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 hypotonia 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 human13,14 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...
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 sinuosoidal 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/μm²) were acquired as previously described.11 Uterine position of each fetus was first identified and ADC of fetal brain was measured.11 The incidence and pattern of motor deficits and hypertonia could not be consistently quantified because of fetal motion.11 The pattern of the ADC response to H-I with ischemia induced by inflation of an intra-aortic balloon catheter, which caused in vivo global fetal H-I,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. 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. T2-weighted and diffusion tensor images (6 diffusion directions, b=0, 0.8 ms/μm²) 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 μm²/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).10 The term (E32) death rate increased with increasing age, suggesting that fetal vulnerability to H-I increases with age (Figure 1A).

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 μm²/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 at E25 (0.78 μm²/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 μm²/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...
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. 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, 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).

**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. 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.

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**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.
similar to that found after H-I at E22.10 The quantitative data corresponding to areas with abnormally low ADC less 0.7 μm²/ms. Color map bar units are μm²/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.

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 hyperintense areas (white arrows), indicative of high water content, and also hypointense 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 μm²/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 μm²/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...
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-
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.

References


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SUPPLEMENTARY MATERIAL
NEAR-TERM FETAL HYPOXIA-ISCHEMIA IN RABBITS: MRI CAN PREDICT MUSCLE TONE ABNORMALITIES AND DEEP BRAIN INJURY

SUPPLEMENTARY METHODS

Prenatal hypoxia-ischemia model.

In vivo global hypoxia–ischemia of fetuses was induced by sustained uterine ischemia at 29 days gestation (90% term) in timed pregnant New Zealand white rabbits (Myrtle’s Rabbits, Thompson Station, Tennessee) as described previously. This scenario models acute placental insufficiency at near term gestation. Briefly, dams were anesthetized and a balloon catheter was introduced into the left femoral artery and advanced into the descending aorta to above the uterine and below the renal arteries. The balloon was inflated for 30, 32, or 40 minutes causing uterine ischemia and subsequent global fetal H-I. Sham animals underwent the same procedure but without balloon inflation. At the end of H-I, the balloon was deflated, resulting in uterine reperfusion. The catheter was then removed, femoral artery repaired, and the dams were allowed to recover. A subset of dam was imaged during H-I and reperfusion in a GE 3T clinical magnet as described earlier. Four hours after H-I fetuses were delivered by laparatomy. Fetal positions in uterus were recorded to identify the position corresponding fetuses on MRI scan during H-I. Surviving kits were kept in a temperature controlled incubator and gavage fed up to E32=P1 (72 hours after H-I) with rabbit milk. At 6, 18, 24 and 72 hours after H-I newborn kits underwent neurological assessment, clinical measurement of tone and muscle tone measurements using a custom built torque-displacement apparatus and a serial MRI scan.

Neurological assessment.

At E32 kits underwent a battery of neurobehavioral tests for the presence of sensory and motor deficits as described previously. The assessments include tests for posture, muscle tone, activity, locomotion, orofacial reflexes, and sense of touch and smell. The test was video recorded and results were evaluated by 2 observers masked to the treatment group assignment. Muscle tone assessment was based on a modified Ashworth scale, that takes into account both hypotonia and hypertonia, as well as difference in forelimb and hind limb tone in control newborns. Presence of motor deficits was finally assessed at 72 h after H-I and kits were labeled as “with no motor deficits” or “with motor deficits”. The latter category included kits having abnormal either/or muscle tone (hypertonia or hypotonia), posture or locomotion.

Setup of a portable muscle tone measurement system for small animals.

Muscle tone is a subjective assessment of resistance to a passive stretch. Muscle tone was assessed in wrist, elbow, shoulder, hip, knee and ankle joints on the left and right sides by both a clinical method of subjective assessment and by quantitative measures of passive resistance to stretch. To quantify muscle tone in small neonatal
animals a portable system was developed to measure passive resistance and stretch angle during sinusoidal joint stretch. The design of the system and principle of measurements was similar to other laboratory muscle tone measurement devices, used to assess muscle tone components in humans and animal studies and adapted for small animal size and small force measurements. The arrangement of this hand-held device and its recording system can be seen in supplementary figure S1.

An animal was positioned on a platform so that the rotational axis of a measured joint was aligned with the rotational axis of a jig and was held in place manually by experimenter. The distal joint part was placed in a U-shaped plastic restrainer connected to the rotating jig and underwent repetitive flexion and extension cycles. The joint was stretched at 4 velocities, 0.5, 1, 1.5 and 2 cycles/sec for 30 sec each, paced with the aid of computer generated cycles. The maximal allowed joint rotation range was ± 40 degrees. Stretching at each velocity was repeated twice in random order. Angle of the joint rotation and applied force (torque) was measured by sensors and recorded using data acquisition card (National Instruments, TX) connected to laptop running custom build program on LabView (National Instruments, TX). The force transducer was calibrated with a set of known weights. The rabbit kits were first subjected to 15–20 cycles so that the kit got used to the apparatus. Recording was started after the kits stopped their voluntary contractions. Care was taken to measure only passive resistance.

Joint stiffness, as a measure of muscle tone, was derived from a linear regression of the displacement–torque curves and was mostly determined by elastic component of muscle resistance. Joint resistance was also decomposed to viscous and elastic components and a complex modulus of resistance was calculated as a measure of total joint resistance.

MR imaging.

Survival in utero MR imaging was performed in 3T GE clinical magnet as described previously. The anesthetized dam instrumented with an aortic catheter (as described above) was positioned in a quadrature extremity coil. An extended catheter connection allowed initiating and ceasing uterine ischemia by inflating and deflating the balloon remotely without moving the animal. Single shot fast spin echo (SSFSE) T2-weighted images were taken for anatomical reference in axial, coronal and sagittal planes, with 25-32 axial slices covering all the fetuses inside dam, slice thickness 4 mm, matrix 256x192, and field of view 16 cm. Anatomical scans were followed by continuous series of diffusion weighted echo-planar images (DWI) with b=0, and 0.8 ms/µm², TR/TE = 7400/70 ms, NEX = 1, and the same slice geometry as in the reference anatomical images during the 5 min before H-I, during H-I and 20 min of reperfusion. Apparent diffusion coefficient (ADC) maps were calculated from DWI series using in-house software, written on Matlab (Natick, MA) and mean ADC values was obtained for each fetal brain for each time point by placing a region of interest on whole fetal brain. ADC nadir was defined as the lowest ADC value during H-I and 20 min of reperfusion. Time to rapid ADC decline was calculated as an intersection of baseline ADC and a regression line with the fastest slope of ADC decline, determined form ADC curve between 15 and 40
min of H-I phase using sliding 5 min time window.

Uterine position of each fetus was identified, to serially follow ADC time course of fetal brains during H-I in utero and postnatally after delivery by hysterotomy. For comparison of H-I between different gestation ages, 4 E29 dams (27 fetuses) were imaged during 32 min H-I, 2 E22 dams (12 fetuses) during 40 min H-I and compared to published data from 8 E25 dams (56 fetuses) during 40 min H-I.

Postnatal MR imaging and data analysis. MR imaging was performed using 4.7T Bruker magnet and 30 mm circular surface coil and multi-slice T2-weighted RARE sequence (TE/TR 80/4000 ms) to determine presence of gross anatomical abnormalities. Diffusion tensor images were acquired with parameters: field of view 2 cm, matrix 128x64, 12 axial slices 1 mm thick, 6 diffusion directions, 6 averages, TR/TE 2000/21 ms, b= 0, 0.8 ms/µm². ADC and FA maps were calculated. Spatial distribution of ADC changes was assessed by the region of interest analysis. The studied regions included cerebral cortex, basal ganglia, thalamus, midbrain and pons, based on anatomical landmarks. Severity of injury was quantified by counting volume of voxels on ADC maps with the value below predefine threshold lower than 0.7 µm²/ms in all slices where the studied regions were present. Volume of injury was quantified as a product of number of voxels below threshold by voxel size. The empirical threshold 0.7 µm²/ms was chosen since the voxels with ADC, lower than this cut-off ADC value, are not normally present in control animal gray and white matter at the studied perinatal period.
Figure S1. Laboratory testing of muscle tone in P1 rabbit kit.

**A.** General view of the torque-displacement apparatus.

**B.** The limb was placed in a U-shaped restrainer (a) connected to a pivoting rig. The joint was rotated at several predefined velocities manually by investigator using handle (b) attached to the rotation axes. Applied force (torque) was measured with a force sensor (c). Joint angular position and applied force (torque) was recorded on computer and analyzed by software written on LabView and Matlab. Care was taken to measure only passive resistance.

**C.** Typical torque - angular displacement curve on P1 rabbit kit. Joint stiffness was estimated from the slope of torque-displacement curve. Hysteresis due to phase shift can between reactive resistance and angular displacement be observed on the plot, which shows the viscous property of the targeted muscle.

**D-E.** Joint stiffness correlated with manual muscle tone assessment according to modified Ashworth scale \(^1\) in forelimbs (R=0.58 for wrist and R=0.48 for elbow) and hind limbs (R=0.36 for knee and R=0.63 for ankle).
Figure S2. ADC time course was obtained in serial imaging on E29 fetuses, starting from in utero imaging at 32 min of H-I (first column) and postnatal imaging in kits after C-section delivery 4 hours after H-I. Representative images are shown for kits with and without muscle hypertonia, assessed at 72 hours after H-I (at E32, corresponding to P1 in naïve controls). After E29 H-I, ADC was lower (deep blue) in basal ganglia and midbrain in hypertonic kit at 6 and 24 hours (white arrows). At 72 hours after H-I, ADC in thalamus/midbrain/basal ganglia increased relative to non-hypertonic kits (black arrow). Cortex was relatively spared in both hypertonic and non-hypertonic kits. Color map bar units are μm^2/ms.
### SUPPLEMENTARY TABLES

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<tr>
<td><strong>Any motor deficits at 72 hour after H-I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logit P = -1.550 + (0.976 * Basal ganglia) + (1.10 * Brain stem)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual negative</td>
<td>12</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual positive</td>
<td>2</td>
<td>17</td>
<td>&lt;0.001</td>
<td>0.86</td>
<td>0.89</td>
</tr>
<tr>
<td><strong>Hypertonia at 72 hour after H-I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logit P = -2.728 + (0.682 * Thalamus) + (0.61 * Brain stem)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual negative</td>
<td>20</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual positive</td>
<td>4</td>
<td>7</td>
<td>0.002</td>
<td>0.83</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table S3. Logistic regression model and results of classification to predict presence of any motor deficits and hypertonia at 72h after H-I by presence of abnormally low ADC regions at 24 hours after H-I. Threshold probability for positive classification: 0.5. The power of the Fisher’s exact test with alpha =0.05 was 0.82 for all motor deficits and 0.78 for hypertonia at 72 hours.

### SUPPLEMENTARY REFERENCES
