Near-Term Fetal Hypoxia–Ischemia in Rabbits
MRI Can Predict Muscle Tone Abnormalities and Deep Brain Injury

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Background and Purpose—The pattern of antenatal brain injury varies with gestational age at the time of insult. Deep brain nuclei are often injured at older gestational ages. Having previously shown postnatal hypertonia after preterm fetal rabbit hypoxia–ischemia, the objective of this study was to investigate the causal relationship between the dynamic regional pattern of brain injury on MRI and the evolution of muscle tone in the near-term rabbit fetus.

Methods—Serial MRI was performed on New Zealand white rabbit fetuses to determine equipotency of fetal hypoxia–ischemia during uterine ischemia comparing 29 days gestation (E29, 92% gestation) with E22 and E25. E29 postnatal kits at 4, 24, and 72 hours after hypoxia–ischemia underwent T2- and diffusion-weighted imaging. Quantitative assessments of tone were made serially using a torque apparatus in addition to clinical assessments.

Results—Based on the brain apparent diffusion coefficient, 32 minutes of uterine ischemia was selected for E29 fetuses. At E30, 58% of the survivors manifested hind limb hypotonia. By E32, 71% of the hypotonic kits developed dystonic hypertonia. Marked and persistent apparent diffusion coefficient reduction in the basal ganglia, thalamus, and brain stem was predictive of these motor deficits.

Conclusions—MRI observation of deep brain injury 6 to 24 hours after near-term hypoxia–ischemia predicts dystonic hypertonia postnatally. Torque-displacement measurements indicate that motor deficits in rabbits progressed from initial hypotonia to hypertonia, similar to human cerebral palsy, but in a compressed timeframe. The presence of deep brain injury and quantitative shift from hypo- to hypertonia may identify patients at risk for developing cerebral palsy. **(*Stroke*. 2012;43:2757-2763.)**

Key Words: diffusion-weighted imaging ■ hypertonia ■ hypoxic–ischemic encephalopathy

Cerebral palsy (CP) is a clinical syndrome commonly designating a group of conditions characterized by chronic motor impairment due to early occurrence of a nonprogressive lesion to the developing brain.[1,2] The type and site of lesions depend on the stage of brain maturation during which pathogenic events occurred.[3–5]

Newborns with hypoxic–ischemic encephalopathy are initially hypotonic and subsequently develop hypertonia after some months.[6] The pathophysiology and evolution of dysfunctional motor control in patients with CP are largely unknown.

In a clinically relevant animal model after fetal hypoxia–ischemia (H-I) at 70% to 79% (E22 and E25) gestation in rabbits, newborn kits at E32 manifest hypertonia, resembling motor deficits of human CP.[7–10] MRI markers on diffusion-weighting imaging during the insult in utero at E25 are predictive of hypertonia.[11] Compared with humans, rabbit motor development is compressed in time. Given the 7- to 10-day period in utero after H-I in the rabbit model, it was possible that the hypotonic period occurred in utero and evolved into hypertonia before birth.

Muscle tone is typically assessed clinically by ordinal measures with distinction into spasticity, rigidity, or dystonia for hypertonia.[12] Laboratory measurements objectively quantify the different types of hypertonia by determining contributions of velocity dependent (viscous) and independent (elastic) components in human[13,14] and animal studies.[15,16]

The hypothesis of this study was that muscle tone in the rabbit would evolve from hypotonia to hypertonia after an H-I insult and that apparent diffusion coefficient (ADC) changes on MRI could be used to predict the eventual motor phenotype. We could not test this hypothesis previously because the preterm fetuses could not be observed ex utero. Sufficient near-term rabbit fetuses survived delivery immediately after H-I to allow immediate neurological examination. The secondary hypothesis was that the pattern of injury

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in near-term H-I would differ from preterm H-I (E22 and E25) with greater involvement of deep brain structures. We used dynamic MRI for evaluation of regional brain injury and torque apparatus measurement of passive joint resistance to stretch for longitudinal and quantitative evaluation of muscle tone changes. The study was designed to investigate short-term outcomes at E32 for comparison with previous studies of earlier gestational ages.

Methods

The Institutional Animal Care and Use Committee of NorthShore University HealthSystem approved all animal experiments.

Prenatal H-I Model and Neurobehavioral Assessment

New Zealand white pregnant rabbits (Myrtle's Rabbits, Thompson Station, TN) at 29 days of gestation (90% term) underwent uterine ischemia induced by inflation of an intra-aortic balloon catheter, which caused in vivo global fetal H-I.[10] Newborn kits were delivered 4 hours after H-I by hysterotomy.[10] Neurobehavioral measurements, tone assessment by a torque-displacement apparatus, and serial MRI were performed at 6, 18, 24, and 72 hours after H-I.

Muscle tone assessment was based on a modified Ashworth scale,[10] which takes into account hypotonia and hypertonia as well as differences in forelimb and hind limb tone. Final assessment of motor deficits was at 72 hours after H-I. Kits were labeled as with or without motor deficits. The former category included kits having abnormal muscle tone (hypertonia or hypotonia), abnormal posture, or locomotion.

Objective Measurements of Muscle Tone

We built a torque-displacement apparatus that measured passive resistance and stretch angle during sinusoidal joint stretch (online-only Data Supplement Methods and Figure I). Joint stiffness (slope of torque-displacement curve), derived from the laboratory muscle tone, was assessed and correlated with manual muscle tone assessment (online-only Data Supplement ID).

Fetal MRI

A subset of dams was studied in a clinical 3-T magnet. T2-weighted and diffusion-weighted imaging of fetal brains (b=0, 0.8 ms/μm^2) were acquired as previously described.[11] Uterine position of each fetus was first identified and ADC of fetal brain was measured.[11] Whole brain ADC could be obtained reliably but regional changes could not be consistently quantified because of fetal motion.

Postnatal MRI

Newborn kits after delivery were studied in an animal 4.7-T magnet and T2-weighted and diffusion tensor images (6 diffusion directions, b=0, 0.8 ms/μm^2) were acquired as previously described.[8] Injury in the region of interest was determined by quantifying the volume of voxels on ADC maps below a predefined threshold value of 0.7 μm^2/ms.

Statistical Analysis

Data were analyzed using SPSS 14.0 software and expressed as means±SEM. Differences between groups were tested using one-way analysis of variance with Tukey post hoc group comparisons. Longitudinal data series were tested with repeated-measures analysis of variance. The cutoff threshold of fetal brain ADC nadir during H-I was determined by linear discriminant analysis, separating hypertonia from nonhypertonia outcomes.

Results

Perinatal Death Increases With Gestation Age for the Same Duration of H-I

We first compared the severity of 40-minute fetal H-I in near-term E29 fetuses with preterm E22 and E25 in terms of mortality and morbidity (E22/E25 data are partly from previously published data[7]). The term (E32) death rate increased with increasing age, suggesting that fetal vulnerability to H-I increases with age (Figure 1A).

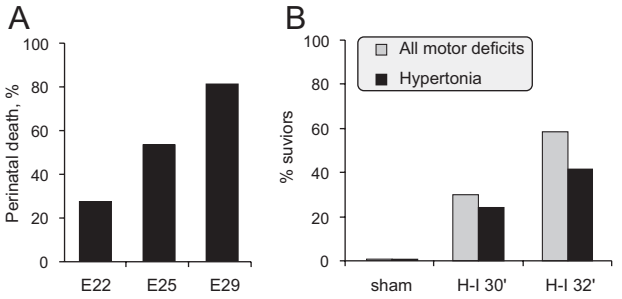

Figure 1. A, Perinatal death increased with age after 40 minutes global fetal H-I at E22 (n=369 fetuses), E25 (186 fetuses), and E29 (89 fetuses; $P<0.001$, χ^2). **B**, Increase of postnatal neurobehavioral deficits with increasing durations of H-I at E29 ($P<0.05$, χ^2 for both motor deficits and hypertonia). Sham controls had no deficits. n=10, 67, 60 for sham; 30 minutes H-I; and 32 minutes H-I correspondingly. H-I indicates hypoxia–ischemia.

Establishing Equipotent Insult

Previously, we showed that 30 minutes H-I at E22 resulted in all normal-appearing kits.[10] The E29 H-I duration was decreased from 40 minutes to 30 or 32 minutes (Figure 1B) resulting in a similar proportion of live-born kits at 72 hours (40.0% and 55.2%, respectively) compared with 59% live kits after 40 minutes H-I at E22.[7] Thirty minutes H-I at E29 resulted in a lower incidence of hypertonia than after 40 minutes H-I at E22,[7] whereas 32 minutes H-I had a similar incidence and was chosen for subsequent studies.

ADC Decline Occurs Faster and Is More Pronounced During H-I at E29 Fetuses

Another parameter to compare brain vulnerability with H-I insult between preterm and near-term fetuses was ADC time course during H-I, because ADC decrease below a threshold level of in E25 fetal brains was predictive of postnatal hypertonia.[11] The pattern of the ADC response to H-I with increasing fetal age for E22 and E29 fetuses was compared with our previous study of E25 fetuses.[11] The fall in ADC at E29 started earlier, was deeper, and recovered more slowly (Figures 2A, 2C,and 2D). The ADC took a median of 28.4 minutes (interquartile range, 25.4–31.3) to fall below the threshold of 0.83 μm^2/ms for E25 fetuses, which distinguished postnatal hypertonia from nonhypertonia. The time of ADC to reach the corresponding mean value at end of 40 minutes H-I in E25 (0.78 μm^2/ms) was 29.6 minutes (median, 28.4; interquartile range, 27.5–32.9) in E29 fetuses.

For 32 minutes H-I in E29 fetuses, the predictive threshold of whole brain ADC for distinguishing hypertonia from nonhypertonia was 0.75 μm^2/ms determined by discriminant analysis. Also, as shown previously, if ADC did not recover in the late reperfusion–reoxygenation period, those fetuses were more likely to die (Figure 2B).

Muscle Tone of After Near-Term Fetal H-I Evolves From Hypotonia to Hypertonia

The incidence and pattern of motor deficits after 32 minutes H-I at E29 were compared with previously published data at

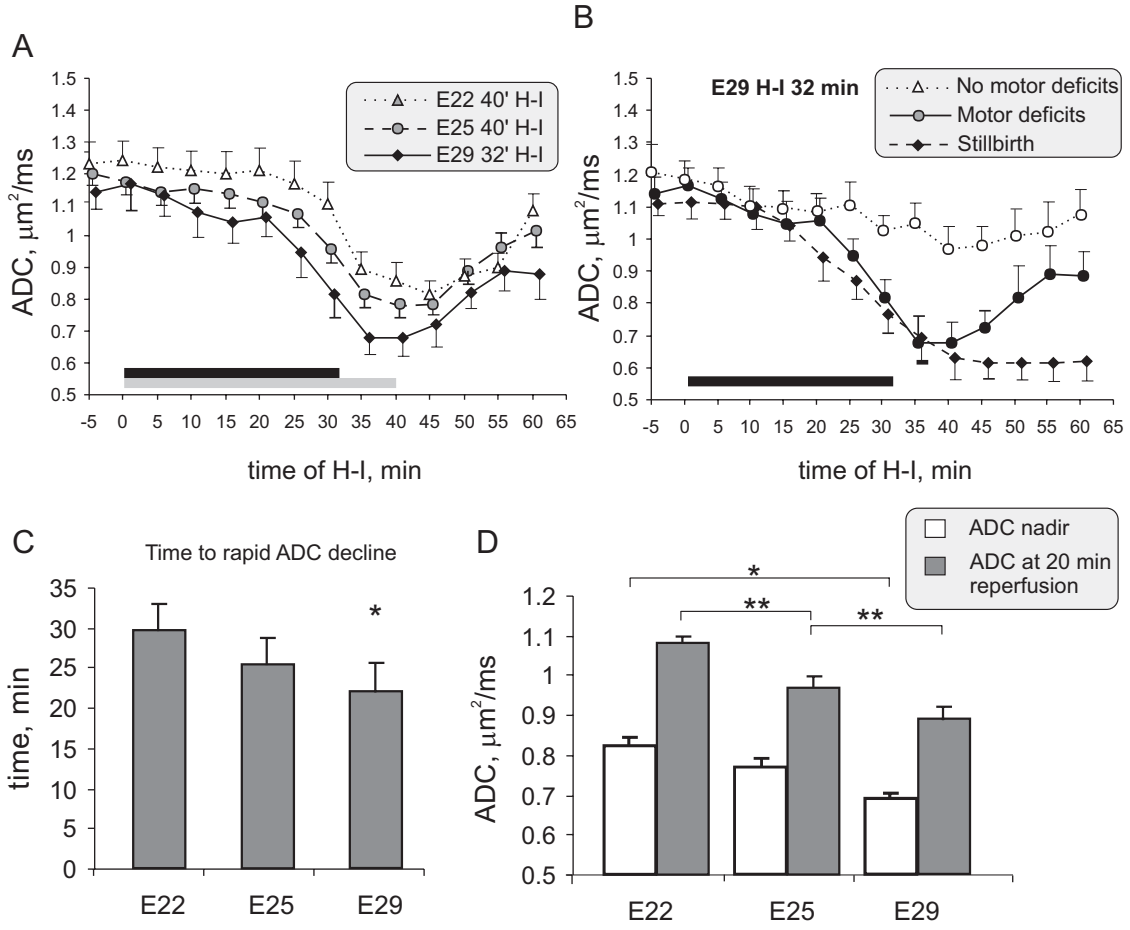

Figure 2. A, Dynamic ADC changes during H-I and reperfusion–reoxygenation in E22, E25, and E29 fetuses that were born alive with motor deficits. Duration of H-I was 40 minutes for E22 and E25 fetuses (gray bar) and 32 minutes for E29 fetuses (black bar). With increasing age there is an earlier and deeper fall in ADC during H-I. **B**, Fetuses that developed motor deficits later showed a pattern of ADC different from sham controls (not shown), nonhypertonic, and stillbirths with 32 minutes H-I at E29. **C**, The onset of rapid phase of ADC decline during H-I occurred significantly earlier at E29 than at E22. *$P<0.05$ in E29 group compared with E22. **D**, The lowest value of ADC during time course (nadir) was lower in E29 compared with E22 (*$P<0.05$). The ADC value at 20 minutes reperfusion progressively decreased with increasing gestational age. **$P<0.05$ in E25 group compared with E22 and E29 groups. Analysis of variance with Tukey post hoc group comparisons. ADC indicates apparent diffusion coefficient; H-I, hypoxia–ischemia.

E22 and E25. The incidence of hypertonia after H-I at E22 was 75% using a clinical score based on modification of the Ashworth scale.[7] In E29 animals, 24 hours after the H-I, 14 of 24 surviving kits (58%) had hypotonia of the hind limbs. Of these hypotonic kits, 10 (71%) subsequently developed hypertonia at 72 hours, 7 in hind limbs (Figure 3A) and 3 in all limbs. The other 4 kits continued to have hind limb hypotonia at 72 hours. In addition to manual evaluation,[7] the qualitative assessment of tone was validated and subsequently quantified by measures of muscle resistance to passive stretch using the torque-displacement apparatus. Kits that developed hypertonia by 72 hour after H-I had initial resistance to stretch that was lower than corresponding age-matched control kits at 24 hour after H-I (Figure 3B). Limb resistance to passive stretch

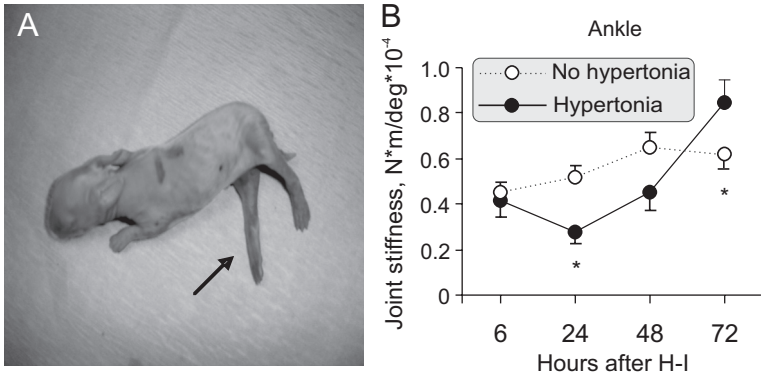

Figure 3. A, Illustrative case of hind limb hypertonia at 72 hours after E29 fetal H-I. Increased tone in knee and ankle extensors (arrow). **B**, Biphasic change of muscle tone in surviving kits after E29 fetal H-I. The tone measure, joint stiffness, was calculated as a slope between joint resistance to passive stretch and joint displacement angle, obtained with a torque-displacement apparatus (online-only Data Supplement Figure I). The hypertonic group (at 72 hours) had initially lower hind limb tone at 24 hours, replaced by elevated tone by 72 hours after H-I. *$P<0.05$ in tone difference between groups, repeated-measures analysis of variance. H-I indicates hypoxia–ischemia.

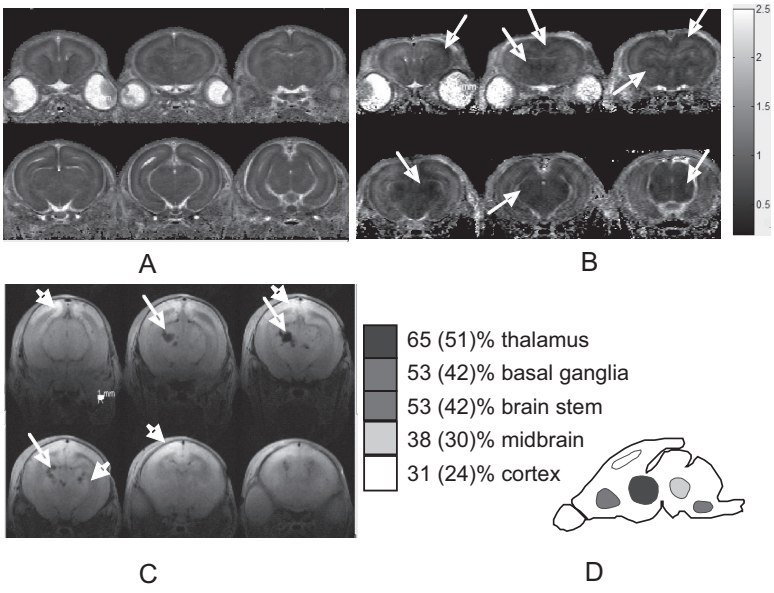

Figure 4. Diffusion weighted images 24 hours after H-I in E29 brain on showing no injury in **A** and severe injury in **B**, in areas of cortex, basal ganglia, thalamus, midbrain, brain stem (arrows indicates areas with abnormally low ADC less 0.7 μm^2/ms). Color map bar units are μm^2/ms. **C**, T2-weighted images 72 hours after H-I in E29 brain (n=19 kits) show ischemic regions (arrowheads, bright image) and hemorrhagic lesions (long arrows, black image) in thalamus and cortex. **D**, The frequency of injury is depicted as a proportion of all kits with any injury on MRI and a proportion of all studied kits after H-I in parenthesis.

did not change with stretch velocity, indicative of dystonic hypertonia and not spastic hypertonia as assessed by human criteria.[12] The dystonic hypertonia after H-I at E29 kits was similar to that found after H-I at E22.[10] The quantitative data confirm that hypertonia is preceded by a period of hypotonia in rabbit kits. The short duration of hypotonia also confirms the rapid development of rabbit motor function, similar to other nonhuman mammals and distinct from the slower human development.

Deep Brain Nuclei Pattern of Injury After E29 H-I

Regional injury could be demarcated by abnormally low ADC at 24 hours after H-I (Figure 4A–B). We also found a spectrum of abnormalities on T2-weighted MRI 72 hours after H-I (Figure 4C), including hyperintensive areas (white arrows), indicative of high water content, and also hypointensive lesions (yellow arrows) in some severely affected kits, suggestive of hemorrhages. The prevalence and locations of brain injury are depicted in Figure 4D. Thalamic injury was most common (65%) and often seen in combination with other regions. In addition, there was frequent injury to the other deep brain structures such as the basal ganglia and brain stem, including the midbrain, pons, and medulla. Cortical injury, predominantly parasagittal, was observed less frequently and mostly in severe cases with pronounced hypertonia of all limbs. There were also cases with predominantly basal ganglia injury without thalamic involvement (31% of cases with any injury on MRI and 24% of all studied E29 kits after H-I).

Persistent Regional Reduction of ADC Within 24 Hours After H-I Is Indicative of Injury

The regions with abnormally low ADC (Figure 4) corresponded to areas with abundant terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, indicative of cell injury (Figure 5A–C). The number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells was significantly higher in regions with ADC <0.7 μm^2/ms (Figure 5D).

The kits that developed hypertonia had a marked and persistent reduction of ADC during acute H-I in the basal ganglia, thalamus, and midbrain compared with nonhypertonic kits (online-only Data Supplement Figure II) and the decrease persisted for 24 hours. At 72 hours after H-I, abnormally high ADC, tissue loss, or hyperintensities on T2-weighted images were observed in the areas of initially decreased ADC (online-only Data Supplement Figure II), similar to the evolution of perinatal brain H-I injury in other animal models and humans.[17]

Deep Brain Nuclear Injury, Diagnosed by ADC Decrease, Is a Biomarker of Motor Deficits

In kits that developed motor deficits, ADC in the thalamus declined at the end of H-I and remained low at 6, 18, and 24 hours (Figure 6A) and this decline was greater than in those kits without motor deficits. A similar pattern of ADC change was observed in the other deep gray matter regions and parietal cortex. ADC changes in the frontal and motor cortex were not significantly different from sham controls. The decrease of ADC values after H-I at 6 and 18 hours was significantly lower than corresponding values in control kits ($P<0.05$, repeated-measures analysis of variance), even on the background of significant maturational changes of ADC in the perinatal period.[18]

The severity of H-I injury was delineated by the volume of voxels with an ADC value <0.7 μm^2/ms. We chose this empirical threshold because gray and white matter ADC in control animals between E29 and E32 were always above this value and because increased cell injury was observed in regions below this threshold (Figure 5). The volume of voxels with ADC below the cutoff value in injured animals decreased from 6 to 24 hours after H-I (Figure 6B) but was still significantly higher at the latter time point in all examined regions (except the cerebral cortex). The ADC normalized to control values beyond 24 hours after H-I in deep gray matter

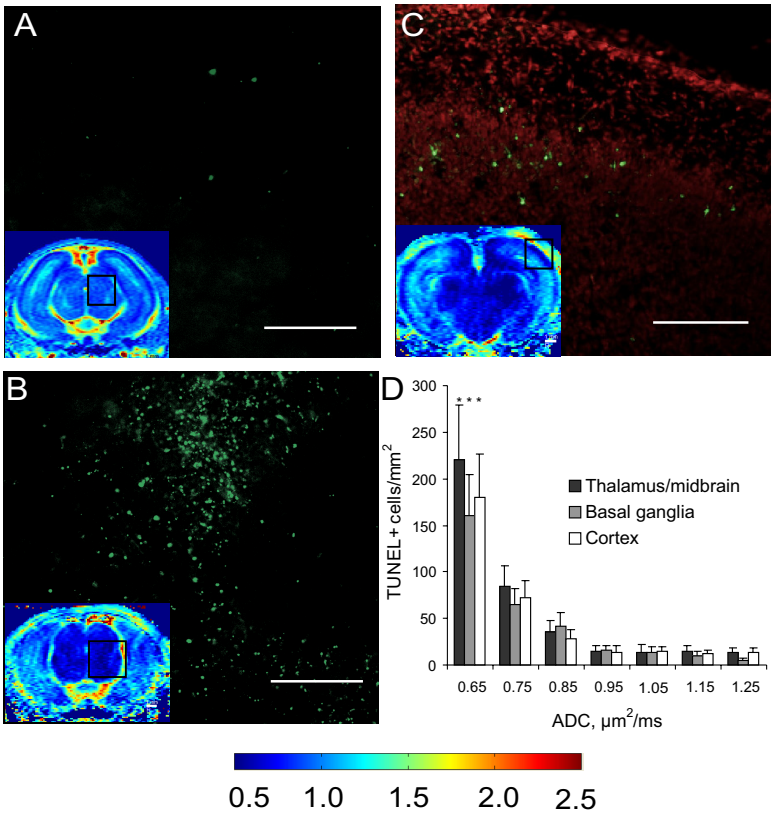

Figure 5. Regions of injury defined by ADC decrease below threshold 0.7 μm^2/ms shown on ADC maps from the same animals (color inserts) on sections at midbrain (**A–B**) and thalamus (**C**) levels in controls (**A**) and hypertonic (**B–C**) kits had numerous injured cells with positive TUNEL staining 24 hours after H-I injury at E29. Overlay TUNEL (green)+propidium iodide (red; **C**) demonstrate that cortical injury was mostly in Layer 4. The number of TUNEL-positive cells was significantly higher in regions with ADC <0.7 μm^2/ms (**D**) in all counted gray matter regions ($P<0.05$, analysis of variance). Color map bar units are μm^2/ms. Scale bar 500 μm. Number of samples n=4/group in H-I and control groups. ADC indicates apparent diffusion coefficient; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; H-I, hypoxia–ischemia.

and the cortex (Figure 6A). Therefore, a persistent ADC decrease between 6 and 24 hours in deep gray matter regions can be used as an early marker of acute hypoxic injury in deep gray matter and is predictive of subsequent motor deficits. Interestingly, T2-weighted images at 72 hours, but not at 24 hours, showed changes that suggest possible hemorrhages in thalamus/midbrains of 2 kits (6%) in areas that had abnormally low ADC at 24 hours after H-I. However, these T2 changes did not possess an independent predictive value of hypertonia and seems to be secondary to the severe injury observed with ADC. To test the association between pattern of injury observed in kits 24 hours and the presence of neurological abnormalities at 72 hours after H-I, a regression analysis was applied. The best combination of predictors for any motor deficits was injury in basal ganglia and in brain stem and for hypertonia in the thalamus and brain stem (online-only Data Supplement Table III).

Discussion

Biphasic Muscle Tone Changes After Antenatal H-I

For the first time in a perinatal H-I model, evolution of motor abnormalities was quantitatively assessed by measuring resistance to passive joint stretch (online-only Data Supplement Figure I). The present study shows the rapid evolution (a few days) from hypotonia to hypertonia in the developing rabbit. Previously only hypertonia was observed after delivery at E32 or P1 after H-I at E22 and E25.[7,9,11] This evolution of hypotonia to hypertonia after E29 H-I is similar to that of humans with H-I injury. Patients with neonatal encephalopathy have mostly hypotonia as an initial finding and hypertonia may develop months or years later with the diagnosis of spasticity or dystonia.[19] This timespan makes causal etiologic linkages difficult in human clinical studies. The rapid transi-

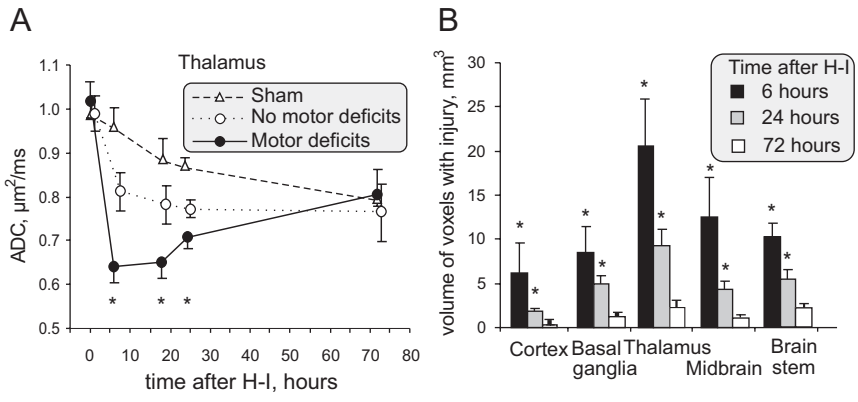

Figure 6. A, Dynamic changes of ADC in the region of thalamus at the level of third ventricle. Significant ADC decrease persisted in the thalamus 6 to 24 hours after H-I and is an early marker of acute hypoxic thalamic injury. *$P<0.05$ comparing motor deficits with group without motor deficits, repeated-measures analysis of variance. **B**, Volume of voxels with ADC <0.7 μm^2/ms in the brains of kits with motor deficits, as an indicator of injury severity, declined from 6 hours to 24 hours after H-I but remained significant at the 24-hour time point. *$P<0.05$, *t* test and Bonferroni correction. ADC indicates apparent diffusion coefficient; H-I, hypoxia–ischemia.

tion in perinatal rabbits makes the model a convenient platform to study the etiology of motor deficits of CP after H-I. Furthermore, we show structural lesions on MRI are predictive of motor deficits postnatally and there is a regional predilection to brain injury.

Sensitivity of Deep Brain Nuclei to H-I Injury and Importance of the Injury in the Pathogenesis of CP

Human term newborns often have cerebral cortical–deep nuclear or deep nuclear–brain stem types of brain injury.[4–6,19] The basal ganglia/thalamus pattern in term and premature infants[5] is associated with the most impaired motor and cognitive outcome at 30 months.[4–5,20] Thalamic motor nuclei are especially injured.[21] Similarly, the near-term rabbit studies at E29 confirm the predictive value of basal ganglia–thalamus–brain stem injury to postnatal motor deficits (online-only Data Supplement Table IV).

The injury also involved cortex and periventricular white matter, albeit to a lesser extent. In human infants after perinatal asphyxia, decreased brain MRI ADC values of the brain stem and basal ganglia correlated with abnormal or adverse outcome.[22] The implication of neuronal loss and gliosis being more common in the thalamus in human periventricular leukomalacia[21,23] is either due to a primary injury or a secondary anterograde or retrograde injury. The secondary consequences of a primary neuronal injury could involve white matter axons with subsequent hypomyelination and impaired development of the cerebral cortex and thalamus/basal ganglia.[24] This study, however, was designed only to study short-term outcome at 72 hours because we wanted to compare with E32 findings of previous studies of earlier gestational ages. Longer-term white matter and cortical/subcortical abnormalities and involvement in motor and cognitive impairment in rabbits require further investigation.

MRI as a Biomarker

A decrease of whole brain ADC $<0.83\ \mu m^2/ms$ in E25[11] and $<0.75\ \mu m^2/ms$ in E29 rabbits during and immediately after H-I (Figure 2) was predictive of motor deficits. We have extended the time window to 6 to 24 hours postnatally after H-I and demonstrated a threshold of ADC decrease $<0.7\ \mu m^2/ms$ to be a reliable diagnostic tool for injury to the deep brain nuclei and was predictive of hypertonia and other motor deficits. The implication is also that MRI indicates a level of regional injury that is significant clinically to cause a motor phenotype. The absence of the specific ADC fall also may be useful prognostically.

Comparison of Timing

Our study underlines the importance of timing in diagnosing perinatal brain injury by diffusion-weighting imaging. In contrast to rodent[25] and piglet[26] neonatal stroke models, we did not observe a secondary decline of ADC 24 hours after H-I. In rabbits, whole brain ADC only partially recovered after 20 minutes of reperfusion–reoxygenation and was consistently low at 6 to 24 hours in deep brain nuclei and cortex. This discrepancy can be attributed to species differences and global nature of H-I and is more analogous to the human situation. In a prospective human longitudinal study with known time of insult, ADC declined and reached a nadir in injured regions of 35% of the control values between 2 and 3 days.[17] ADC returned to normal levels by 7 days. In the present study, the corresponding nadir of ADC occurred around 6 hours and returned to normal levels at approximately 3 days, suggesting the evolution of H-I injury follows the human patterns but occurs 4 to 6 times faster. This may correspond to the relatively short timeframe of neurobehavioral evolution from hypotonia to hypertonia in rabbits.

Summary

The near-term rabbit model may be a useful platform to test mechanisms for evolution of muscle tone changes. We have shown that quantification of lesions is important both for prediction and studying evolution of muscle tone. Abnormally low ADC values suggestive of deep brain injury 6 to 24 hours after near-term H-I predicts dystonic hypertonia postnatally. The first torque-displacement studies to be done in perinatal models indicate that motor deficits in rabbits progressed from initial hypotonia to hypertonia, similar to human CP but in a compressed timeframe. The presence of deep brain injury and quantitative shift from hypo- to hypertonia may identify patients at risk for developing CP.

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Disclosures

None.

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