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Moderate Dietary Restriction Reduces p53-Mediated Neurovascular Damage and Microglia Activation After Hypoxic Ischemia in Neonatal Brain

Yi-Fang Tu, MD; Pei-Jung Lu, PhD; Chao-Ching Huang, MD; Chien-Jung Ho, BS; Ya-Ping Chou, BS

Background and Purpose—Neurovascular damage, including neuronal apoptosis and blood–brain barrier (BBB) damage, and microglia activation account for the hypoxic-ischemia (HI) susceptibility in neonatal brain. The p53 upregulation is involved in apoptosis, endothelial cell damage, and microglia activation. We hypothesized that underweight induced by dietary restriction (DR) protects against HI in rat pups by attenuating p53-mediated neurovascular damage.

Methods—Male rat pups were grouped as normal litter (NL) size (12 pups/dam), DR (18 pups/dam), and extreme DR (24 pups/dam) from postnatal day 1 and subjected to HI on postnatal day 7. Immunohistochemistry and immunoblotting were used to determine p53, phospho-murine double minute-2, caspases, BBB damage and microglia activation, and immunofluorescence to determine the cellular distribution of p53. Pharmacological approaches were used to regulate p53.

Results—The NL, DR, and extreme DR pups had similar TUNEL-positive cells and caspases on postnatal day 7 and comparable learning performance at adulthood. After HI, the DR-HI, but not extreme DR-HI, pups had significantly lower p53, higher phospho-murine double minute-2, lower cleaved caspases, less BBB damage and microglia activation, and less brain volume loss than NL-HI pups. In NL-HI pups, p53 expression was located mainly in the neurons, endothelial cells, and microglia. The p53 blockage by pifithrin- α in NL-HI pups decreased apoptosis, BBB damage, and microglia activation, and was neuroprotective. In contrast, upregulating p53 by nutlin-3 in DR-HI pups increased apoptosis, BBB damage, and microglia activation, and worsened brain damage.

Conclusions—Moderate DR, but not extreme DR, reduces p53-mediated neurovascular damage after HI and confers long-term protection in neonatal brain. (*Stroke*. 2012;43:491-498.)

Key Words: dietary restriction ■ hypoxic-ischemia ■ neonatal brain ■ underweight

Perinatal hypoxic-ischemia (HI) is a major cause of mortality and neurological disabilities in infants. Approximately 30% to 40% of them die at birth, and 20% to 40% of surviving infants have development of significant neurological deficits.^{1,2} The major target of ischemic-reperfusion injury in the central nerve system is the neurovascular unit, which is composed of neurons, microvessels, and microglia.^{2,3} Neuronal apoptosis and microvessels damage, ie, blood–brain barrier (BBB) disruption, has been linked to the severity of HI injury in the neonatal brains.^{1–5} Activated microglia are the hallmark of neuroinflammation and exacerbate brain injury through production of proinflammatory cytokines.^{4,5} Therefore, neurovascular damage, such as neuronal apoptosis, BBB damage, and microglia activation, may account for the HI susceptibility of the neonatal brain.^{4,5}

The transcription factor p53 is a key regulator of cell-cycle progression, DNA repair mechanisms, and apoptosis.⁶ After brain ischemia, the p53 was upregulated and contributed to

neuronal apoptosis.⁶ Inhibition of p53 after HI in neonatal rats decreased infarct size and improved functional outcome.⁷ Also, p53 plays a role in vascular endothelial cell apoptosis and microglia activation.^{8–9} Increased p53 expression after hypoxia in human umbilical vein endothelial cells accelerated cell death after hypoxia.⁸ In vitro studies showed that p53 expression was increased after activation of primary rat microglia, and blockage of p53 prevented microglia neurotoxicity.⁹ Whether p53 is the shared injurious pathway affecting the neurons, endothelial cells, and microglia after HI in the neonatal brain remains unknown.

In human newborns, the term small-for-gestational-age refers to a low birth weight with respect to gestational age. Small-for-gestational-age infants are at higher risk for perinatal asphyxia but are at lower risk for incidence of cerebral palsy consequence than infants who are appropriate for gestational age.¹⁰ In rat pups, underweight can be achieved by increasing the litter size, ie, restricting access to breast milk

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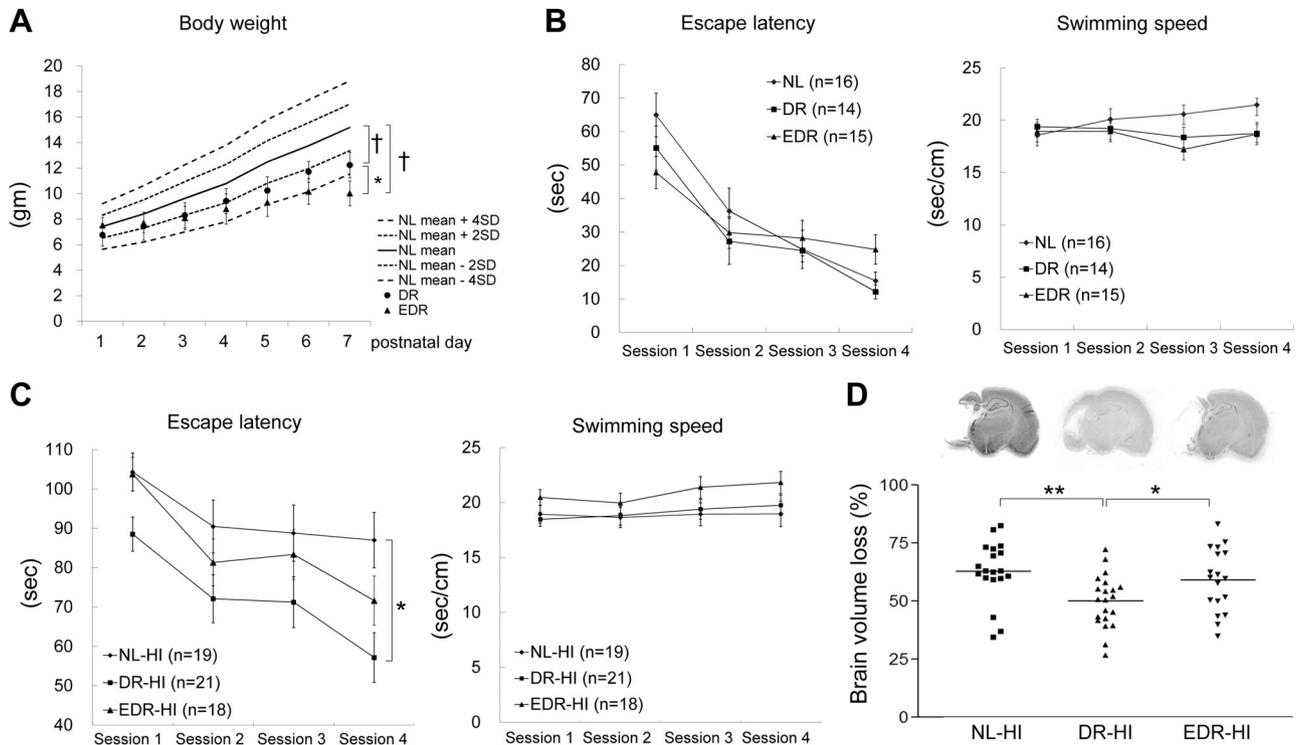


Figure 1. **A**, Relative to the growth curve of normal litter (NL) pups (means±standard deviation [SD], n=26), the dietary restriction (DR) pups (n=24) weighed between 2 and 4 SD, and the extreme DR (EDR) pups (n=25) weighed <4 SD on postnatal day 7. **B**, Morris water maze test in postnatal day 42 showed similar escape latency and swimming speed between NL, DR, and EDR groups. **C**, The DR-hypoxic-ischemia (HI) but not EDR-HI rats spent significantly less escape latency than NL-HI rats despite similar swimming speed between the 3 HI groups. **D**, On P84, DR-HI rats had significantly less brain volume loss than NL-HI and EDR-HI rats. Data in **B–D** are means±standard error of the mean. * $P<0.05$; ** $P<0.01$; † $P<0.001$.

during the suckling period.¹¹ Previous work has demonstrated that maternal care is adequate in the pups from large litter sizes,¹² and restricting access to breast milk in rat pups mimics dietary restriction (DR).¹³ DR increases the length of life in several species.¹³ However, it remains unclear whether being underweight via DR protects against neurovascular injury after HI in the neonatal brain. Thus, we hypothesized that underweight induced by DR protects against HI injury in rat pups by attenuating p53-mediated neuronal apoptosis, BBB damage, and microglia activation.

Materials and Methods

Animal Experiments

This study was approved by our university's Animal Care Committee. Sprague-Dawley rat pups (Charles River Laboratories, Wilmington, MA) were housed with their dams until weaning on postnatal day 21. Only male pups were used for experiments. Litters born on the same day were randomly assigned into 3 groups from postnatal day 1: normal litter (NL) size (12 pups/dam); moderate DR (18 pups/dam); and extreme DR (EDR; 24 pups/dam) groups. Pup body weight was measured daily until postnatal day 7 and weekly thereafter. Plasma glucose was determined after 1-hour fasting on postnatal day 7 using Glucose Kit Reagent (Biosystem). The interscapular subcutaneous and perirenal fat pads were dissected and weighed.¹⁴

On postnatal day 7, after being anesthetized with 2.5% halothane, the right common carotid artery was permanently ligated. The pups were returned to their dams for a 1-hour recovery and then were placed in air-tight 500-mL jars filled with humidified 8% oxygen for 2 hours. After hypoxia, the pups were returned to their dams.¹⁵ Rats with HI were defined as NL size after HI (NL-HI), moderate DR after HI (DR-HI), and EDR after HI (EDR-HI) groups.

Drug Administration

Pifithrin- α (3 mg/kg; Sigma-Aldrich) or vehicle (DMSO; Sigma-Aldrich) was administered intraperitoneally to NL or DR pups 30 minutes before carotid artery ligation, whereas nutlin-3 (100 or 250 mmol; Cayman) or vehicle (DMSO) was administered intracerebroventricularly 30 minutes before ligation. Intracerebroventricular injection in right cerebral hemisphere was performed (in relation to the bregma: 2.0 mm posterior to, 1.5 mm lateral to, and 2.0 mm beneath the skull surface) using a 30-gauge needle.¹⁵ The outcome was determined by brain volume losses on postnatal day 21.

Morris Water Maze Test

A circular water pool (160-cm diameter×50-cm height) was divided into 4 quadrants and an 8×8-cm platform was positioned 1 cm below the water surface in the center of 1 of the quadrants. On days 1 and 2, rats were subject to 4 training sessions (2 per day) to escape onto the submerged platform. The time for the rat to escape onto the platform (escape latency) was recorded by a computer program (EthoVision; Wageningen).¹⁵

Infarct Volume Evaluation

Brains embedded in paraffin blocks were sectioned coronally (10- μ m-thick). One of every 20 sections was stained with cresyl violet (Nissl stain), and a total of at least 13 sections per rat were used to measure the brain volume loss. Brain volume loss in the lesioned versus the nonlesioned hemisphere was calculated: (nonlesioned hemisphere volume–lesioned hemisphere volume)÷(nonlesioned hemisphere volume).¹⁴

TUNEL Stain

At 24 hours after HI, brain paraffin sections were prepared for TUNEL reaction (Fluorescein-FragEL kit; Oncogene).¹⁴ Digitally captured images were analyzed using Imaging Software NIS-

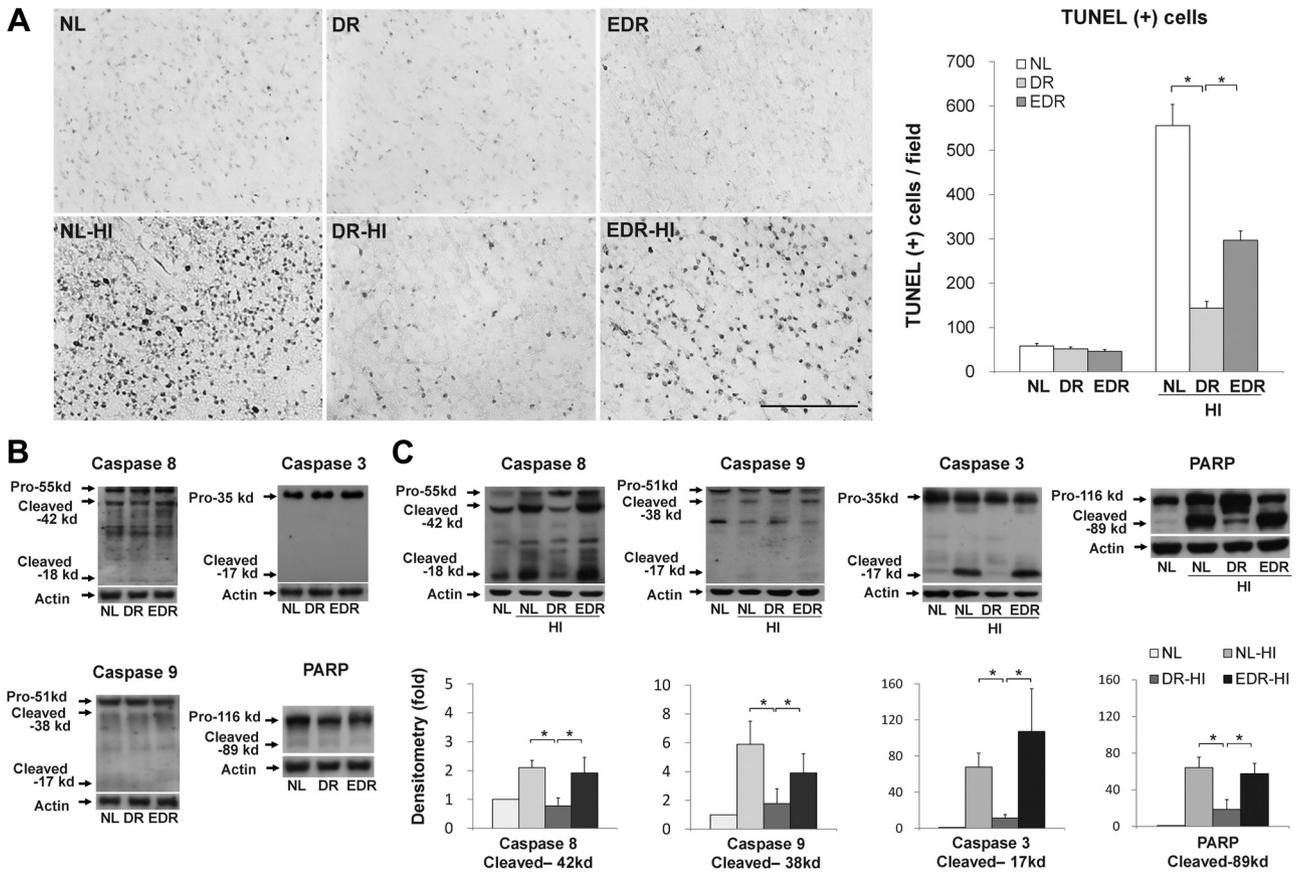


Figure 2. A, The normal litter (NL), dietary restriction (DR), and extreme DR (EDR) pups showed similar TUNEL-positive cells on postnatal day 7 before hypoxic-ischemia (HI), whereas the DR-HI pups had significantly fewer TUNEL-positive cells 24 hours after HI than NL-HI and EDR-HI pups. Scale bar=125 μ m, n=4. The NL, DR, and EDR pups showed similar cleaved caspase 8 (18 and 42 kd), caspase 9 (17 and 38 kd), caspase 3 (17 kd), and PARP (89 kd) on postnatal day 7 (B), whereas the DR-HI pups had significantly lower cleaved levels of caspases and PARP 24 hours after HI than the other 2 HI groups (C). n=6; * P <0.05. Data are means \pm standard error of the mean.

Elements (Nikon). In each brain, TUNEL-positive cells were measured at 3 200 \times magnification visual fields (0.145 mm²) in the ischemic cortex of 3 brain sections as previously described.¹⁴ The numbers of TUNEL-positive cells were expressed as the average number of TUNEL-positive cells per visual field.¹⁴

Western Blot Analysis

The cortex was homogenized, resolved using 10% SDS-PAGE, and blotted electrophoretically to polyvinylidene fluoride membranes.¹⁴ Primary antibodies (all 1:1000) included anti-p53, phospho-murine double minute-2 (pMDM2) Ser166, poly (ADP-ribose) polymerase (PARP), Bax, caspase 3, caspase 9 (Cell Signaling), and caspase 8 (Calbiochem). The horseradish peroxidase-conjugated immunoglobulin G secondary antibodies (1:5000; Jackson) were used. Immunoreactivity was visualized using enhanced chemiluminescence (Millipore). Vision-Works LS (Ultra-Violet Products) analysis software was used for densitometry.

Immunohistochemistry

Immunohistochemistry for p53, microglia activation, and BBB damage was performed 24 hours after HI. Frozen sections were probed with primary antibody: p53 (1:100; Cell signaling) or ED1 (1:100; Chemicon), followed by horseradish peroxidase-conjugated anti-IgG secondary antibodies (1:200; Chemicon). Biotin-peroxidase signals were detected and images were acquired.¹⁴ The number of ED1-positive microglia was measured as TUNEL-positive cells.

For BBB permeability measurement, frozen sections were incubated with 0.3% H₂O₂/methanol for 30 minutes, and then anti-IgG antibody (1:200; Chemicon) for 2 hours. Biotin-peroxidase signals were detected and images were acquired. Measurement of the

integrated optical density of IgG signals in the ischemic cortex was analyzed using imaging software (ImagePro Plus 6.0) at 200 \times magnification per visual field (0.145 mm²). The mean integrated optical density was measured in the same areas as TUNEL. The data for each experimental group were compared to those of the control group and expressed as relative integrated optical density ratios.¹⁴

Immunofluorescence Staining

Frozen sections were probed with primary antibody p53 (1:100; Cell Signaling), NeuN (1:200; Chemicon), RECA1 (1:100; Abcam), and ED1 (1:100; Chemicon) in phosphate-buffered saline/0.01% Triton X-100 at 4 $^{\circ}$ C overnight. The sections were then incubated with Alexa Fluor 488 goat IgG and Alexa Fluor 594 goat IgG (Invitrogen) secondary antibodies for 2 hours. Images were acquired as described previously.¹⁴

Statistical Analysis

Data were means \pm SD, unless indicated otherwise. Data were analyzed using Student *t* test. Repeated measure in a general linear model was applied to compare escape latency of the water maze test. We used Tukey method for post hoc comparisons. P <0.05 was considered to be statistically significant, and all probabilities were 2-tailed.

Results

Effects of DR on Rat Pups

The NL pups steadily increased in body weight: 7.4 \pm 0.6 g/rat on postnatal day 1 and 15.2 \pm 0.9 g/rat on postnatal day 7. Relative to the growth curve of NL pups, the DR pups

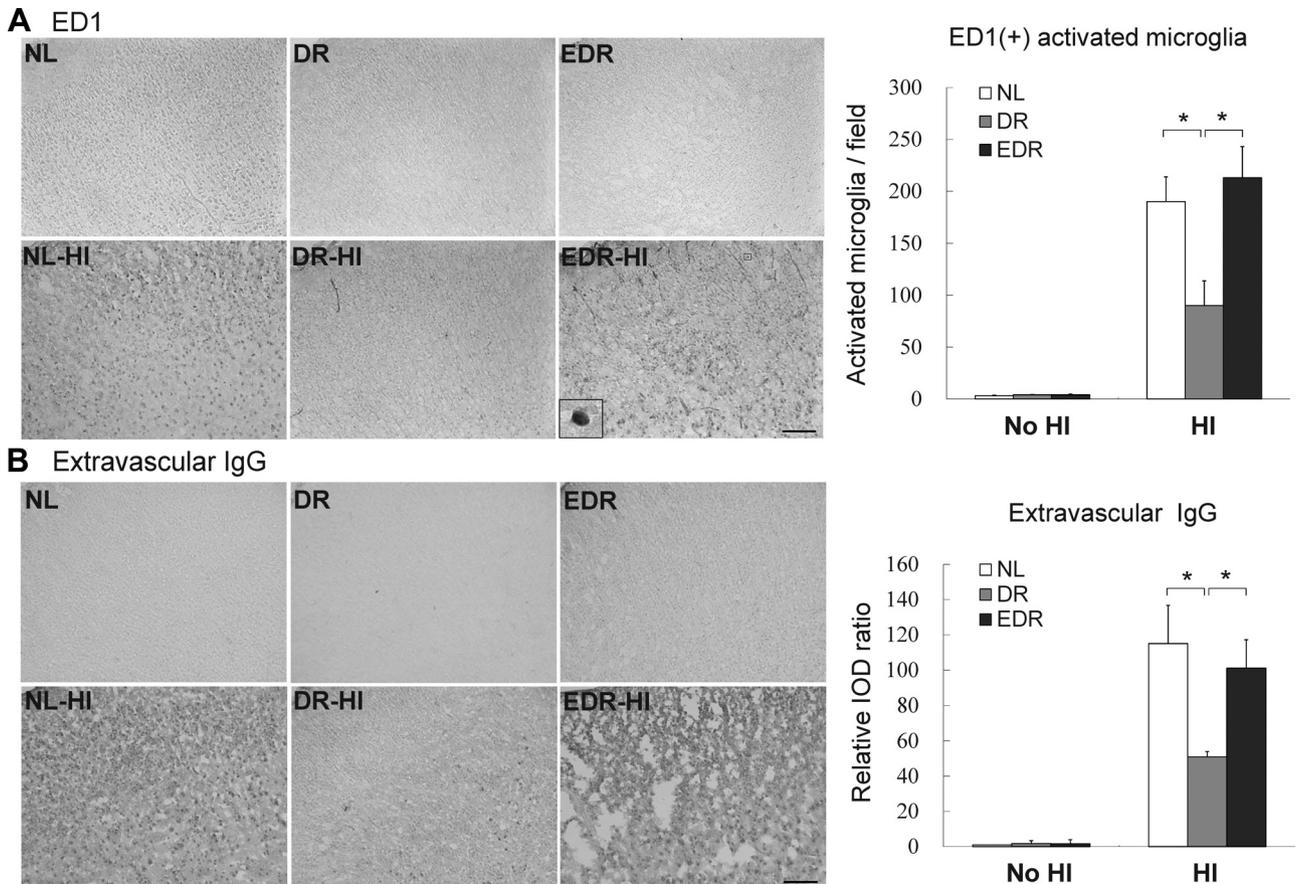


Figure 3. The representative immunohistochemistry pictures of ED1 (**A**) and extravascular IgG (**B**) before and 24 hours after HI. Dietary restriction (DR)-hypoxic-ischemia (HI) pups had significantly fewer ED1-positive activated microglia and blood-brain barrier (BBB) damage than normal litter (NL)-HI and extreme DR (EDR)-HI pups. Scale bar=100 μ m. Inset, A high-magnification picture of round ED1-positive activated microglia in EDR-HI pups. IOD, integrated optical density; n=4, * P <0.05. Data are means \pm standard error of the mean.

(12.2 \pm 1.2 g/rat) weighed between 2 and 4 SD, and the EDR pups weighed (10.0 \pm 1.1 g/rat) <4 SD on postnatal day 7 (Figure 1A). The body weights of DR and EDR pups were lower than that of NL pups (both P <0.001), and EDR pups also weighed significantly lower than DR pups (P <0.05). The total fat weights in the interscapular and perirenal area in DR (8.6 \pm 0.8 g/rat) and EDR (4.0 \pm 0.4 g/rat) pups were lower (all P <0.001) than those in NL (31.6 \pm 1.7 g/rat) pups. The EDR pups had even lower body fat weights than the DR pups (P <0.05). The plasma glucose levels were similar between NL (126.5 \pm 16 mg/dL), DR (121.2 \pm 12.9 mg/dL), and EDR (119 \pm 12.3 mg/dL) pups.

On postnatal day 42, Morris water maze test showed that the NL, DR, and EDR rats made progress and gradually reduced escape latency from session 1 to session 4 (P <0.001; F =45.00; Figure 1B). The 3 groups had similar performance in escape latencies of session 4 (P =0.715; F =0.619) and swimming speed.

Moderate DR Attenuated Neurovascular Damage After HI and Had Better Long-Term Outcome

On postnatal day 7 before HI, the NL, DR, and EDR pups showed no difference in TUNEL-positive cells (Figure 2A) and the cleaved levels of caspases and PARP (Figure 2B). During HI, 9% (3/32) of NL, 3% (1/32) of DR, and 12% (6/49) of EDR pups died. After HI, no additional NL or DR

pups died before weaning, but 18% (8/43) of EDR pups did. The body temperatures were similar between the 3 groups (NL: 33.6 \pm 0.7 $^{\circ}$ C; DR: 33.7 \pm 0.8 $^{\circ}$ C; EDR: 34.2 \pm 0.8 $^{\circ}$ C) after HI. At 24 hours after HI, DR-HI pups had significantly fewer TUNEL-positive cells (Figure 2A) and lower cleaved levels of caspase 8, caspase 9, caspase 3, and PARP than NL-HI and EDR-HI pups (Figure 2C). There were no significant differences in these proapoptosis markers between NL-HI and EDR-HI pups. In addition, immunohistochemistry showed DR-HI pups had significantly fewer ED1-positive activated microglia (Figure 3A) and less extravascular IgG (Figure 3B) than NL-HI and EDR-HI pups.

On postnatal day 42, the escape latency was different (P <0.001; F =16.198) between the 3 post-HI groups (Figure 1C). Post hoc multiple comparisons revealed that DR-HI rats had shorter escape latency than NL-HI rats, whereas EDR-HI rats had similar escape latency as NL-HI rats. Swimming speed was comparable between the 3 HI groups. Brain volume losses assessed on postnatal day 84 showed DR-HI rats had significantly less volume loss than NL-HI and EDR-HI rats (Figure 1D).

Moderate DR Attenuated p53 Upregulation in the Neurovascular Unit After HI

The postnatal day 7 NL, DR, and EDR pups had similar levels of pMDM2 and p53 (Figure 4A). At 24 hours after HI, NL-HI

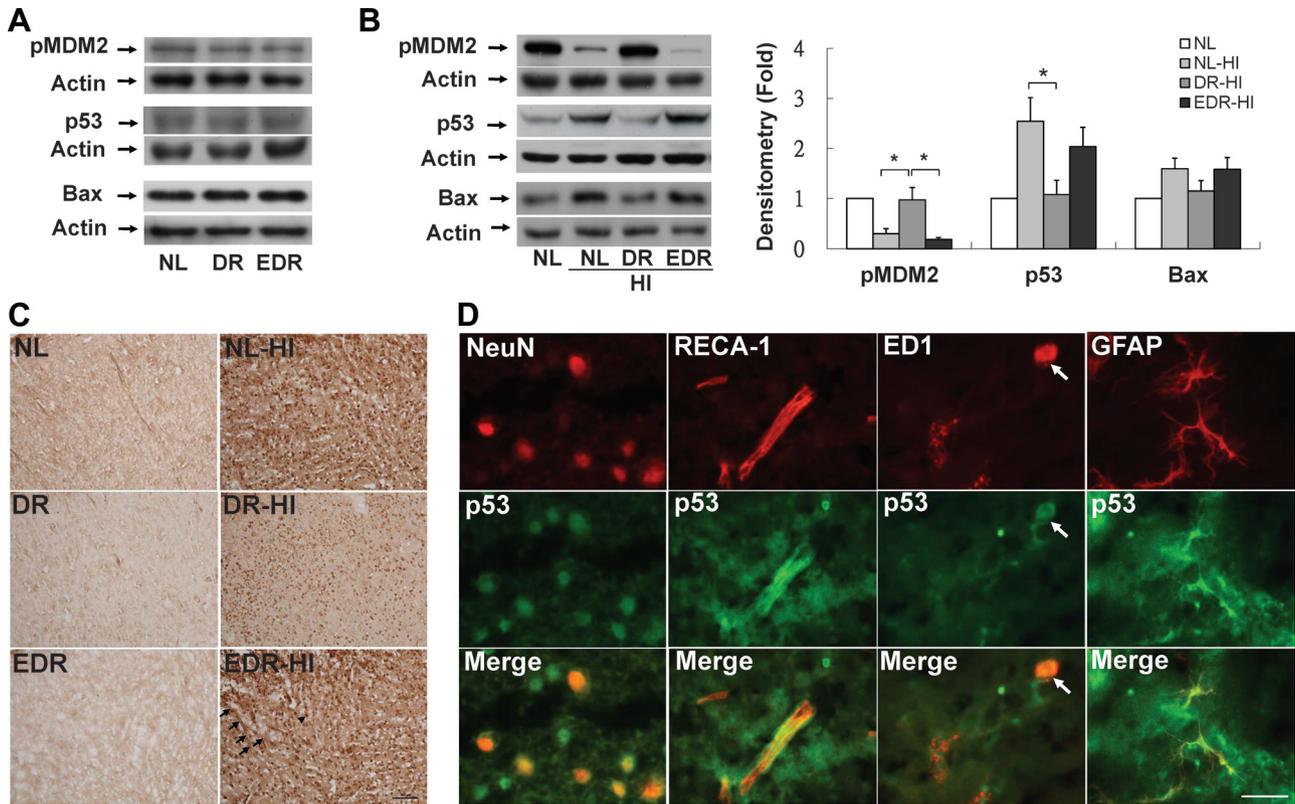


Figure 4. Immunoblotting showed the normal litter (NL), dietary restriction (DR), and extreme DR (EDR) pups had similar pMDM2 and p53 levels on postnatal day 7 (A). In contrast, the NL-hypoxic-ischemia (HI) and EDR-HI pups had significantly increased p53 and decreased pMDM2 levels than DR-HI pups did 24 hours after HI (B; n=5). Immunohistochemistry at 24 hours after HI showed increased p53 expression in vascular (arrows) and nonvascular (arrowhead) cells in NL-HI and EDR-HI pups (C; scale bar=200 μ m). D, Immunofluorescence in NL-HI pups showed HI-induced p53 upregulation in neurons (NeuN), endothelial cells (RECA-1), activated microglia (ED1; arrow), and astrocyte (GFAP). p53 is expressed mainly in the nucleus (scale bar=100 μ m).

and EDR-HI pups had significantly increased p53 levels than DR-HI pups. The pMDM2, the known regulator of p53, was decreased in NL-HI and EDR-HI pups but was preserved in DR-HI pups (Figure 4B). Immunohistochemistry at 24 hours after HI showed increased p53 expression in NL-HI and EDR-HI, but not in DR-HI pups, and p53 was upregulated in the vascular and nonvascular cells (Figure 4C). Further immunofluorescence staining in the NL-HI pups confirmed that p53 expression was located in the neurovascular unit, which included neurons (NeuN), vascular endothelial cells (RECA-1), activated microglia (ED1), and astrocytes (GFAP; Figure 4D).

Moderate DR Protected Against HI Through Reducing p53 Expression

We first examined whether p53 was involved in neurovascular damage and brain injury in NL-HI pups by using pifithrin- α , a known inhibitor of p53-dependent transactivation.⁷ Compared with DMSO, pifithrin- α did not reduce the p53 expression but significantly decreased p53 downstream protein Bax and the cleaved levels of caspase 3 and PARP in NL-HI pups 24 hours after HI (Figure 5A). Pifithrin- α treatment in NL-HI pups also significantly reduced ED1-positive activated microglia and IgG extravasation after HI (Figure 5B, C). On postnatal day 21, the pifithrin- α -treated NL-HI pups had significantly less brain volume loss than the DMSO-treated NL-HI pups (Figure 5D). In DR-HI pups,

however, the reduction of apoptotic markers, ED1-positive activated microglia, and IgG extravasation was not significant between pups treated with pifithrin- α and pups treated with DMSO (Figure 5A–C). Pifithrin- α treatment did not provide further neuroprotection in DR-HI pups (Figure 5D).

We then upregulated p53 by using a MDM2 inhibitor, nutlin-3, in DR-HI pups to examine whether moderate DR protected against HI-induced neurovascular damage and provided neuroprotection via p53 inhibition. Compared with DMSO, nutlin-3 dose-dependently decreased pMDM2 and increased p53 levels in DR-HI pups 24 hours after HI (Figure 6A). Correlatively, nutlin-3-treated DR pups had markedly increased cleaved caspase 3 and PARP levels than DMSO-treated pups (Figure 6A). The nutlin-3-treated DR pups also showed significant increases of ED1-positive activated microglia and extravasation of IgG and had more brain volume loss than the DMSO-treated DR pups (Figure 6 B–D). In contrast, in NL-HI pups, nutlin-3 treatment did not significantly reduce pMDM2 or increase the apoptotic markers ED1-positive activated microglia and IgG extravasation compared with DMSO (Figure 6 A–C). Nutlin-3 treatment did not provide further brain volume loss in NL-HI pups (Figure 6D).

Discussion

We examined the impact of underweight induced by DR on neonatal HI brain damage. Two different degrees of DR

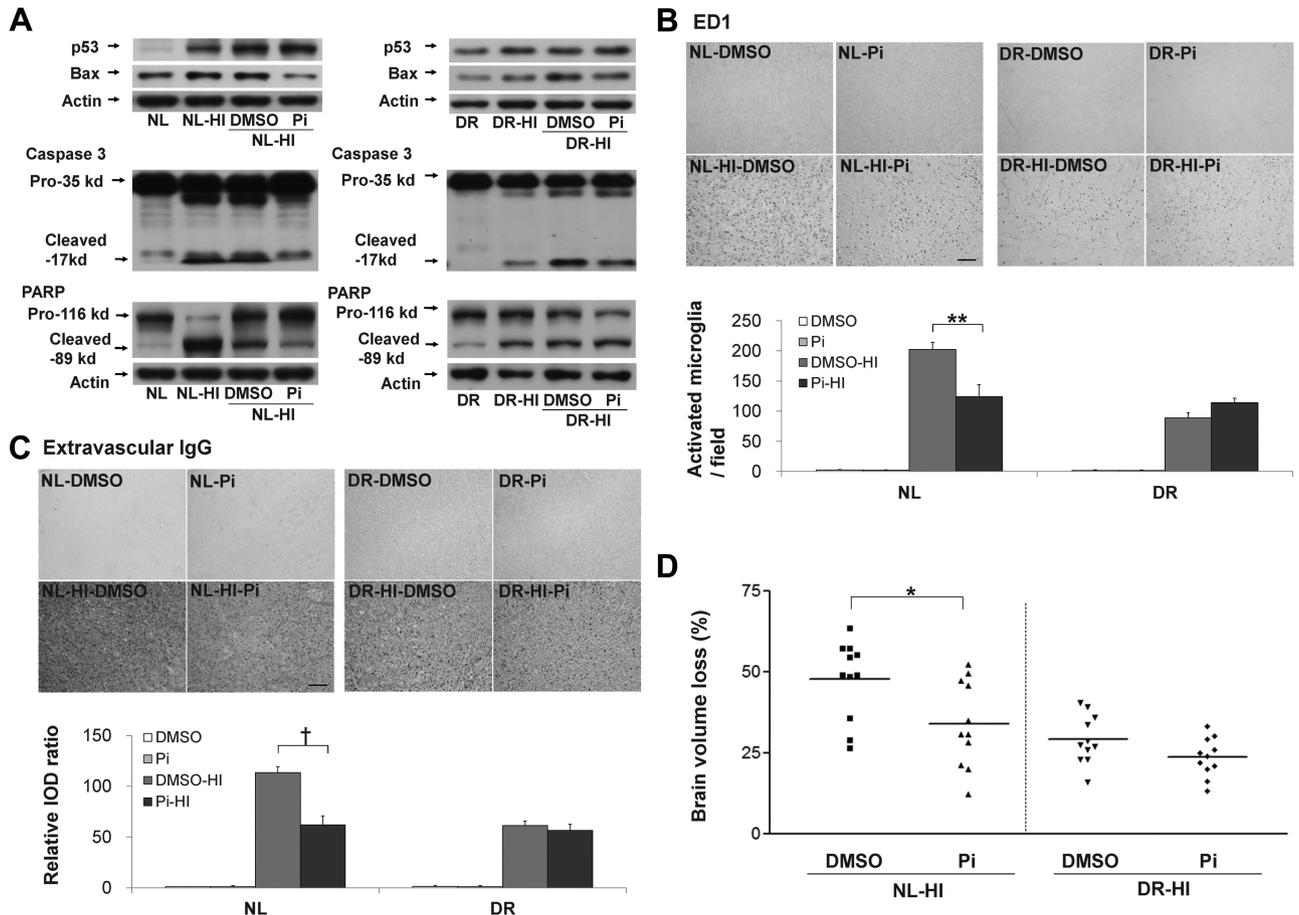


Figure 5. Effects of p53 inhibition by pifithrin- α (Pi) on normal litter (NL)-hypoxic-ischemia (HI) and dietary restriction (DR)-HI pups. **A**, Immunoblotting showed that Pi-treated NL-HI pups had decreases of Bax and cleaved caspase 3 and PARP levels 24 hours after HI than DMSO-treated NL-HI pups ($n=3$). In contrast, the effects of Pi on Bax and cleaved caspase 3 and PARP levels in DR-HI pups were not significant ($n=2$). The Pi-treated NL-HI pups (NL-HI-Pi) showed decreases of ED1-positive activated microglia (**B**) and blood-brain barrier (BBB) damage (extravascular IgG; **C**) 24 hours after HI than DMSO-treated pups (NL-HI-DMSO). In contrast, Pi-treated DR-HI pups had similar ED1-positive activated microglia and BBB damage as DMSO-treated DR-HI pups (**B** and **C**, Scale bar = 100 μm , $n=4$). **D**, Brain volume loss measurement on postnatal day 21 revealed NL-HI-Pi rats had significantly less brain damage than NL-HI-DMSO pups. In DR-HI rats, the brain volume loss was not significantly different between pups treated with Pi and those with DMSO. * $P<0.05$, ** $P<0.01$, † $P<0.001$. Data are means \pm standard error of the mean.

(moderate DR and EDR) induced by increasing litter sizes during the suckling period caused significant reduction of body weight without compromising blood glucose levels. DR or EDR per se caused no changes in neuropathology or proapoptosis markers in the cortex before HI on postnatal day 7, and also did not impair learning performance at adulthood. After HI on postnatal day 7, the DR-HI but not the EDR-HI pups had significantly less neurovascular injury, which included attenuation of neuronal apoptosis and BBB damage, and microglial activation, and had better long-term neurobehavioral and pathological outcome than the NL-HI pups. Moreover, HI upregulated p53 expression in the neurovascular unit (neurons, endothelial cells, and microglia) in NL-HI and EDR-HI pups but not in DR-HI pups. Blockage of p53 in NL-HI pups significantly reduced neurovascular damage and microglia activation and provided neuroprotection. In contrast, upregulation of p53 expression in DR-HI pups by inhibiting pMDM2 increased neurovascular damage and microglia activation and decreased the neuroprotective effect of moderate DR. Taken together, our findings suggest that

moderate DR, but not EDR, provides neuroprotection through downregulating HI-induced p53 expression in the neurovascular unit.

Brain dysfunction may arise from the complex interactions of multiple cell types within the neurovascular unit.² HI not only causes neuronal and endothelial cell damages but also activates microglia, resulting in disruption of the neurovascular unit.^{2,14} Disruption of neurovascular unit may lead to more BBB perturbation and microglia activation that further exacerbates neuronal damage.^{2,4} Activation of microglia exacerbates neuronal damage, and inhibiting microglial activation reduces HI injury.^{4,5} The vulnerability of endothelial cells and BBB may be related to microglia activation, which contributes to BBB disruption through matrix protease generation.² Treatment designed to target on protective pathways that simultaneously affect neurons, vascular endothelial cells, and microglia may provide powerful therapeutic strategies against HI. DR to a level of $\approx 70\%$ of what animals would eat ad libitum increases the length of life span in several species.¹³ Evidence has shown that DR stimulates neurogen-

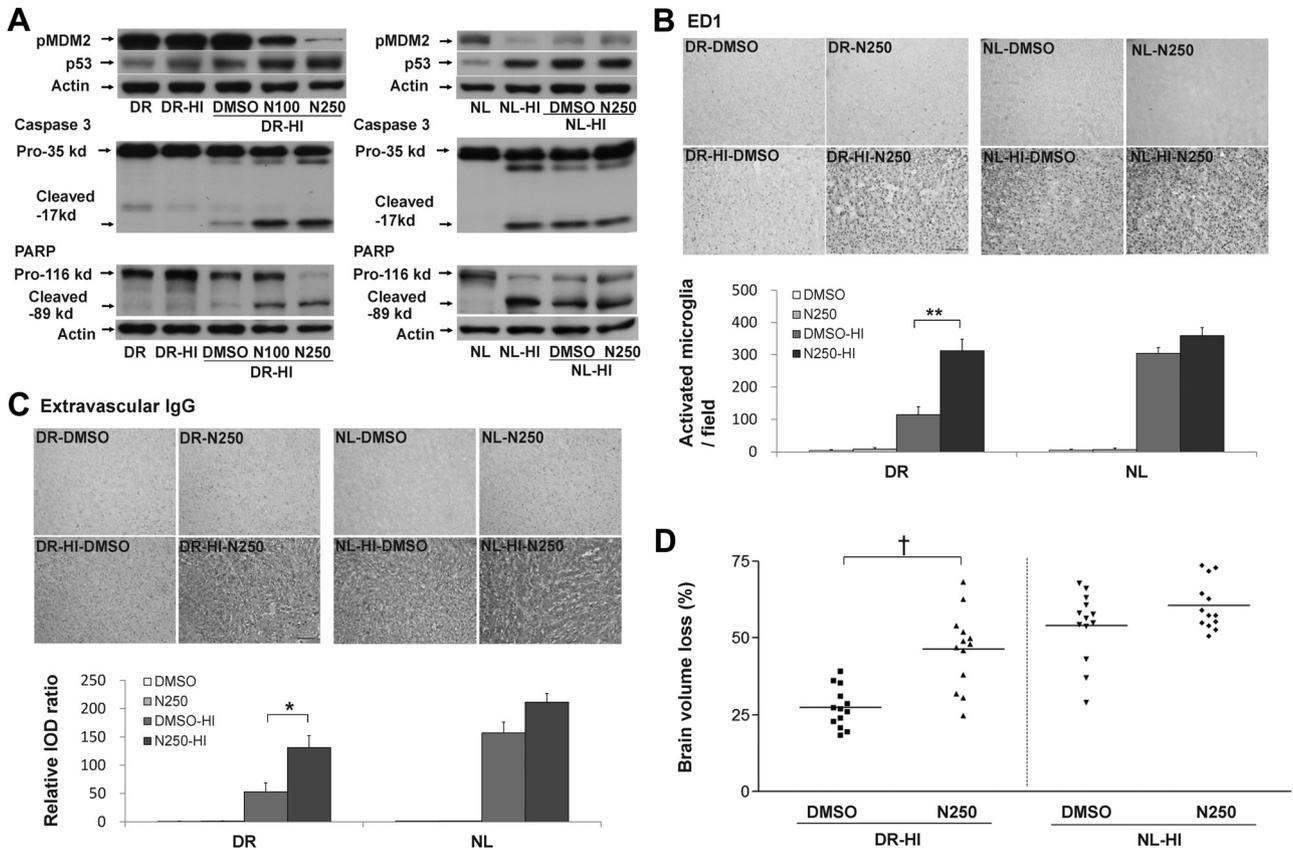


Figure 6. Effects of p53 upregulation by nutlin-3, a MDM2 inhibitor, on dietary restriction (DR)-hypoxic-ischemia (HI) and normal litter (NL)-HI pups. **A**, Immunoblotting showed nutlin-3 (N100 and N250 mmol) dose-dependently decreased pMDM2 and increased p53 levels 24 hours after HI compared with DMSO in DR-HI rats. The nutlin-3-treated DR-HI pups also had increased cleaved caspase 3 and PARP levels than the DMSO-treated DR-HI pups (n=3). In contrast, N250 was unable to further reduce pMDM2 or to increase p53, cleaved caspase 3, and PARP compared with DMSO in NL-HI rats (n=2). In the DR-HI group, N250 mmol-treated pups (DR-HI-N250) elicited significantly more ED1-positive activated microglia (**B**) and blood-brain barrier damage (**C**) 24 hours after HI than DMSO-treated pups (DR-HI-DMSO). In NL-HI rats, the effects of N250 on microglia and BBB damage were not significant. Scale bar=100 μm, n=4. **D**, Brain volume loss measurement demonstrated more brain damage in DR-HI-N250 pups than in DR-HI-DMSO pups. It was not significantly different between NL-HI-N250 pups and NL-HI-DMSO pups. *P<0.05, **P<0.01, †P<0.001. Data are means±standard error of the mean.

esis, enhances synaptic plasticity, resists aging-related cognitive decline, and protects against ischemic stroke.^{13,16} In addition to affecting neurons, DR also decreased ED1-positive microglia infiltration in the brain of the stroke-prone spontaneously hypertensive rat and reversed obesity-induced endothelial dysfunction through suppressing oxidative stress.^{17,18} We showed that moderate DR, but not EDR, protected against HI injury in neonatal brain by attenuating neurovascular injury and microglia activation in the neurovascular unit.

Apoptotic machinery is an important mechanism of cell death after HI in the developing brain.¹ Compared with NL-HI pups, DR-HI but not EDR-HI pups had decreased TUNEL-positive cells and reduced cleaved caspase levels. This finding suggests that moderate DR, but not EDR, protects against HI in rat pups by raising the antiapoptotic brake that inhibits apoptotic machinery to promote neuronal survival. Numerous studies have shown the importance of p53 in both intrinsic and extrinsic apoptotic cascade.^{6,7} MDM2 is a key regulator of p53 stability. The phosphorylation of MDM2 at Ser 166 increases its interaction with p300, allowing MDM2-mediated ubiquitination and degradation of

p53.¹⁹ Our study showed that p53 was upregulated, whereas pMDM2 was downregulated, in NL-HI and EDR-HI pups. In contrast, p53 and pMDM2 levels were not altered in DR-HI pups. These findings suggest that moderate DR downregulates p53 after HI via maintaining the pMDM2 level, which leads to mitigation of both intrinsic and extrinsic apoptosis machinery in the neonatal brain.

When p53 was increased by protease inhibition or adenovirus transduction, the apoptotic death of human umbilical vein endothelial cells were exacerbated after hypoxia.⁸ Blockage of p53 by activated protein C prevented apoptosis in hypoxic human brain endothelial cells.²⁰ Moreover, studies has shown that p53 expression increased after microglia activation, and blockage of p53 significantly prevented microglia-mediated neurotoxicity.⁹ Upregulation of proinflammatory genes induced by interferon gamma was suppressed in p53^{-/-} microglia.²¹ These findings indicated that p53 is also involved in endothelial cell injury and microglia activation after brain damage. We found that HI-induced upregulation of p53, BBB damage and microglia activation were attenuated by moderate DR. Inhibiting p53 decreased BBB damage and microglia activation in NL-HI pups,

whereas upregulating p53 increased BBB damage and microglia activation in DR-HI pups. Moreover, it did not significantly further increase BBB damage and microglia activation when p53 was upregulated in NL-HI pups or decrease BBB damage and microglia activation when p53 was inhibited in DR-HI pups. These suggested the critical role of p53 in moderate DR on HI-induced BBB damage and microglia activation in neonatal brain.

There were differences in the mortality during and after HI among the NL, DR, and EDR pups. The DR pups had the lowest mortality, whereas the EDR group showed the highest mortality during HI, and only the EDR pups showed further mortality after HI. These findings might not only underestimate the brain damage outcome in the EDR group but also support our conclusion that the EDR rats did not have the neuroprotective benefits of DR rats. There may be a concern regarding whether early-life DR might have long-term adverse effect on development. The findings that similar neuronal apoptosis and water maze performance between the NL, DR, and EDR rats without HI suggest that DR during lactation may not have long-term adverse impacts on neurodevelopment. The mechanism underlying why moderate but not extreme DR is neuroprotective remains unknown. The interaction of p53 with MDM2 is a key step that regulates p53 degradation. Another well-known key molecule associated with DR in mammals is sirtuin type 1 protein.²² Sirtuin type 1 also regulates the activities of p53.²² Therefore, further studies are needed to examine the upstream pathways that differentially regulate MDM2-p53 signaling and the role of sirtuin type 1 and p53 after HI in DR and EDR pups.

A diagram (Supplemental Figure; <http://stroke.ahajournals.org>) is proposed to show that p53 downregulation in the neurovascular unit (neurons, endothelial cells, and microglia) after HI may be the potential link between being underweight from a larger litter size and neuroprotection in the neonatal brain. Elucidating the pathway leading to p53 downregulation after HI in rat pups with moderate DR may yield neuroprotective drugs that mimic the beneficial effects of DR for treating HI brain injury in newborns at high risk.

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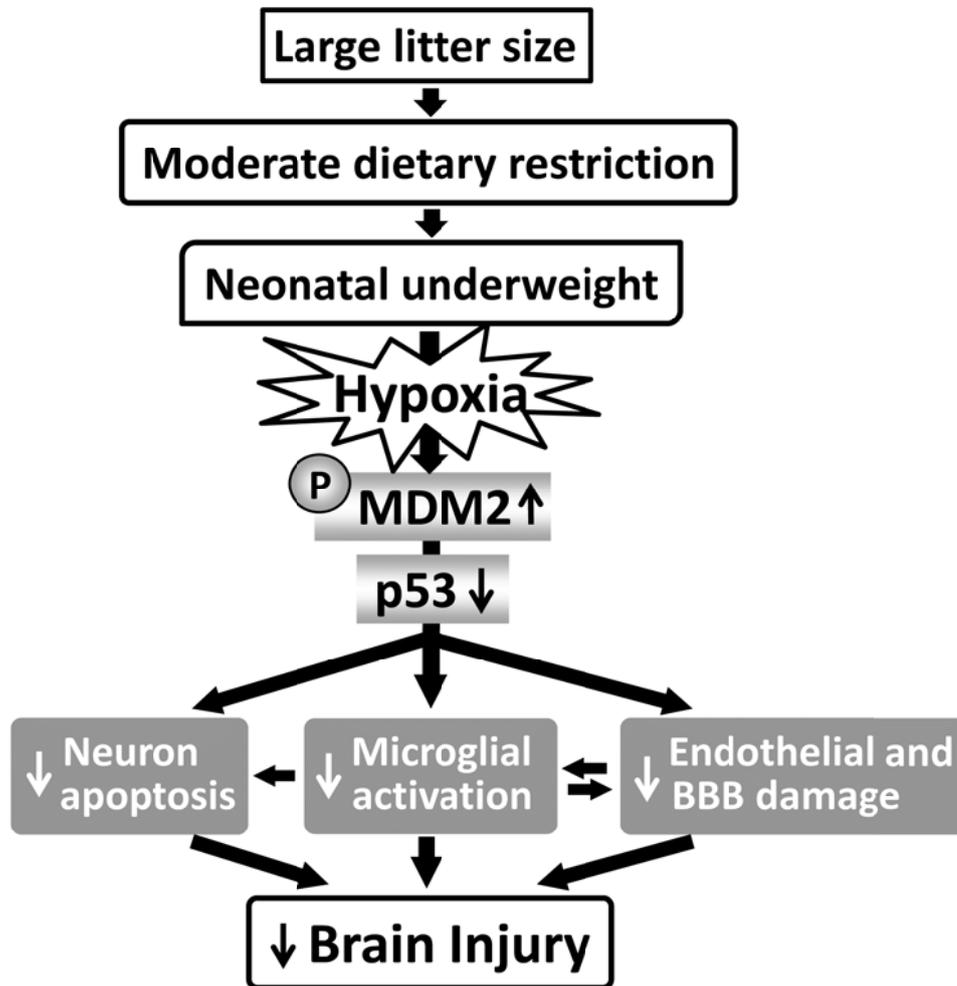
Disclosures

None.

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SUPPLEMENTAL MATERIAL



Supplement figure.

A proposed diagram showing that the decreased of p53 on the neurovascular unit (neurons, endothelial cells and microglia) after HI may be the potential link between dietary restriction from a large litter size and reduced HI injuries in the neonatal brain.