**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[1]. 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[2]. 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[1]. 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[1] , 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[3-4] and animal studies[5-6] 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[4].

*MR imaging.*

*Survival in utero MR imaging* was performed in 3T GE clinical magnet as described previously[2]. 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/$\mu m^2$, 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[2] 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-wheighted 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/$\mu m^2$. ADC and FA maps were calculated[7]. 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 $\mu m^2$/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 $\mu m^2$/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.

**SUPPLEMENTARY FIGURES AND FIGURE LEGENDS**

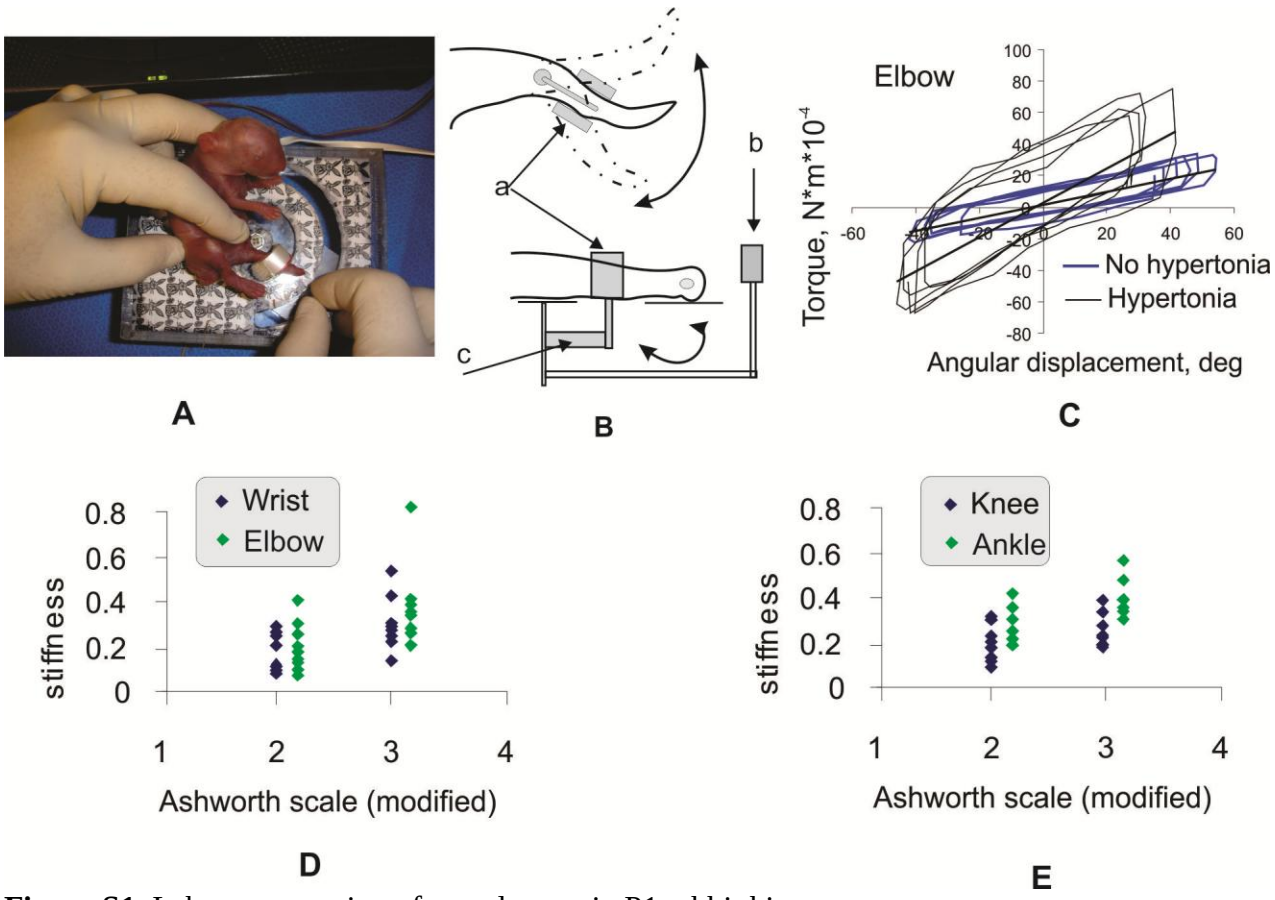


**Figure S1.** Laboratory testing of muscle tone in P1 rabbit kit.

**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).

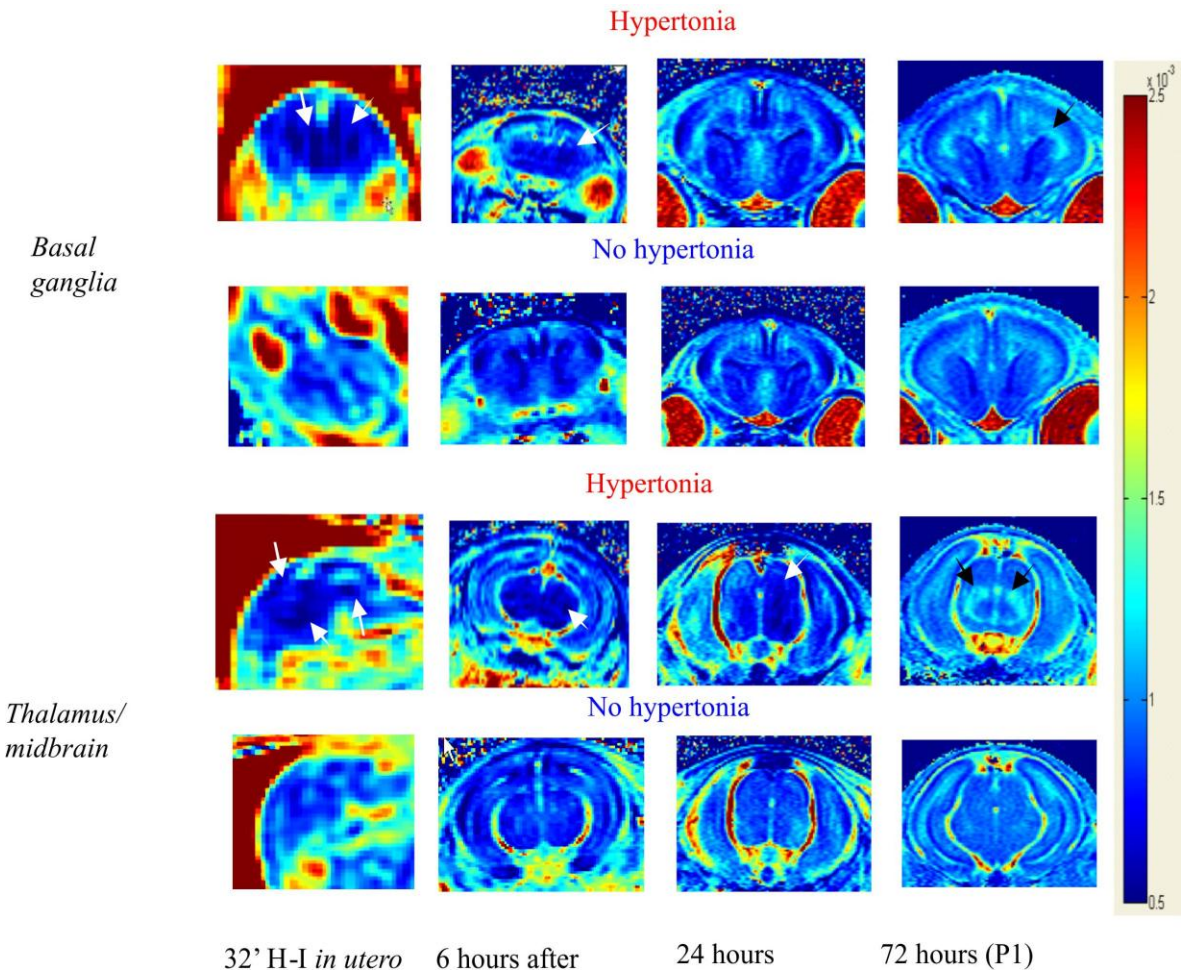


**Figure S2**. ADC time course was obtained in serial imaging on E29 fetuses, starting from in utero imaging at 32 min of H-I (first column) and postnatal imaging in kits after C-section delivery 4 hours after H-I. Representative images are shown for kits with and without muscle hypertonia, assessed 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 $\mu m^2/ms$.

## SUPPLEMENTARY TABLES

| | Predicted negative | Predicted positive | Fisher's exact test, p-value | Sensitivity | Specificity |
|---|---|---|---|---|---|
| **Any motor deficits at 72 hour after H-I** | | | | | |
| Logit P = -1.550 + (0.976 * Basal ganglia) + (1.10* Brain stem) | | | | | |
| Actual negative | 12 | 2 | | | |
| Actual positive | 2 | 17 | <0.001 | 0.86 | 0.89 |
| **Hypertonia at 72 hour after H-I** | | | | | |
| Logit P = -2.728 + (0.682 * Thalamus) + (0.61 * Brain stem) | | | | | |
| Actual negative | 20 | 2 | | | |
| Actual positive | 4 | 7 | 0.002 | 0.83 | 0.78 |

**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


1. Derrick M, Luo NL, Bregman JC, Jilling T, Ji X, Fisher K, et al. Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: A model for human cerebral palsy? *J. Neurosci.* 2004;24:24-34
2. Drobyshevsky A, Derrick M, Prasad PV, Ji X, Englof I, Tan S. Fetal brain magnetic resonance imaging response acutely to hypoxia-ischemia predicts postnatal outcome. *Ann. Neurol.* 2007;61:307-314
3. Chen JJ, Wu YN, Huang SC, Lee HM, Wang YL. The use of a portable muscle tone measurement device to measure the effects of botulinum toxin type a on elbow flexor spasticity. *Arch. Phys. Med. Rehabil.* 2005;86:1655-1660
4. Lee HM, Chen JJ, Ju MS, Lin CC, Poon PP. Validation of portable muscle tone measurement device for quantifying velocity-dependent properties in elbow spasticity. *J. Electromyogr. Kinesiol.* 2004;14:577-589
5. Kakinohana O, Hefferan MP, Nakamura S, Kakinohana M, Galik J, Tomori Z, et al. Development of gaba-sensitive spasticity and rigidity in rats after transient spinal cord ischemia: A qualitative and quantitative electrophysiological and histopathological study. *Neuroscience*. 2006;141:1569-1583
6. Wu YN, Hyland BI, Chen JJ. Biomechanical and electromyogram characterization of neuroleptic-induced rigidity in the rat. *Neuroscience*. 2007;147:183-196
7. Drobyshevsky A, Derrick M, Wyrwicz AM, Ji X, Englof I, Ullman LM, et al. White matter injury correlates with hypertonia in an animal model of cerebral palsy. *J. Cereb. Blood Flow Metab.* 2007;27:270-281