/kg on PD5 and 0.5mg/kg on PD6
Experiment 2. Protocol B (PD5-6): DEX—0.5mg/kg on PD5, 0.1mg/kg on PD6; HC—5.0mg/kg on PD5, 1.0mg/kg on PD6.

Plasma CORT levels measured after 6 hours and 24 hours of last GC injection and quantified by RIA. ANOVA revealed significant difference in plasma CORT levels for DEX injection on PD5 and 6. Post-hoc Fisher PLSD showed significant difference in DEX treated animals (*p<0.0001 DEX vs. VEH)

Figure 4 – SP-A mRNA levels for Protocol A and B

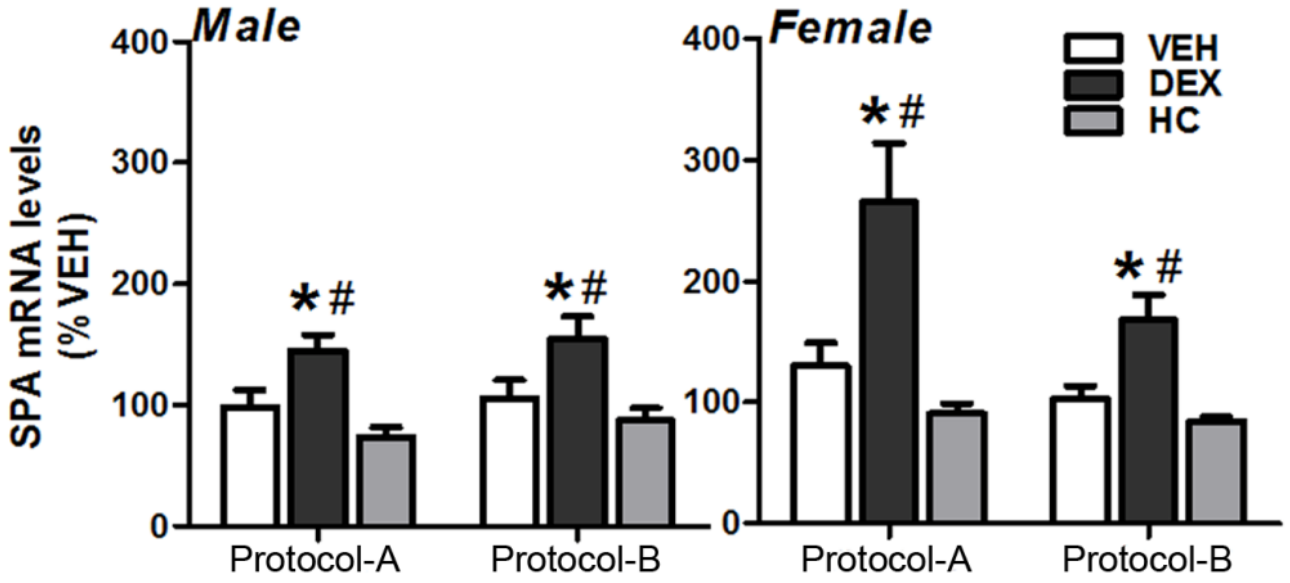


Fig 4. SPA mRNA levels. Protocol -A (PD3-6): DEX - 0.5 mg/kg on PD3, 0.25 mg/kg on PD4, 0.1 mg/kg on PD5, 0.05 mg/kg on PD6 and HC – 5 mg/kg on PD3, 2.0 mg/kg on PD4, 1.0 mg/kg on PD5, 0.5 mg/kg on PD6 ; Protocol-B (PD5-6): DEX - 0.5 mg/kg on PD5, 0.1 mg/kg on PD6 and HC - 5 mg/kg on PD5, 1 mg/kg on PD6. *: $p<0.05$ DEX vs VEH, #: $p<0.05$ DEX vs HC

ANOVA showed significant treatment effects (ANOVA with post-hoc, $p<0.0001$, n=7 for each group) and sex effects ($p=0.0097$, n=7 for each group) with SP-A mRNA levels higher in female samples. Post-hoc Fisher PLSD showed significant difference in DEX treated animals for both protocols A and B in male and female samples compared to VEH and HC groups. There was no difference in the HC treated animals compared to VEH.

Figure 5 – 11-β-HSD1 mRNA levels for Protocol A and B

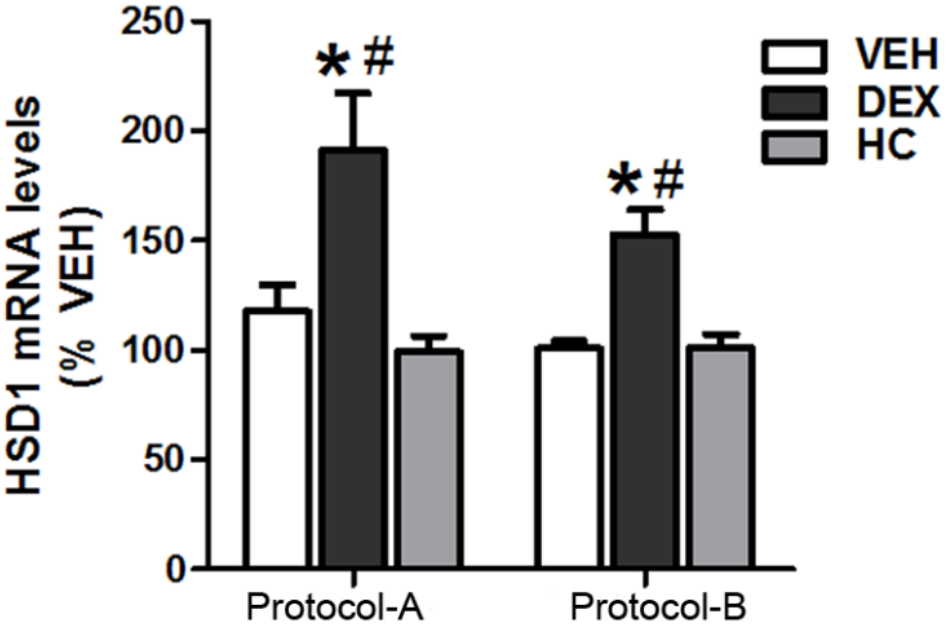


Fig 5. HSD1 mRNA levels. Protocol-A (PD3-6): DEX - 0.5 mg/kg on PD3, 0.25 mg/kg on PD4, 0.1 mg/kg on PD5, 0.05 mg/kg on PD6 and HC – 5 mg/kg on PD3, 2.0 mg/kg on PD4, 1.0 mg/kg on PD5, 0.5 mg/kg on PD6 ; Protocol-B (PD5-6): DEX - 0.5 mg/kg on PD5, 0.1 mg/kg on PD6 and HC - 5 mg/kg on PD5, 1 mg/kg on PD6. *: $p<0.05$ DEX vs VEH, #: $p<0.05$ DEX vs HC

The two way ANOVA showed that there is a significant treatment effects (ANOVA with post-hoc, $p<0.0001$, n=7 for each group) and sex effects ($p=0.0097$, n=7 for each group). SP-A mRNA levels were significantly increased in DEX treated animals for both protocols A and B in male and female samples compared to VEH and HC groups. The levels of the SP-A mRNA were much higher in female samples. There was no difference in SPA mRNA levels in the HC treated animals compared to VEH groups.

Figure 6 – CORT and SP-A Protein Levels for Protocol B

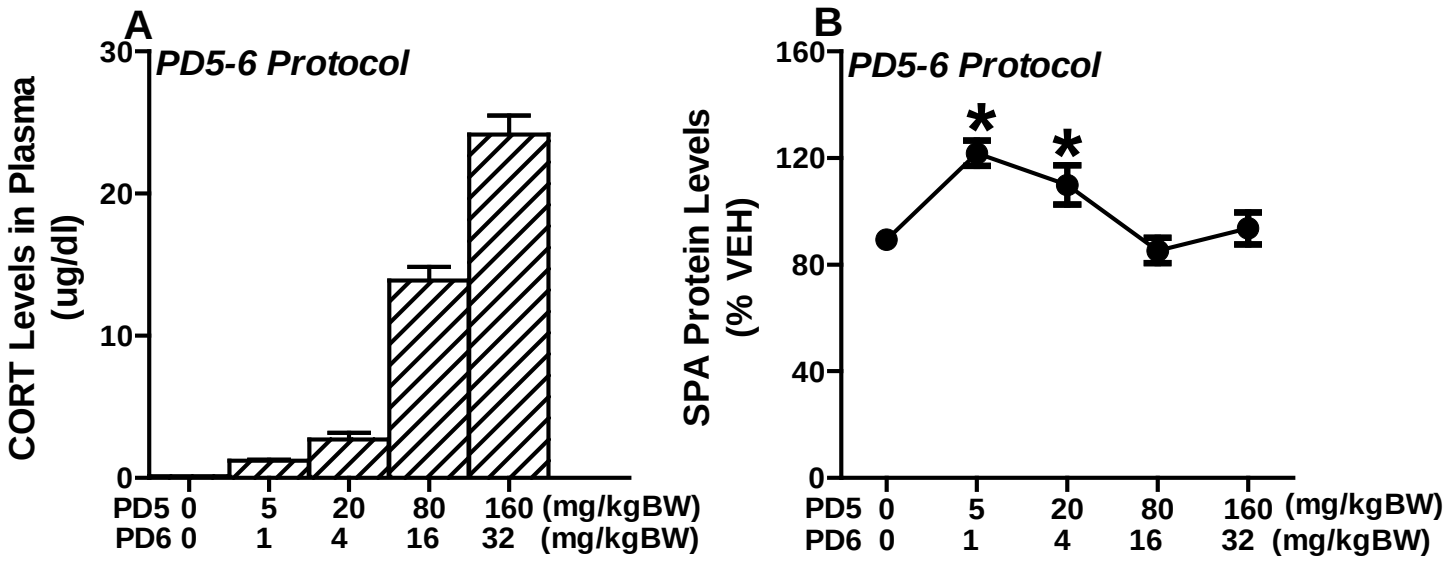


Fig 6. CORT levels in plasma (Panel A) and SPA protein levels in lung tissue (Panel B), which obtained from animals treated with HC using a serial doses of 5, 20, 80, 160 m/kg on PD5 and 5x less doses on PD6. ANOVA revealed a dose effect ($p<0.0001$), 5&20 mg/kg starting doses were significantly different from VEH.

ANOVA revealed treatment effect ($p=0.0001$). Post-hoc Fisher PLSD showed two significant dosing protocols different from VEH ($p<0.05$): 5mg/kg on PD 5 followed by 1mg/kg on PD6, 20 mg/kg on PD5 followed by 4 mg/kg on PD 6.
As HC injection dose increased, plasma CORT levels increased. This led to a decrease in SP-A protein production, perhaps due to cell toxic effects or receptor down-regulation due to a negative feedback.

Tables

Table 1 – Experiment 1 and 2 treatment doses

Table 1. Injection doses for Protocol A and B

	DEX (mg/kg)				HC (mg/kg)			
Protocol	PD3	PD4	PD5	PD6	PD3	PD4	PD5	PD6
A	0.5	0.25	0.125	0.05	5.0	2.0	1.0	0.5
B			0.5	0.1			5.0	1.0

Experiment 1: for Protocol A, pups in the DEX group received intramuscular injection of 0.5mg/kg DEX on PD3, 0.25mg/kg on PD4, 0.125mg/kg on PD5 and 0.05mg/kg on PD6; HC group received 5mg/kg of HC on PD3, 2.0mg/kg on PD4, 1.0mg/kg on PD5 and 0.5mg/kg on PD6.
Experiment 2: Protocol B, pups in the DEX group received 0.5mg/kg of DEX on PD5 and 0.1mg/kg on PD6; HC group received 5.0mg/kg of HC on PD5 and 1.0mg/kg on PD6.
For both experiments, VEH group served as controls receiving the equivalent volumes of sterile saline.

Table 2 – Experiment 3 treatment doses

Table 2. HC doses for Plasma Hormone Assay

	PD5 (mg/kg)	PD6 (mg/kg)
Group 1	5	1
Group 2	20	4
Group 3	80	16
Group 4	160	32

HC doses were injected for two days on PD5 (5mg/kg, 20mg/kg, 80mg/kg or 160mg/kg) followed by a serial of doses that were 5x less given on PD6 (1mg/kg, 4mg/kg, 16mg/kg or 32mg/kg correspondingly).

Table 3 – Sequences of All Primers for RT-PCR

Primer Name	Primer Label	Sequences
GR	GR-L	GACATGTGGAAGCTGCAAAGTA
	GR-R	TCGTTTCTTCCAGCACAAAG
MR	MR-L	CCTGGCAGCGAAACAGAT
	MR-R	TCCTCGAGAGGCAAGTTTTT
11-Beta-HSD1	HSD1-L	CTACAAATGAAGAGTTCAGACCAGA
	HSD1-R	GCCCCAGTGACAATCACTTT
SP-A	SP-A-L	CCTGGAGGAATGCCAGGTCT
	SP-A-R	GAGCTCCTCATCCAGGTAAGC

The table lists sequences of forward primer and reverse primer for all genes that were studied by RT-PCR, including SP-A, 11-β-HSD1, GR and MR.