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Original Article
Combined antenatal and postnatal steroid effects on fetal and postnatal growth, and neurological outcomes in neonatal rats

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Abstract: Preterm infants are often exposed to both antenatal and postnatal glucocorticoids (GCs). We tested the hypothesis that combined antenatal and postnatal GCs have long-lasting adverse effects on fetal and neonatal growth, growth factors, and neurological outcomes. Pregnant rats were administered a single IM dose of betamethasone (0.2 mg/Kg, AB), dexamethasone (0.2 mg/Kg, AD), or equivalent volumes of saline (AS) at 17 & 18 days gestation. Following delivery, pups from each treatment group were sacrificed at P0, and the remainder was treated with a single IM dose of either betamethasone (0.25 mg/Kg, PB), dexamethasone (0.25 mg/Kg, PD), or equivalent volumes of saline (PS) on P5, P6, and P7. Somatic growth, neurological status, and growth factors were determined at P14, P21, and P45. At birth, AD resulted in decreased somatic growth. AB advanced the hopping reflex associated with spinal rhythmic mechanisms. At P21, all GC groups were growth suppressed, but only the AS/PD group had deficits in brain weight and delayed plantar reflex associated with brainstem function. By P45, sustained reductions in body and brain weight occurred all combined antenatal and postnatal GC groups, as well as elevated ACTH and corticosterone. Retardation in plantar reflex occurred in all AD groups. IGF-I, GH and insulin levels were elevated at all ages with dexamethasone. Combined antenatal and postnatal GCs has persistent detrimental lasting effects on growth, growth factors, neurological outcomes, and HPA axis activity. Whether these effects persist in adult life and are risk factors for insulin resistance, remains to be elucidated.

Keywords: Betamethasone, dexamethasone, growth hormone, insulin, insulin-like growth factor-I, neurological outcomes

Introduction

Glucocorticoids (GCs) such as betamethasone and dexamethasone are used antenatally to reduce the risk of complications associated with preterm delivery [1] and prevent or treat chronic lung disease [2]. Dexamethasone and betamethasone are the two most commonly used corticosteroids for antenatal therapy. These drugs are identical in biological activity and readily cross the placenta in their active forms. Treatment of 2 doses of 12 mg of betamethasone given intramuscularly (IM), every 24 hours, or four doses of 6 mg of dexamethasone given IM every 12 hours has been shown to deliver concentrations to the fetus that are comparable to physiologic stress levels of cortisol after birth. These levels are estimated to occupy 75% of available corticosteroid receptors, which should induce maximal antenatal

corticosteroid receptor-mediated response in the fetal target tissues [3]. The effects of antenatal GCs are maximal at 48 hours after administration, and decrease by 7 and 10 days after administration therefore repeated courses are given on a weekly basis when there is a high risk of preterm birth [4]. Although a single dose of antenatal GCs may provide levels equivalent to cortisol levels, repeated doses can and do result in supraphysiologic levels that are harmful to the fetus [5, 6]. Clinical studies demonstrate decreased head size and visual memory with antenatal betamethasone, and abnormal neurological outcomes such as rate of neuromotor abnormalities and cerebral palsy with postnatal dexamethasone [7].

The relationship between antenatal GCs and reduced fetal growth has been documented in human and animal studies [8, 9]. Single courses of betamethasone treatment cause suppression of the human fetal adrenal gland for 4 days [5]. A follow-up study for 3 years of preterm infants have shown that repeated corticosteroid courses were associated with decreased size at birth [10]. The effects of antenatal corticosteroid treatment on body and brain growth may be due to its effects on insulin-like growth factors (IGFs-I and -II). Small for gestational age (SGA) babies who fail to demonstrate catch-up growth are at increased risk of developing insulin resistance and cardiovascular disease in later life [11]. In preterm lambs, prenatal exposure to GCs given at mid-gestation resulted in growth retardation and changes in lung structure [12]. Other studies have demonstrated that short courses and low doses of GCs can alter brain development and cause growth failure [13], particularly growth of the cerebellum [14-19]. Immature rats treated with a single dose of dexamethasone (1 mg/kg body weight, or 20 mg/kg body weight) had impaired growth of the whole body, brain, and thymus [20]. These negative effects on fetal growth may be due to alterations in insulin-like growth factor (IGF)-I, which plays a critical role in fetal and postnatal growth and development [21-23].

IGFs are growth-promoting peptides that are structurally homologous with insulin [24]. The insulin-IGF family comprises insulin, insulin-like growth factor-1 (IGF-1), IGF-II, and IGF binding proteins [25]. IGFs exert their action by binding to receptors. Availability to their receptors is

regulated by IGF binding proteins (IGFBP 1-6) [26]. Binding proteins can cause either inhibitory or stimulatory effects on IGF action. IGF binding protein-3 (IGFBP-3) promotes IGF-I activity, prolongs its half-life, and transports it to target cells. IGFBP-3 is the predominant binding protein promoting growth in the early neonatal period. Conversely, IGFBP-1 suppresses IGF-I and is associated with decreased somatic growth and insulin resistance [24]. While insulin is known to augment fetal growth by stimulating the production of IGF-I, insulin deficiency is associated with high IGFBP-1 and low IGF-I levels [24, 27, 28]. In intrauterine growth restricted fetuses, marked elevations in serum IGFBP-1 and deficits in IGFBP-3 have been noted [24, 29, 30]. Insulin is the major regulator of IGFBP-1 and plays a key role in fetal and early postnatal growth [24].

Postnatal steroids are used in preterm infants to prevent or treat bronchopulmonary dysplasia (BPD), for hemodynamic support, and to facilitate early extubation [31, 32]. However, early exposure to GCs cause many complications, such as high blood pressure and blood glucose, gastrointestinal (GI) bleeding, spontaneous (GI) perforation, increased risk for cerebral palsy, and growth failure [32, 33]. Given that a significant number of premature infants are exposed to both antenatal and postnatal GCs, there is an imperative to determine whether dual exposure will further exacerbate postnatal growth and neurological outcomes. We therefore hypothesized that combined antenatal and postnatal GCs have long-lasting adverse effects on fetal and neonatal growth, growth factors, and neurological outcomes.

We examined and compared the effects of antenatal and/or postnatal GCs on fetal and postnatal growth, factors that influence growth, and neurological outcomes in newborn, weaned, and adolescent rats.

Materials and methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee, Long Beach Memorial Medical Center, Long Beach, CA. Animals were cared for according to the guidelines outlined by the Association for the Assessment and Accreditation of Laboratory

Animal Care (AAALAC) and the Guide for the Care and Use of Laboratory Animals (National Research Council) (National Research Council). Euthanasia of the animals was conducted according to the guidelines of the American Veterinary Medical Association (AVMA Panel). Timed pregnant Sprague Dawley rats (200-300 grams body weight) were purchased from Charles River (Hollister, CA) at 15 days gestation. The pregnant rats were allowed to stabilize for 48 hours under controlled environmental conditions with free access to food and water.

Experimental design

At 17 days gestation (E17) and E18, the pregnant dams were randomized to receive either a single injection of betamethasone (0.2 mg/kg) IM diluted in sterile 0.9% saline to a volume of 0.25 mL (n=6), or single injections of dexamethasone (0.2 mg/kg) IM diluted in sterile 0.9% saline to a volume of 0.25 mL (n=6). Controls animals were administered equivalent volume saline IM (n=6). The animals remained undisturbed until delivery of their pups. At birth (P0) 10 pups/group were euthanized and the remainder was pooled and randomly distributed to matched treated dams. At P5, the pups were randomly assigned to receive dexamethasone (D, 0.25 mg/Kg, IM), betamethasone (B, 0.25 mg/Kg), or equivalent volume saline (S) on P5, P6, and P7, resulting in antenatal/postnatal treatment groups: 1) S/S; 2) S/D; 3) S/B; 4) D/S; 5) D/B; 6) D/D; 7) B/S; 8) B/D; and 9) B/B (n=10 pups/group). Treatment at P5 is in accord with the delayed dexamethasone treatment of BPD [34]. The animals were monitored for body weight, anthropomorphic growth, and neurological development. At euthanasia (term, P21 and P45), blood samples were analyzed for insulin-like growth factor (IGF)-I, growth hormone (GH), and insulin levels. Due to the small blood volume at birth, the expression of IGF-I, IGF-IR, IGFBP-1 and IGFBP-3 was determined in the cerebral cortex of term animals to correlate with neurodevelopment outcomes.

Neurodevelopment testing

Rats were assessed for neurological development at P0, P14, P21 and P45 using reflex-stimulus-responses as previously described [35]. All neurological tests were conducted in a masked manner by two individuals. The tests were conducted on all animals in each group.

Responses were recorded as 0 (no response), 1 (mild response), or 2 (full response).

Assay of serum IGF-I and GH levels

Levels of IGF-I and GH in serum samples were determined at P14, P21 and P45 (n=6/group) using commercially available enzyme immunoassay kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's protocol.

Assay of plasma insulin

Insulin levels in the plasma were determined at P14, P21 and P45 (n=6/group) using enzyme immunoassay kits purchased from Cayman Chemicals (Ann Arbor, MI, USA) according to the manufacturer's protocol.

Assay of plasma corticosterone

Plasma corticosterone levels were determined at P45 (n=6/group) using enzyme immunoassay kits purchased from Enzo Life Sciences (Farmingdale, NY, USA) according to the manufacturer's protocol.

Assay of plasma ACTH

Plasma ACTH levels were determined at P45 (n=5/group) using enzyme immunoassay kits purchased from Sigma-Aldrich (St. Louis, MO, USA) according to the manufacturer's protocol.

Isolation of total RNA

Total cellular RNA in the cerebral cortex of term animals (n=4/group; 2 males and 2 females) was extracted by homogenization using a polytron homogenizer (Brinkman Instruments, Inc., Westbury, N.J.) as previously described [36].

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out using cDNA amplification kits purchased from Perkin Elmer, Norwalk, CT, USA and sense and antisense primers for rat GAPDH, IGF-I receptor, IGFBP-1 and IGFBP-3 (Life Technologies, Carlsbad CA, USA), and according to previous reports [36-38].

Densitometric scanning

Gel electrophoresis of the PCR products was performed on 1.5% agarose gels stained with EtBr. The intensities of the bands were meas-

Table 1. Effects of antenatal steroids on growth and neurological status in rats at birth (P0)

Growth Parameters	Saline	Betamethasone	Dexamethasone
Total Body Weight (g)	6.00 ± 0.22	6.55 ± 0.12**	5.73 ± 0.12*
Linear Growth			
Tail length (cm)	1.74 ± 0.04	1.80 ± 0.03	1.57 ± 0.03**
Tibia length (cm)	1.15 ± 0.04	1.22 ± 0.02	1.19 ± 0.06
Organ Weights (g)			
Brain	0.22 ± 0.01	0.26 ± 0.01**	0.23 ± 0.003
Heart	0.05 ± 0.005	0.038 ± 0.002	0.03 ± 0.002*
Lungs	0.13 ± 0.005	0.11 ± 0.006	0.12 ± 0.005
Liver	0.26 ± 0.02	0.24 ± 0.01	0.22 ± 0.011
Kidneys	0.07 ± 0.005	0.07 ± 0.003	0.07 ± 0.003
Pancreas	0.03 ± 0.004	0.02 ± 0.002	0.01 ± 0.002**
Spleen	0.02 ± 0.003	0.02 ± 0.003	0.02 ± 0.002

Neurological tests (% of animals responding)

	None	Mild	Full	None	Mild	Full	None	Mild	Full
Palmar	10%	90%	0%	10%	60%	30%	10%	90%	0%
Plantar	70%	30%	0%	50%	50%	0%	80%	20%	0%
Hopping	80%	20%	0%	0%**	80%*	20%	50%	50%	0%
Tactile	50%	50%	0%	10%	60%	30%	20%	80%	0%
Negative geotaxis	20%	60%	20%	0%	80%	20%	10%	90%	0%
Freefall righting	80%	10%	10%	50%	0%	50%	100%	0%	0%

Pregnant rats were administered saline, betamethasone (0.2 mg/kg/day) or dexamethasone (0.2 mg/kg/day) intramuscularly (IM) on 17 and 18 days of gestation. Data are expressed as mean ± SEM where applicable (n=30/group). Data for neurological tests were examined using the Fisher's exact test (none =0; Mild =1; Full =2). *$P<0.05$, **$P<0.01$ vs. Saline.

st. Data for neurological development were analyzed using the Fisher exact test. Data are expressed as mean ± SEM. A *p*-value of less than or equal to 0.05 was considered significant. Statistical analyses were accomplished with the use of SPSS, version 21; and all graphs were prepared with the use of GraphPad Prism, version 7.

Results

Antenatal steroids, fetal growth and neurological outcomes

Table 1 shows the effect of antenatal steroids on fetal growth and neurological tests. All animals were born at E22 (term). Exposure to betamethasone resulted in increased mean body weight and brain weights, while exposure to dexamethasone reduced me-

ured with the use of a GelDoc 1000 Darkroom Imager and Molecular Analyst software (BioRad Laboratories, Hercules, CA). The PCR fragments were identified according to their molecular mass using a DNA mass ladder (Perkin Elmer, Norwalk, CT). The amount of DNA in each specimen was quantitated by the integrated density of the product bands within a closed rectangle, which was then normalized to the density of the GAPDH bands. The data are expressed as mean IGF-IR, IGFBP-1 and IGFBP-3 to GAPDH ratio ± SEM (n=4 samples/group; 2 males and 2 females).

Statistical analyses

Analysis of variance was used to examine differences among the treated and control groups for the each measured variable. Post hoc test for significance was determined using the Student-Newman-Keuls test. Non-normally distributed data was analyzed using Kruskall Wallis test with Dunn's multiple comparison te-

an body weight, tail length, and heart and pancreas weights compared to placebo saline. Antenatal betamethasone improved the hopping reflex with 80% demonstrating mild response and 20% exhibiting full responses compared to the placebo saline and dexamethasone groups.

Postnatal steroids, postnatal growth and neurological outcomes

At P5 (before postnatal steroid treatment), animals that received antenatal steroids had reduced body weight and linear growth compared to placebo saline (data now shown). At P14 (one week after postnatal steroid treatment), there were no differences in anthropomorphic growth, but there were significant effects on neurological outcomes. In the animals that received postnatal steroids only, neurological testing showed major reductions in plantar, tactile, and negative geotaxis reflexes in the group AS/PB group; and reductions in the

Table 2. Effect of combined antenatal and postnatal steroids on neurological status at P14

	AS/PS	AS/PB	AS/PD	AB/PB	AB/PS	AB/PD	AD/PD	AD/PS	AD/PB
(Mild response) % of animals responding									
Palmar	0	0	0	60%*	0	0	0	0	0
Plantar	10%	30%	0*	40%*	10%	10%	30%*	0*	40%*
Hopping	0	0	0	10%	0	10%	0	0	10%
Tactile	20%	40%*	0*	30%	0*	20%	0*	10%	20%
Negative geotaxis	10%	20%	30%*	100%*	40%*	40%*	10%	60%*	40%*
Freefall righting	0	0	20%*	0	0	60%*	20%*	20%*	0
(Full response) % of animals responding									
Palmar	100%	100%	100%	40%	100%	100%	100%	100%	100%
Plantar	90%	70%*	100%	60%	90%	90%	70%*	100%	60%
Hopping	100%	100%	100%	90%	100%	90%	100%	100%	90%
Tactile	80%	60%*	100%	70%	100%	80%	100%	90%	80%
Negative geotaxis	90%	80%	70%*	0*	60%*	60%*	90%	40%*	60%*
Freefall righting	100%	100%	80%*	100%	0*	40%*	80%*	80%*	100%

For antenatal steroid exposure, pregnant rats were administered antenatal saline, betamethasone (0.2 mg/kg/day) or dexamethasone (0.2 mg/kg/day) intramuscularly (IM) on 17 and 18 days of gestation. For postnatal steroids, pups were administered saline, betamethasone (0.25 mg/kg) or dexamethasone (0.25 mg/kg on postnatal day 5, 6, and 7. AS/PS (antenatal saline+postnatal saline); AS/PB (antenatal saline+postnatal betamethasone); AS/PD (antenatal saline+postnatal dexamethasone); AB/PB (antenatal betamethasone+postnatal betamethasone); AB/PS (antenatal betamethasone+postnatal saline); AB/PD (antenatal betamethasone+postnatal dexamethasone); AD/PD (antenatal dexamethasone+postnatal dexamethasone); AD/PS (antenatal dexamethasone+postnatal saline); and AD/PB (antenatal dexamethasone+postnatal betamethasone). None of the pups exhibited 0 response. *$P<0.05$ vs. AS/PS (Fisher's exact test).

Table 3. Effect of combined antenatal and postnatal steroids on neurological status at P21

	AS/PS	AS/PB	AS/PD	AB/PB	AB/PS	AB/PD	AD/PD	AD/PS	AD/PB
(Mild response) % of animals responding									
Palmar	0	0	0	0	0	0	10%	0	0
Plantar	0	20%*	10%	0	0	0	30%*	10%	40%*
Hopping	0	0	0	0	0	0	0	0	0
Tactile	0	0	0	0	0	0	10%	0	0
Negative geotaxis	30%	20%	10%*	0	0	0	10%	10%	0
Freefall righting	0	0	0	0	0	0	0	0	0
(Full response) % of animals responding									
Palmar	100%	100%	100%	100%	100%	100%	90%	100%	100%
Plantar	100%	80%*	90%	100%	100%	100%	70%*	90%	60%*
Hopping	100%	100%	100%	100%	100%	100%	100%	100%	100%
Tactile	100%	100%	100%	100%	100%	100%	90%	100%	100%
Negative geotaxis	70%	80%	90%*	100%*	100%*	100%*	90%*	90%*	100%*
Freefall righting	100%	100%	100%	100%	100%	100%	100%	100%	100%

For antenatal steroid exposure, pregnant rats were administered antenatal saline, betamethasone (0.2 mg/kg/day) or dexamethasone (0.2 mg/kg/day) intramuscularly (IM) on 17 and 18 days of gestation. For postnatal steroids, pups were administered saline, betamethasone (0.25 mg/kg) or dexamethasone (0.25 mg/kg on postnatal day 5, 6, and 7. AS/PS (antenatal saline+postnatal saline); AS/PB (antenatal saline+postnatal betamethasone); AS/PD (antenatal saline+postnatal dexamethasone); AB/PB (antenatal betamethasone+postnatal betamethasone); AB/PS (antenatal betamethasone+postnatal saline); AB/PD (antenatal betamethasone+postnatal dexamethasone); AD/PD (antenatal dexamethasone+postnatal dexamethasone); AD/PS (antenatal dexamethasone+postnatal saline); and AD/PB (antenatal dexamethasone+postnatal betamethasone). None of the pups exhibited 0 response. *$P<0.05$ vs. AS/PS (Fisher's exact test).

negative geotaxis and freefall righting reflexes in the AS/PD group. None of the animals exhibited 0 responses (**Table 2**).

In the animals that received antenatal betamethasone, reductions in palmar, plantar, hopping, tactile and negative geotaxis reflexes oc-
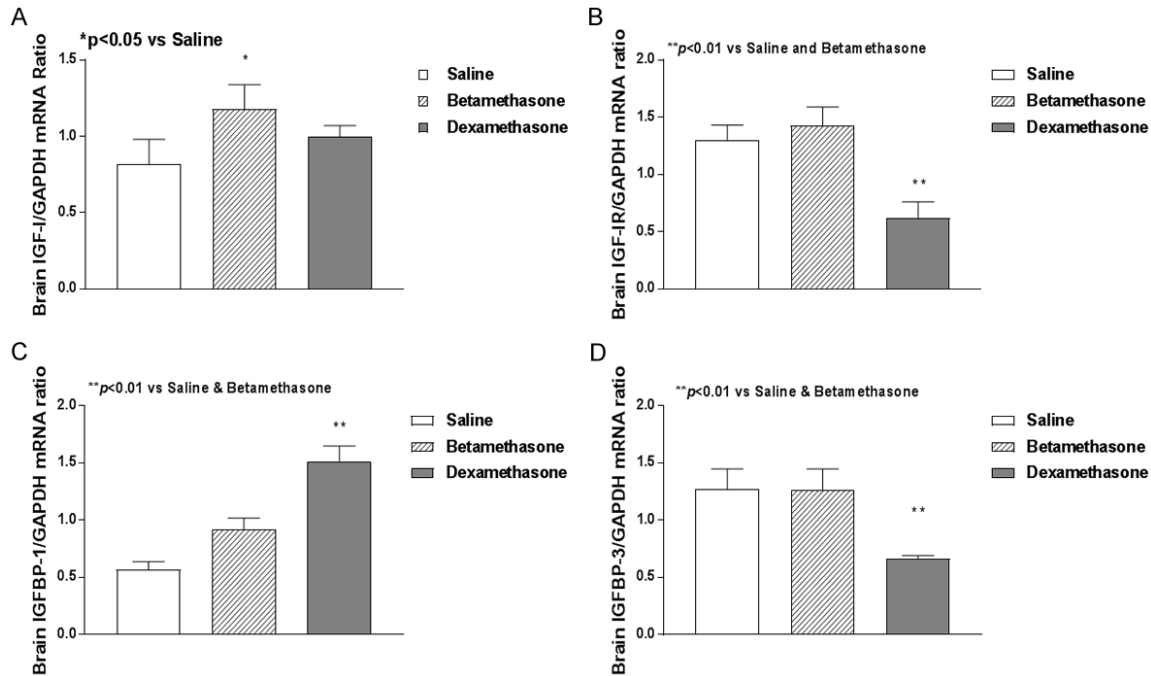
Figure 1. Messenger RNA expression of IGF-I (A), IGFIR (B), IGFBP-1 (C) and IGFBP-3 (D) in the newborn rat brain at birth (P0). Pups were exposed antenatally to 0.25 mL betamethasone or dexamethasone (0.2 mg/kg in sterile normal saline), injected intramuscularly to the pregnant dam on E17 and E18. Control pups were exposed to equivalent volume sterile normal saline. Data are expressed as mean ratio of genes/b-actin (n=4 samples/group; 2 males and 2 females). *P<0.05; **P<0.01 vs. placebo saline.

curred in the AB/PB group; reductions in negative geotaxis and freefall righting reflexes occurred in the AB/PS group; and reductions in hopping, negative geotaxis and freefall righting occurred in the AB/PD group. In the animals that received antenatal dexamethasone, reductions in plantar and freefall righting occurred in the AD/PD group; reductions in negative geotaxis and freefall right occurred in the AD/PS group; and reductions in plantar, hopping and negative geotaxis occurred in the AD/PB group.

At P21, a time of weaning from the dams and when the pups begin eating solid food, animals in the AS/PB (41.6 ± 0.8, P<0.01) AS/PD (43.4 ± 1.5, P<0.01), AB/PS (45.0 ± 0.81, P<0.01), AD/PD (45.3 ± 3.0, P<0.01) and AD/PS (42.1 ± 0.52, P<0.01) groups had lower body weights while animals exposed to AB/PB (53.5 ± 0.9, P<0.05) had higher body weight compared to placebo saline (49.7 ± 0.7). There were no differences in body weight noted in the AB/PD and AD/PB groups. None of the groups exhibited differences in body length. Brain weight was reduced in the AS/PD (1.31 ± 0.02 vs. 1.47 ± 0.03, P<0.01) group, while midbrain (0.34 ±

0.02 vs. 0.2 ± 0.01, P<0.01) and cerebellum (0.21 ± 0.006 vs. 0.17 ± 0.01, P<0.01) weights were increased with AB/PB and AD/PB groups, respectively, compared to controls. Neurological outcomes showed sustained plantar reflex reductions in the AS/PB, AS/PD, AD/PD, AD/PS, and ADPB groups; reduced palmar reflex in the AD/PD group; and reduced tactile reflex in the AD/PD group (**Table 3**).

At P45, lower body weight was noted in the AB/PS (175.4 ± 6.0 vs. 216 ± 12.1, P<0.05) group as was lower tail length in the AS/PB (14.7 ± 0.3, P<0.01), AB/PS (14.0 ± 0.1, P<0.01), AD/PD (14.8 ± 0.4, P<0.01), AD/PS (15.2 ± 0.2, P<0.05), and AD/PB (14.7 ± 0.3, P<0.01) groups compared to control (16.6 ± 0.4). Lower tibia length was noted in the AS/PB (4.1 ± 0.08, P<0.05) and AD/PB (4.2 ± 0.05, P<0.05) groups compared to control (4.5 ± 0.1). Brain weight was lower in the AS/PB (1.7 ± 0.02, P<0.01), AB/PB (1.6 ± 0.05, P<0.01), AB/PS (1.6 ± 0.15, P<0.01), AB/PD (1.56 ± 0.04, P<0.01), AD/PD (1.7 ± 0.03, P<0.01), and AD/PB (1.7 ± 0.03, P<0.01) groups compared to control (1.9 ± 0.02). Cerebellum and lung weights were lower in the AB/PD (0.18 ± 0.03 vs. 0.29 ± 0.01) and
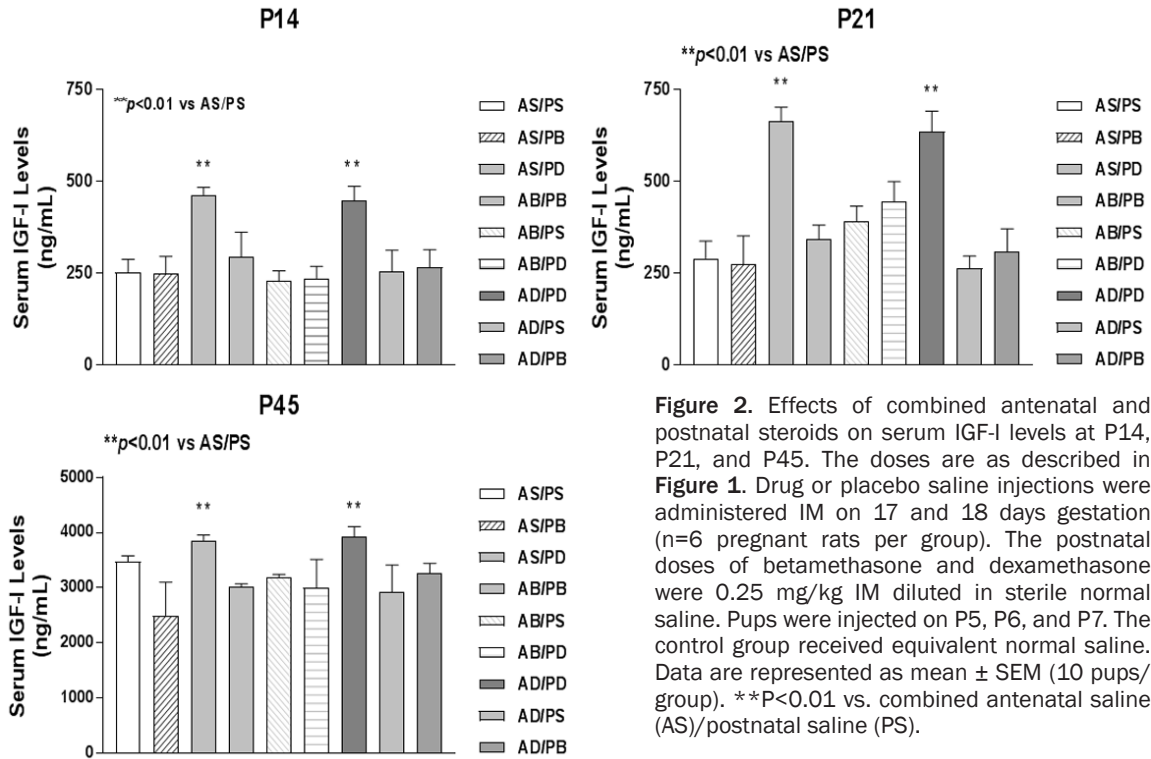
P14

P21

P45

Figure 2. Effects of combined antenatal and postnatal steroids on serum IGF-I levels at P14, P21, and P45. The doses are as described in **Figure 1**. Drug or placebo saline injections were administered IM on 17 and 18 days gestation (n=6 pregnant rats per group). The postnatal doses of betamethasone and dexamethasone were 0.25 mg/kg IM diluted in sterile normal saline. Pups were injected on P5, P6, and P7. The control group received equivalent normal saline. Data are represented as mean ± SEM (10 pups/group). **P<0.01 vs. combined antenatal saline (AS)/postnatal saline (PS).

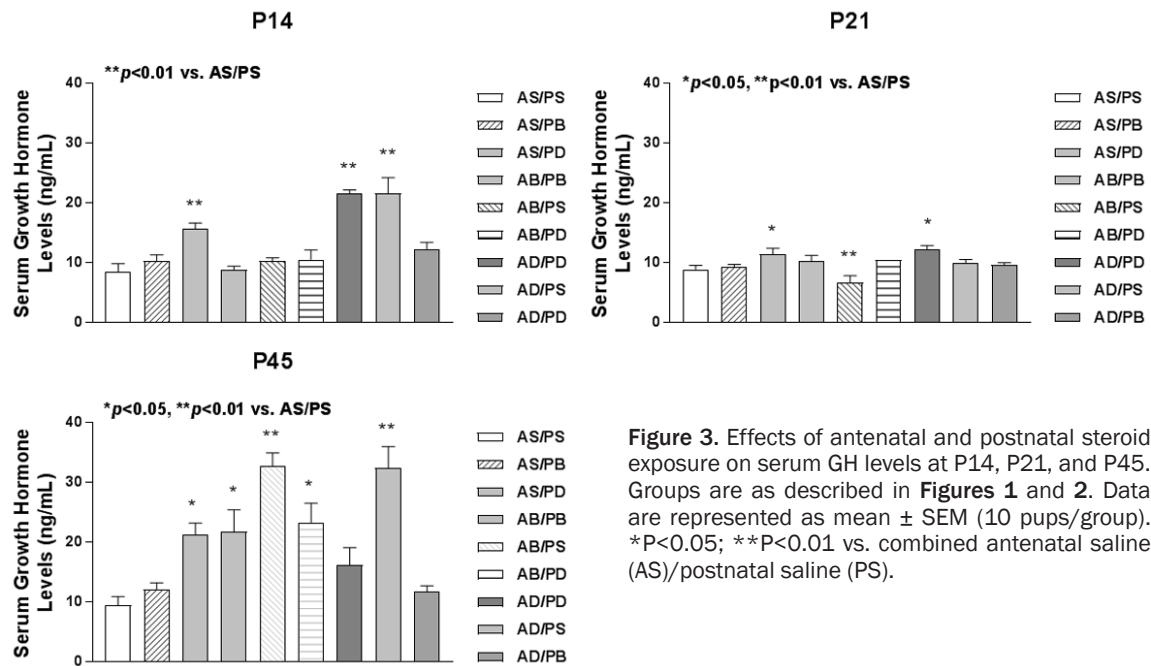## P14

P21

P45

Figure 3. Effects of antenatal and postnatal steroid exposure on serum GH levels at P14, P21, and P45. Groups are as described in **Figures 1** and **2**. Data are represented as mean ± SEM (10 pups/group). *P<0.05; **P<0.01 vs. combined antenatal saline (AS)/postnatal saline (PS).

AS/PB (1.02 ± 0.05 vs. 1.37 ± 0.07, P<0.01) groups, respectively. Neurological tests at P45 showed sustained reductions in full plantar reflex in the AS/PB (80%, P<0.05), AD/PD (70%, P<0.05) and AD/PB (60%, P<0.05) groups, and a non-significant lower negative geotaxis reflex in the AS/PD (90%).

Antenatal steroids, fetal brain IGF-I signaling

Figure 1 represents the expression of IGF-I, IGF-IR, IGFBP-1 and IGFBP-3 in the neonatal rat brain at birth. Brain IGF-I mRNA was elevated in the betamethasone-treated group (**Figure 1A**). Treatment with dexamethasone sig-
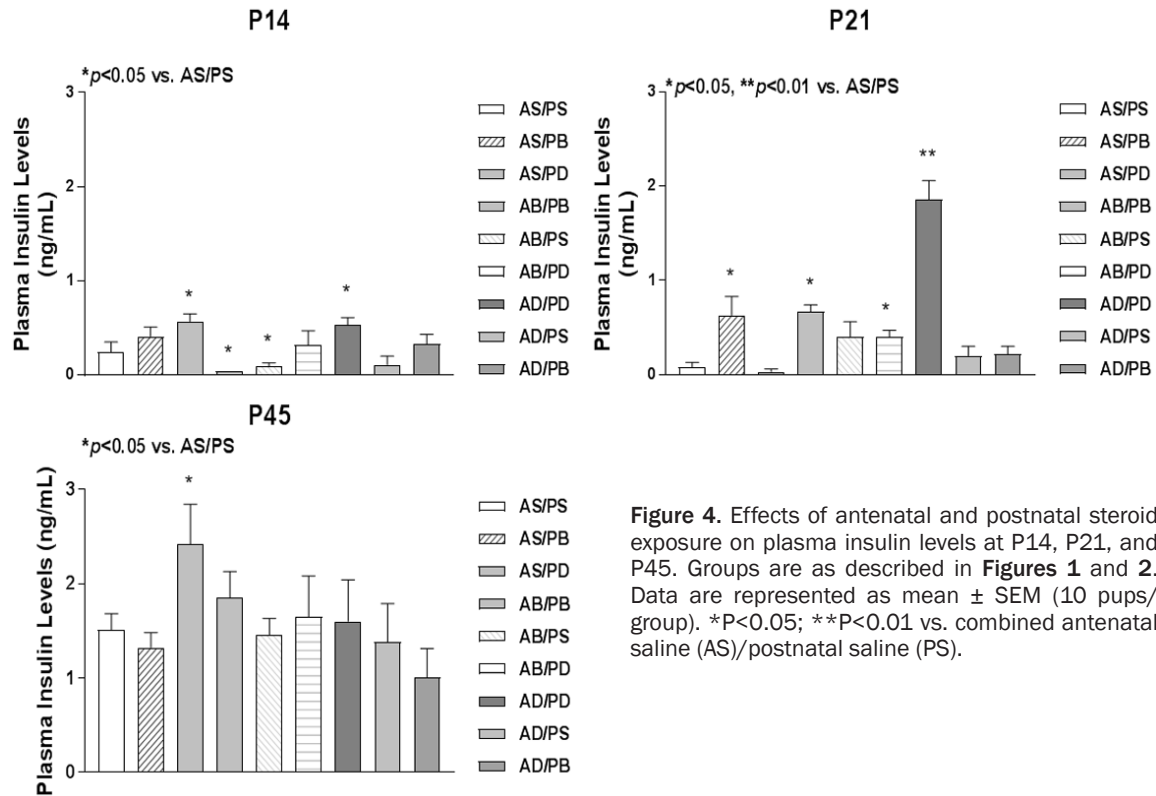
Figure 4. Effects of antenatal and postnatal steroid exposure on plasma insulin levels at P14, P21, and P45. Groups are as described in **Figures 1** and **2**. Data are represented as mean ± SEM (10 pups/group). *P<0.05; **P<0.01 vs. combined antenatal saline (AS)/postnatal saline (PS).

nificantly downregulated IGF-IR mRNA (2-fold), and IGFBP-3 (2-fold), but elevated IGFBP-1 mRNA (3-fold) with no change in IGF-I expression (**Figure 1B-D**).

Combined antenatal and postnatal steroids, serum IGF-I

Figure 2 shows the serum levels of IGF-I at P14, P21 and P45. At P14, IGF-I levels were significantly higher in the AS/PD and AD/PD groups. At P21, serum IGF-I levels were higher in the AB/PB group. By P45, no differences were noted among the groups.

Combined antenatal and postnatal steroids, serum GH

Figure 3 shows serum GH levels at P14, P21, and P45. At P14, only the AD/PS group had higher GH levels, but by P21 the levels increased in the AS/PD group and decreased in the AB/PS group. By P45, higher GH levels were noted in AS/PD, AB/PB, AB/PS, AB/PD and AD/PS groups compared to the AS/PS group.

Combined antenatal and postnatal steroids, plasma insulin

Figure 4 shows plasma insulin levels at P14, P21 and P45. Insulin levels declined at P14 in

the AB/PB and AB/PS groups, but increased at P21 in the AS/PB, AB/PS, AB/PD, and AD/PD groups. By P45 only treatment with AS/PD had sustained higher insulin levels.

Combined antenatal and postnatal steroids, plasma ACTH and corticosterone

Plasma ACTH and corticosterone levels at P45 are presented in **Figure 5**. Treatment with AB/PS and AD/PB resulted in higher ACTH levels while only AD/BP resulted in a robust increase in corticosterone levels.

Discussion

The present study tested the hypothesis that the adverse effects of antenatal steroids on fetal and neonatal growth are mediated in part, by their influence on the IGF system. The first test of our hypothesis was demonstrated by the effects of dexamethasone on brain IGF-IR and IGFBP-3 mRNA expression, with negligible effects on IGF-I mRNA expression and brain weight. The results of our findings demonstrated that deficits in IGF-IR and IGFBP-3 mRNA approached 50% in the dexamethasone group compared to brain/body weight deficits of only 10%, whereas actual brain weight remained
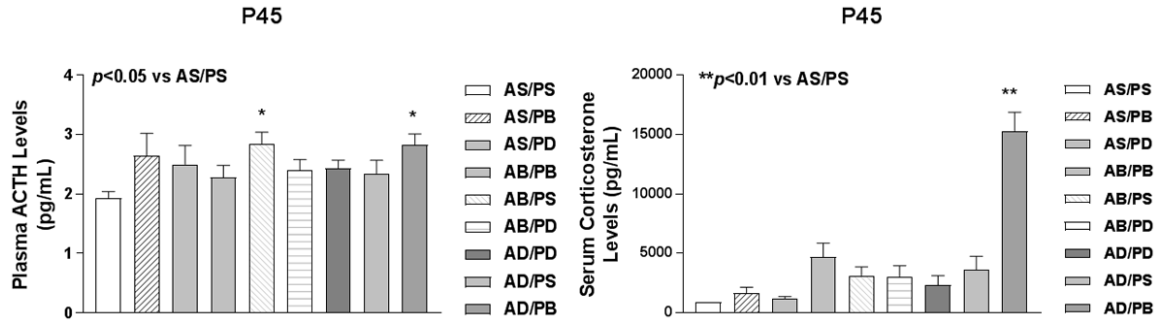
Figure 5. Effects of antenatal and postnatal steroid exposure on plasma ACTH and corticosterone levels at P45. Groups are as described in **Figures 1** and **2**. Data are represented as mean ± SEM (10 pups/group). *P<0.05; **P<0.01 vs. combined antenatal saline (AS)/postnatal saline (PS).

unchanged. This is also in contrast to the minimal effects of betamethasone.

IGFs are potent mitogens that promote cell growth and anabolism in many tissues including the central nervous system [39-41]. Stimulation of neurite growth including the neurotrophic actions has been attributed to IGF-I activity. Considering the important role of IGF-I in brain development, and growth promoting role of IGFBP-3 (the most abundant binding protein in the late fetal and early postnatal age) [24] in facilitating IGF-I bioactivity, these findings may suggest that dexamethasone-induced brain growth suppression can occur even when the actual brain weight is spared. It is important to note that IGF-I protein levels in the brain were not measured, but the mRNA expression level which does not necessarily reflect corresponding protein levels. Moreover, decreased brain to body weight ratio by treatment with dexamethasone, suggests that the brain cells might have been targeted. Our findings on IGF-I mRNA are in contrast to a previous in-vitro study which showed that dexamethasone reduced IGF-I mRNA levels in the primary cultures of neuronal and glial cells from rat brain [42]. These discrepancies may be attributed to differences between the in-vivo and in-vitro models and influence of IGFBPs.

Although IGF-IR mRNA has been found in the fetal rat at day 14, and is expressed at high levels through perinatal age [43], its mRNA expression is maximal at fetal day 15 and 20, whereas the maximal mRNA expression for the insulin receptor is at fetal day 20 and the day of birth. It is well known that the biological effects of IGF-I, 2 and insulin are mediated through their interactions with specific cell membrane receptors. In a study by Yamamoto et al. [44], a decrease in specific IGF-I binding was directly related to the ligand concentration and was dependent on the duration of pituitary cell exposure to IGF-I. Chronic exposure as well as high levels caused a down regulation of the IGF-I receptor number with no change in receptor affinity. IGFBP-3 increases the half-life of IGFs in circulation [43]. When IGF-I and -II are injected into normal rats, they bind to IGFBP-3 increasing their stability and half-life to 4 hours compared to 20 minutes in hypophysectomized rats [24, 45]. Guler et al. [46] determined the half-lives of free and IGFBP bound IGF-I and -II, and their findings further support the role of IGF binding proteins in augmenting the bioactivity of IGFs. The most significant finding is the effect of antenatal dexamethasone on IGF-IR, IGFBP-1 and IGFBP-3 mRNA expression in the brain. In a previous study by Villafuerte et al. [47], dexamethasone reduced the production of IGFBP-3 on cultured hepatocytes by inhibition of IGFBP-3 gene transcription, which may be the same mechanism in our study. However, this could not explain the unchanged mRNA expression levels of brain IGF-I, and may suggest that IGF-I must be produced at higher rates in the antenatal dexamethasone group due to a decreased bioavailability of IGFBP-3. Nevertheless, it seems that the effect of antenatal dexamethasone on IGF signaling (i.e. reduced mRNA expression levels of IGF-IR and IGFBP-3) in the brain did not adversely affect the actual brain size.

Rat neurons have the same composition and electrical properties as human neurons [7]. In rats, the brain growth spurt occurs after term.

In humans, neuronal division except for the cerebellum and dentate gyrus, is completed before the 24th week of gestation and the peak brain growth spurt occurs around term. Thus a rat at 8 days of age is roughly equivalent to a full term infant in terms of growth, periventricular germinal matrix, neurochemical data, electroencephalographic pattern, and synapse formation [7, 48]. It was interesting to note that animals that were antenatally exposed to betamethasone had higher brain weights and IGF-I mRNA expression, and exhibited advanced hopping reflexes in a greater percentage of the rats compared to placebo saline and dexamethasone, suggesting maturation of spinal mechanisms involved in rhythmic stepping responses [35].

At P14, all groups exposed to antenatal and/or postnatal steroids exhibited retarded negative geotaxis and free-fall righting reflexes both of which have been attributed to vestibular function [35]. Vestibular function is associated with cerebellar development which has been shown to be negatively affected by GCs [49]. At P14, the cerebellum plays an important role in the regulation of complex movement patterns [50], therefore, the delayed effect of GCs on negative geotaxis and free-fall righting reflexes suggest abnormal cerebellum function [35]. This delayed effect was not seen at P21. Instead, delayed plantar and palmar reflexes were noted in the postnatal GC and antenatal dexamethasone groups, suggesting delayed sensory thresholds and signaling from the brainstem [51]. At P45, brain weight was lower predominantly in the animals exposed to combined antenatal and postnatal GCs. Delayed plantar reflexes persisted in the AS/PB, AD/PD and AD/PB groups, while delayed negative geotaxis reflexes persisted in the AS/PD group. These findings provide evidence that the combined use of antenatal and postnatal GCs have lasting detrimental effects on neurological outcomes.

In the suckling rats at P14, it was interesting to note the higher serum IGF-I levels in the AS/PD and AD/PD groups compared to all other groups despite no change in body weight or linear growth. The GH/IGF-I/insulin system is important for postnatal growth and development. Particularly, IGF-I is a major stimulus for postnatal growth [52]. It is induced by pituitary GH in the liver to promote growth, along with

its binding proteins [53], and accounts for about 75% of all circulating IGFs [54]. Together with its receptor, it is expressed in almost all tissues for autocrine/paracrine purposes [55]. Although IGF-I is predominantly produced by the liver, studies have shown that liver-derived IGF-I is not required for postnatal growth, suggesting that local production of IGF-I may be more important than liver-derived circulating IGF-I for body growth [56]. IGF-I availability is tightly regulated by its binding proteins which increase IGF-I half-life from minutes to hours, and shuttles IGF-I to specific target tissues [57]. IGF-I is present in high concentrations in serum, and is mostly protein bound [58]. Approximately 90% of IGF-I is bound to IGFBP-3, the primary hepatic-derived IGFBP [59]. In rats, the fetal serum profile, characterized by high IGF-II and IGFBP-2, is replaced around the third week of life by the adult-type profile of high IGF-I and IGFBP-3, with a dramatic reduction in IGF-II and IGFBP-2 [60]. Evidence suggest that dexamethasone causes marked increases in serum IGF-I and insulin levels, whereas IGF-I bioactivity was significantly decreased [61, 62]. Thus, the high serum levels noted in this study with dexamethasone is in agreement with those previous findings and may suggest reduced bioavailability. This effect of dexamethasone was long lasting and remained sustained until adolescent, despite normal food intake as rats are weaned at P21, and may account for the lower brain weight as well as the sustained retardation in plantar and palmar reflexes which are representative of delayed sensory thresholds and signaling from the brainstem [51].

The responses of serum GH to dexamethasone were similar to IGF-I at P14 and P21. Dexamethasone has been shown to augment the insensitivity to GH and IGF-I by reducing GHR and IGF-IR expression [63]. Although we did not measure GHR expression, our data showed reductions in IGF-IR in the brain of term rats with dexamethasone (**Figure 1B**). Dexamethasone-induced elevations in serum IGF-I and GH concurrent with reduced brain and body weight concur with previous reports of GH insensitivity. Similar elevations in serum GH levels and growth retardation were also noted at P45 in the AS/PB, AB/PB, and AB/PS groups suggesting that betamethasone may also promote GH insensitivity, but the effect is

latent. Studies suggest that during the perinatal period, GH does not appear to play a major role in growth and IGF-I secretion but may play a more important role in regulation of glucose metabolism [64]. Ovine fetuses antenatally-treated with dexamethasone had higher glucose and insulin levels than controls, suggesting adverse metabolic effects due to hyperglycemia and hyperinsulinemia [65].

In our study, plasma insulin levels also increased with dexamethasone at P14, and with combined antenatal and postnatal dexamethasone at P21. The effect of antenatal dexamethasone on plasma insulin remained sustained until P45, providing further evidence that combined antenatal and postnatal GCs result in hormonal changes that may have long-lasting detrimental effects. We also observed elevated levels of ACTH and corticosterone in the AB/PS and AD/PB animals at P45. In these same groups, body weight, brain weight, and tail length were reduced. Studies in human newborns show similar elevations in cord blood ACTH and cortisol with reduced fetal growth [66]. We now show sustained elevations in serum ACTH and corticosterone levels with combined antenatal and postnatal GCs suggesting permanent programming of hypothalamic-pituitary-adrenal (HPA) axis activity and function.

While this study provides important and clinically-relevant information regarding, one limitation was the use of semi-quantitative RT-PCR to assess the mRNA expression of IGFs and IGFBPs in the brain. Real-time PCR would have provided more quantitative assessments. However, the biomolecular and neurological outcomes, taken together, point to the significance of combined exposure to antenatal and postnatal GCs. While caution is necessary when extrapolating data from animals to the human situation, there are still developmental similarities between species, that can provide valuable information.

In conclusion, betamethasone and dexamethasone are both synthetic GCs that are widely used during the perinatal period. The present study showed differential beneficial effects of antenatal betamethasone over dexamethasone with respect to body growth and the IGF-I system in the brain. Antenatal beta-methasone produced no significant effects on the IGF system in the brain or neurological deficits at birth or adolescence compared to the antenatal dexamethasone. Combined antenatal and postnatal GCs has lasting effects on body and brain growth, factors that influence growth and glucose homeostasis, neurological outcomes, and HPA axis activity. Whether these effects persist in adult life and are risk factors for insulin resistance, remains to be elucidated.

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Disclosure of conflict of interest

None.

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