Effect of Prenatal Glucocorticoids on Cerebral Vasculature of the Developing Brain

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Background and Purpose—Prenatal glucocorticoids prevent germinal matrix hemorrhage in premature infants. The underlying mechanism, however, is elusive. Germinal matrix is enriched with angiogenic vessels exhibiting paucity of pericytes and glial fibrillary acidic protein-positive astrocyte end feet. Therefore, we asked whether glucocorticoid treatment would suppress angiogenesis and enhance periendothelial coverage by pericytes and glial fibrillary acidic protein-positive end feet in the germinal matrix microvasculature.

Methods—We treated pregnant rabbits with intramuscular betamethasone and delivered pups prematurely by cesarean section at E29 (term = 32 days). Endothelial turnover, vascular density, pericyte coverage, glial fibrillary acidic protein-positive end feet, cell death, and growth factors orchestrating angiogenesis, including vascular endothelial growth factor, angiopoietins, transforming growth factor-β, and platelet-derived growth factor-B, were compared between betamethasone-treated and untreated pups. Similar comparisons were done between autopsy materials from premature infants exposed and unexposed to prenatal glucocorticoids.

Results—Antenatal glucocorticoid treatment reduced endothelial proliferation, vascular density, and vascular endothelial growth factor expression in the germinal matrix of both rabbits and humans. The pericyte coverage was greater in glucocorticoid-treated rabbit pups and human infants than in controls, but not the glial fibrillary acidic protein-positive end feet coverage. Transforming growth factor-β, but not angiopoietins and platelet-derived growth factor-B, were elevated in glucocorticoid-treated rabbit pups compared with controls. Betamethasone treatment induced apoptosis, neuronal degeneration, and gliosis in rabbit pups. However, there was no evidence of increased cell death in glucocorticoid-exposed human infants.

Conclusions—Prenatal glucocorticoid suppresses vascular endothelial growth factor and elevates transforming growth factor-β levels, which results in angiogenic inhibition, trimming of neovasculature, and enhanced pericyte coverage. These changes contribute to stabilizing the germinal matrix vasculature, thereby reducing its propensity to hemorrhage. Prenatal glucocorticoid exposure does not induce neural cell death in humans, unlike rabbits. (Stroke. 2010;41:1766-1773.)

Key Words: betamethasone ■ germinal matrix hemorrhage—intraventricular hemorrhage ■ germinal matrix ■ glucocorticoids ■ pericyte ■ TGF-β ■ vasculature

Prenatal glucocorticoids (GCs) prevent respiratory distress syndrome and intraventricular hemorrhage (IVH) in preterm infants.1,2 Indeed, the National Institutes of Health Consensus Development Panel on the “Effect of corticosteroids for fetal maturation on perinatal outcomes” has recommended use of prenatal GC in preterm labor.3 In the United States, the preterm birth rate is 12.5%, and 75% of women in preterm labor with gestational age of ≤34 weeks are treated with GC.4 Because approximately 13 million babies are born premature worldwide every year, a huge number of preterm infants are exposed to prenatal steroids. This increases their survival and reduces both the incidence and severity of IVH.5,6 Yet, the molecular mechanism by which GCs prevent IVH is elusive. Therefore, we asked how prenatal GC would reduce the incidence of IVH.

IVH typically initiates in the germinal matrix (GM). This periventricular region, located on the head of the caudate nucleus and underneath the ventricular ependyma, is a richly vascularized collection of neural precursor cells and is selectively vulnerable to hemorrhage. IVH is attributed to intrinsic fragility of the GM vasculature and disturbance in cerebral blood flow. Our previous work has shown that a rapid angiogenesis in the GM, induced by high vascular endothelial growth factor (VEGF) and angiopoietin (ANGPT)-2 levels, contributes to increased vascular fragility and vulnerability to hemorrhage and that angiogenic inhibition reduces the occur-
rence of IVH in a rabbit pup model. Furthermore, angiogenic vessels of the GM exhibit paucity of pericytes, deficiency of fibronectin in the basal lamina, and reduced perivascular coverage by glial fibrillary acidic protein (GFAP)-positive astrocyte end feet. Hence, the fragility of GM microvasculature is attributed to immature basal lamina and reduced perivascular coverage by pericytes as well as GFAP-positive end feet.

Prenatal GC betamethasone and its stereoisomer, dexamethasone, are used in preterm labor. They exhibit a wide range of pharmacological effects and toxicities on the brain of premature infants. However, little is known about the effects of GCs on the morphology and molecular components of the developing cerebral vasculature. The GC downregulates VEGF in an in vitro model of the blood–brain barrier and cultured cells of various origin and, accordingly, the GC treatment effectively suppresses angiogenesis in various disease models. The blockade of VEGF signaling primes the nascent, immature, and pericyte-deficient microvasculature of tumors. In addition, this remodels the remaining vasculature, which results in less dilated blood vessels exhibiting enhanced pericyte coverage. Other than VEGF, growth factors angiopoietin-1, platelet-derived growth factor-B (PDGF-B), and transforming growth factor-β (TGF-β) play a key role in maturation of the vasculature, particularly in the assembly of pericytes around the immature blood vessels. Therefore, we hypothesized that prenatal GC would suppress angiogenesis by downregulation of angiogenic growth factors, including VEGF and ANGPT-2, and enhance pericyte recruitment by inducing distinctive changes in the regulating growth factors angiopoietin-1, PDGF-B, and TGF-β.

There is increasing evidence that the GC treatment affects the phenotype and function of astrocytes. For example, the dexamethasone treatment in the astrocyte cultures and triamcinolone intravitreal injection in a mouse model of laser retinal photocoagulation enhance GFAP levels in the astrocytes. Importantly, a high dose of dexamethasone and methylprednisolone induces apoptotic cell death in rats raising safety concerns with prenatal GC treatment. Thus, we postulated that prenatal GC treatment might mature the cerebral vasculature by increasing GFAP-positive perivascular end feet but might cause undesirable adverse effects such as neural cell death and gliosis.

**Materials and Methods**

**Animal Experiment**

The animal protocol was approved by Institutional Animal Care and Use Committee of New York Medical College, Valhalla, NY. We obtained 8 timed pregnant New Zealand rabbits from Charles River Laboratories (Wilmington, Mass.). The rabbits were sequentially assigned to receive either intramuscular betamethasone (n = 4) or saline (n = 4). The dose of betamethasone in pregnant women is 12.5 mg once daily for 2 days and the average weight of pregnant women is approximately 60 kg. On this basis, we calculated a dose of 0.2 mg/kg daily for 2 days in pregnant rabbits. Thus, betamethasone (Celestone; Schering Corporation, Kenilworth, NJ) was administered 0.2 mg/kg per dose every 24 hours on gestational Days 27 and 28 for a total of 2 doses.

Cesarean section was performed at Day 29 of gestational age to deliver rabbit pups prematurely (term = 32 days). Pups were dried immediately and were kept warm in an infant incubator at 35°C. After stabilization of their conditions, they were weighed and fed with puppy formula (Esbilac; Petag, Hampshire, III). Pups were euthanized at 3 epochs: 2, 6, and 48 hour of age. Brain was then dissected and cut into 2-mm coronal slices on brain matrix. All the histological evaluations were done from coronal sections taken at the level of midseptal nucleus. The comparison groups were balanced with respect to the body weight and gender of rabbit pups.

**Laser Capture Microdissection**


**Human Tissue Collection and Processing**

The Institutional Review Board of New York Medical College approved the use of human autopsy materials for this study. Women in preterm labor receive either betamethasone (12.5 mg once daily for 2 days) or dexamethasone (6 mg twice daily for 2 days) to prevent respiratory distress syndrome in premature infants. Approximately 2- to 3-mm thick coronal slices were taken at the level of thalamostriate groove from the frontal lobe. The coronal blocks included the frontal lobe cortex, white matter, and GM. The samples were fixed in 4% paraformaldehyde in phosphate-buffered saline for 18 hours and then were cryoprotected by immersing into 20% sucrose in phosphate-buffered saline buffer. The tissues were frozen after embedding them into optimum cutting temperature compound. Frozen coronal blocks were cut into 15-μm sections using a cryostat and saved at −80°C until use.

**Immunohistochemistry, Neuronal Degeneration (Fluoro-Jade B), Fluorescent In Situ Detection of DNA Fragmentation (Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling), Western Blot Analyses, and Quantitative Real-Time Polymerase Chain Reaction**

The techniques are illustrated in the Supplemental Methods.

**Quantification of Vascular Density, Endothelial Proliferation, Cell Death, Pericyte, and Astrocyte Coverage**

These are described in the Supplemental Methods.

**Statistical Analysis**

To determine differences in the endothelial proliferation, vascular density, vessel area, and cell degeneration between GC-exposed and unexposed human infants, 2-way analysis of variance with repeated measures was used. The repeated factor was applied to the 3 brain regions: cortex, white matter, and GM. To assess differences in endothelial proliferation, vessel density, vessel area, pericyte coverage, and growth factors in rabbit pups, 2-way analysis of variance was used for each of the brain regions (cortex, white matter, and GM) separately. The independent factors in 2-way analysis of variance were postnatal age (2 hours versus 48 hours) and treatment (betamethasone versus no treatment). All post hoc comparisons to test for differences between means were done using Tukey multiple comparison test at the 0.05 significance level. Student t test was used to compare between 2 groups (Western blot analyses data).
Results

Glucocorticoids Reduce Endothelial Proliferation in Both Rabbits and Humans

Because GC suppresses angiogenesis in various disease models,\(^{16,17}\) we asked whether GC would reduce endothelial proliferation in the GM. To this end, we double-labeled the brain sections with Ki67 (proliferation marker) and CD31 (endothelium in rabbit) or CD34 (endothelium in human) -specific antibodies and evaluated x-z and y-z (orthogonal views) reconstructions of stacks of confocal images to verify Ki67 immunoreactivity embedded into CD34 (Figure 1A, C). The endothelial proliferation index was significantly lower in the GM of betamethasone-treated pups than in controls at both 2 and 48 hours of age (**\(P<0.001\), ***\(P<0.01\)); and this decreased at 48 hours than at 2 hours of age in both control and treated pups (###\(P<0.001\), †\(P<0.05\), C). Cryosections from 23-week premature infants exposed and unexposed to GC labeled for Ki67 and CD34 antibodies. Note endothelial proliferation (Ki67 overlapping CD31, arrowhead) more frequent in GC-treated infants than in controls. Scale bar, 50 \(\mu m\).

Figure 1. GC suppresses endothelial proliferation. A, Representative immunofluorescence of cryosections from E29 rabbit pups stained with Ki67 and CD31 antibodies. Note CD31 staining vessels with Ki67 signals indicating endothelial proliferation (arrowhead). Vessels with Ki67 labeling were fewer in the betamethasone-treated pups than controls. Above and right to the image are orthogonal views in x-z and y-z planes of a composite of z-stack of a series of confocal images taken 0.6 \(\mu m\) apart. Scale bar, 20 \(\mu m\). B, Data are mean±SEM (\(n=5\) at each point). Endothelial proliferation was lower in betamethasone-treated pups than in controls at both 2 and 48 hours of age (**\(P<0.001\), ***\(P<0.01\)); and this decreased at 48 hours than at 2 hours of age in both control and treated pups (###\(P<0.001\), †\(P<0.05\)). C, Cryosections from 23-week premature infants exposed and unexposed to GC labeled for Ki67 and CD34 antibodies. Note endothelial proliferation (Ki67 overlapping CD31, arrowhead) more frequent in GC-treated infants than in controls. Scale bar, 50 \(\mu m\). D, Data are mean±SEM (\(n=5\) at each point). Endothelial proliferation was less in GM of GC-treated infants relative to untreated controls (**\(P<0.05\)).
significantly less in the GC-treated infants than in untreated controls ($P<0.04$ and $0.008$, respectively; Figure 2C–D). In the cortex and white matter, these metrics were comparable between the treatment and control groups. Hence, GC trims the angiogenic GM vasculature in the premature rabbit pups and human infants.

**Betamethasone Suppresses VEGF But Not ANGPT-2**

Because prenatal GC pruned GM neovasculature in our experiments, we asked whether prenatal betamethasone would suppress the angiogenic growth factors, VEGF and ANGPT-2, in the GM. To this end, we measured protein levels of these 2 growth factors in homogenates from a coronal slice taken at the midseptal nucleus level; and we assayed mRNA expression in the laser dissected samples from the 3 brain regions: GM, cerebral cortex, and white matter. Western blot analysis revealed that 24 kDa VEGF was significantly less abundant in betamethasone-treated rabbit pups compared with untreated controls ($P=0.04$; Figure 3A). Accordingly, real-time polymerase chain reaction showed that betamethasone treatment reduced VEGF mRNA level in the GM ($P=0.02$; Figure 3B) but not in the cortex and white matter at 2 hours of age. At 6 hours of age, VEGF mRNA expression was significantly reduced in all the 3 brain regions—cortex, white matter, and GM—of the betamethasone-treated pups compared with controls ($P=0.01$, 0.04 and 0.02, respectively). However, prenatal
Betamethasone treatment did not affect VEGF mRNA levels at 48 hours of age in any of the brain regions. Importantly, ANGPT-2 protein and mRNA accumulation were similar in the treated and control pups (Figure 3C–D). Together, betamethasone treatment suppressed VEGF but not ANGPT-2.

**Betamethasone Enhances Pericyte Coverage in the GM Vasculature**

There is paucity of pericytes in the GM vasculature; and VEGF inhibition prunes the immature vessels lacking pericytes. Therefore, we postulated that betamethasone treatment would enhance pericyte coverage in the GM vasculature. Thus, we assessed coronal brain sections double-labeled with NG2 (pericyte marker) and CD31 antibodies. In rabbits, we found that betamethasone enhanced pericyte vascular coverage at both 2 and 48 hours of age (P<0.001 each; Figure 4A–B) in the GM, but not in the cortex or white matter. However, the pericyte coverage remained significantly less in the GM than in the other brain regions in the treated pups.

We next evaluated pericyte coverage in human autopsy materials from premature infants and obtained similar findings as in rabbit pups. The pericyte coverage was higher in the GM of premature infants exposed to prenatal GC compared with untreated GC (P=0.016; Figure 4C–D). GC exposure did not affect pericyte coverage in the cortex and white matter vasculature. Collectively, GC treatment enhanced pericyte coverage in the GM vasculature.

**Betamethasone Elevates TGF-β But Not ANGPT-1 or PDGF-B Levels**

The ligand receptor systems that recruit pericytes include TGF-β, ANGPT-1, PDGF-B, and their receptors. Because betamethasone treatment augmented pericyte coverage in the GM vasculature, we determined whether TGF-β, ANGPT-1, and PDGF-B levels were higher in the GM of GC-treated pups compared with untreated controls. Real-time polymerase chain reaction showed that ANGPT-1 and PDGF-B levels were comparable between betamethasone-treated and control groups at all epochs (Figure 5A–B). However, TGF-β mRNA expression was elevated in the rabbit GM exposed to betamethasone compared with unexposed controls at both 2 and 6 hours (P=0.04 and 0.03, respectively), but not at 48 hours of age. TGF-β was also higher in the cortex of treated pups compared with controls at 2 hours (P<0.05) but not at 6 and 48 hours of age (Figure 5C). To confirm elevation in TGF-β levels, we measured its protein expression by Western blot analyses and found that TGF-β protein level was higher in betamethasone-treated rabbit pups compared with untreated controls (P<0.05; Figure 5D). Hence, betamethasone treatment upregulates TGF-β levels but not ANGPT-1 or PDGF-B expression.

We also assessed the receptors of VEGF, ANGPT, and PDGF-B by real-time polymerase chain reaction. We found no significant difference in mRNA expression of VEGFR2, Tie-2, and PDGFRα receptors between betamethasone-treated and control groups (data not shown).

**Betamethasone Enhances GFAP-Positive Astrocytes in the GM**

Because GC treatment enhances GFAP in astrocytes, we assessed GFAP expression in the GM using immunohistochemistry. Immunolabeling revealed that GFAP-positive astrocytes were more abundant in the GM of betamethasone-treated pups compared with untreated controls at both 2 and 48 hours of age (Figure 6A). We next performed Western blot analyses on the homogenates from 1-mm thick brain slice at the level of midseptal nucleus. GFAP protein expression was significantly greater in the betamethasone-treated pups compared with untreated controls (P<0.05, n=5 in each group; Figure 6B).

We then compared GFAP-positive perivascular end feet coverage in the GC-exposed and unexposed human infants. The percent GFAP-positive astrocyte end feet was 1.5-fold greater in the betamethasone-treated pups compared with...
untreated controls (32.5±9.6% versus 20.2±4.3%). The difference, however, was not statistically significant. In conclusion, prenatal GC significantly enhances GFAP expression in the astrocytes of the rabbit GM but not GFAP-positive end feet coverage in the GM of premature infants.

Betamethasone Induces Apoptosis, Neuronal Degeneration, and Growth Retardation in Rabbits

Because betamethasone treatment can induce cell death, we assessed apoptosis and neuronal degeneration in rabbit pups treated with prenatal betamethasone compared with untreated controls at 2 hours postnatal age. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive cells were more abundant in the GM, cortex, and white matter of the treated pups compared with untreated controls (P<0.001, 0.025, and 0.026, respectively; Figure 6 and Supplemental Figure I, available online at http://stroke.ahajournals.org). Accordingly, Fluoro-Jade B-positive neurons were in larger number in the GM, cortex, and white matter of pups treated with prenatal betamethasone compared with untreated controls (P=0.001, 0.022, and 0.003 respectively; Figure 6D, Supplemental Figure I). We next assessed apoptosis and neuronal degeneration in human premature infants exposed and unexposed to antenatal GC. In contrast to rabbits, density of TUNEL-positive neural cells and Fluoro-Jade B-positive neurons were remarkably comparable between the 2 groups of human infants (Supplemental Figure I).

The betamethasone-treated pups were markedly smaller in weight (29.3±1.6 g versus 47.1±0.9 g; P<0.001, n=12 pups each) compared with untreated controls. Hence, prenatal GC...
induces cell death in premature rabbit pups but not in preterm infants.

Discussion

IVH is the most common neurological complication of prematurity affecting approximately 12 000 infants each year in the United States alone.25 Of note, prematurity rate is escalating26; survival of premature infants has remarkably increased with advances in the medical care; and the IVH rate among preterm infants has remained almost stationary during the last 10 years.27 Thus, IVH and the attendant complications, including cerebral palsy, posthemorrhagic hydrocephalus, and cognitive deficits, have emerged as global health problems. No treatment of IVH is currently available. The only widely practiced preventive strategy is the use of prenatal GC in women in preterm labor, which reduces the occurrence of IVH in preterm infants by >50%.5 In this study, we determined the mechanistic basis of the use of prenatal GC to prevent IVH in preterm infants. We found that the prenatal GC suppressed angiogenesis, pruned the neovascularure, and enhanced pericyte coverage, thereby stabilizing the GM vasculature. We then observed that prenatal GC increased apoptotic neural cell death and neuronal degeneration in premature rabbit pups but not in human premature infants.

In the present study, the GC suppressed VEGF expression in the forebrain, particularly in the GM; and accordingly, endothelial proliferation was diminished in the GM of GC-treated pups compared with untreated controls. Consistent with our findings, the GC downregulates VEGF in a culture model of the blood—brain barrier as well as in other cell types; and GC also suppresses tumor angiogenesis in animal models.14–17 Importantly, our previous study has shown that GM has high VEGF and angiopoietin-2 levels inducing rapid endothelial proliferation in the microvasculature and that the suppression of VEGF by celecoxib, a cyclo-oxygenase-2 inhibitor, or ZD6474, a VEGFR2 blocker, minimizes both the incidence and severity of IVH.7 Hence, this is plausible that the prenatal GC confers protection against IVH by downregulation of VEGF and suppression of angiogenesis.

Another key finding in our study was that the GC treatment reduced vascular density and enhanced pericyte coverage in the GM microvasculature. Because angiogenic inhibitors suppress VEGF levels resulting in apoptosis of endothelial cells not protected by pericytes, the increase in pericyte coverage might assist in suppression of angiogenesis and contribute to pericyte recruitment in the GM vasculature.

Our previous work has shown that perivascular coverage by GFAP-positive end feet was decreased in the GM compared with the cerebral cortex and white matter in premature infants 23 to 34 weeks.8 We expected that GC exposure will increase GFAP-positive end feet in the GM. However, although GFAP-positive end feet perivascular coverage tended to be elevated in the GM of infants exposed to GC, the difference was not statistically significant. These studies performed on human autopsy material of a short postmortem interval are invaluable. Nevertheless, the limitations of such studies are exposure of infants to a number of prenatal and postnatal variables, including mechanical ventilation, medications, and others, which can potentially confound the results. We also found elevation in GFAP levels in the rabbit brain, demonstrated by immunolabeling and Western blot analyses. The increase in GFAP on GC treatment might be attributable to an elevation in TGF-β levels. Several other studies have also shown an elevation in GFAP levels in astrocytes on steroid treatment.20,21 Together, GC treatment enhances GFAP levels in the astrocytes, but this elevation may not be significant in the perivascular end feet.

Of note, we observed abundance of apoptotic neural cells and neuronal degeneration in the rabbit pups exposed to prenatal GC. High doses of dexamethasone and methylprednisolone also induce apoptotic cell death in hippocampal culture experiments and in rats and monkeys.22,28,29 The apoptosis is typically mediated by GC receptors through genomic or nongenomic pathways, and these effects differ with respect to GC preparation, dose and duration of treatment as well as with the stage of neural cell maturation.29 In contrast to rabbit pups, cell death was comparable in premature infants exposed and unexposed to prenatal GC. Similar to humans, prenatal dexamethasone exposure does not affect neural cell death in ovine fetuses at 90% gestation.30 However, at 70% gestational age, prenatal dexamethasone reduces apoptosis and caspase activity in the ovine fetal cerebral cortex.30 This marked discrepancy in the effect of GC on cell death among the human, rabbit, and sheep fetuses could be attributed to distinctive maturation and susceptibility of neural cells to GC as well as to the differences in the pharmacokinetics of GC among the species. All the mothers of the infants included in the present study completed the GC course within 72 hours of the delivery of their infants; and these premature infants died at 6 to 72 hours postnatal age. Therefore, it is less likely that we missed the window of apoptotic cell death and neuronal degeneration after prenatal steroid treatment. In this context, it is important to link intrauterine cerebral development of rabbits to humans. The E29 rabbit pups (term=32 days, E29=85% to 90% gestation) could be considered equivalent to 33-week premature infant. However, previous studies indicate that cortical and noncortical development of E29 rabbits equates to approximately 20 weeks of gestational age in humans and myelination initiates in the early third trimester in humans and at postnatal Day 4 to 7 in rabbits.31 Thus, E29 pups might be similar to premature infants of 30±4 weeks gestational age.
This article presents the mechanistic basis of GC treatment in the prevention of IVH. Obtaining autopsy materials from premature infants of a short postmortem interval with comparable demographics for the GC treatment and a control group was a result of our diligently made, unremitting effort of several years. The infants in the 2 groups were of short postnatal age to reflect the effect of prenatal steroid. However, the limitations of human studies are exposure of premature infants to a number of pre- and postnatal variables, including mechanical ventilation, exposure to medications, and others, that can potentially impact the data. The data in both rabbits and humans showed that GC augmented the perivascular pericyte coverage. However, despite the enhancement in pericyte coverage after GC treatment, this remained less in the GM than in the other brain regions: cerebral cortex and white matter. Because pericytes are the providers of structural integrity to the vasculature, strategies to further enhance the pericyte coverage might offer greater protection against the development of IVH in premature infants.

In conclusion, prenatal GC suppressed VEGF levels and elevated TGF-β, which resulted in inhibition of angiogenesis, trimming of the neovascularization, and enhancement in the pericyte coverage. These morphological and molecular changes would stabilize the GM vasculature, thereby reducing its vulnerability to hemorrhage. Prenatal glucocorticoid exposure did not induce neural cell death in premature human infants, unlike rabbit pups.

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Disclosures
None.

References


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