anomalies ($n = 11$), and Apgar scores of 0 at 1 min, thus requiring epinephrine administration and/or chest compression ($n = 4$). Parents of three infants did not consent to participation in this study. At 6 h after birth, infants with IVH detected by head ultrasonography were excluded ($n = 3$) because early onset IVH is more likely to develop because of perinatal factors rather than postnatal factors (29). Eighty-three infants were consequently included in the study.

All infants with hypotension received cardiac support. In the NICU, hypotension was defined as MAP below the lower 95% confidence intervals found in a population study (30) or below the numerical number of the given patient's gestational age, which were almost equal. For infants with hypotension, dopamine or a single-volume expander (10 ml·kg$^{-1}$) was initially administered at the attending physician's discretion according to the infants' circulatory data such as heart rate, urine output, blood lactate level, and echocardiographic parameters. Dopamine was continuously infused via a peripherally inserted central catheter or umbilical venous line and usually initiated at a low to moderate dose (3–10 μg·kg$^{-1}$·min$^{-1}$). If blood pressure was not sufficiently restored, the dose of dopamine was incrementally increased by 2–5 μg·kg$^{-1}$·min$^{-1}$ up to 20 μg·kg$^{-1}$·min$^{-1}$. In some cases, an equal dose of dobutamine was alternatively added or an additional single-volume expander was administered. When all the above treatments were ineffective, epinephrine and/or corticosteroids were administered. All neonates were placed in an incubator during the study period. Skin temperature was maintained at 37.0 ± 0.5 °C.

### Blood Flow Measurement

A noninvasive continuous monitoring technique was used for measuring skin and subcutaneous blood flow, as described in our previous studies (20,31). In brief, the system comprised a laser Doppler flowmeter (CDF-2000; Nexis, Fukuoka, Japan), a probe, and a computer. The probe was easily attached on the patients' skin using double-sided adhesive tape. The laser beams penetrated 3–4 mm into the skin, and the system measured the blood flow in the capillary vessels, venules, and arterioles within a specified hemispherical region. Probes were attached to the dorsum of the right or left foot, avoiding visible vessels. LBF and FBF were measured, and the data were stored in a computer for a 48-h period. Once the data were collected, blood flow values were extracted at 10-s intervals 30 min before and after each data point between 6 and 48 h at 6-h intervals and averaged using 360 measurements for each.

### Cardiovascular Parameters

Blood pressure was measured in all neonates. Systolic and diastolic pressure and MAP were recorded by nursing staff every hour for continuous blood pressure measurement ($n = 50$) and every 3 h using oscillometric methods ($n = 33$), confirming that the infants were at rest and free of any difficulties associated with the catheter line. Following this, data for every 6 h (one data point for each measurement) up to 48 h were collected for analysis.

### Head Ultrasonography

Head ultrasonography was performed at 1–3, 6, 24, and 48 h after birth and on postnatal days 4 and 7 using SONOS 5500 (Philips Medical Systems, Andover, MA) to confirm the presence and severity of IVH. In addition to these examinations, ultrasonography was also performed when attending physicians considered it necessary for follow-up of IVH or due to sudden changes in cardiovascular or respiratory conditions. Only IVH of grade 2 or more according to the Papile classification was defined as IVH in this study. Grade 3 or 4 IVH was defined as severe IVH.

### Data Analysis and Statistics

Infants were divided into the following two groups: those with IVH and those without IVH. Background characteristics were compared between the two groups by the Mann–Whitney *U*-test or the $\chi^2$ test. A *t*-test with the Bonferroni correction was applied for multiple comparisons between groups. Comparisons of temporal changes in LBF, FBF, and MAP in each group were performed using one-way repeated ANOVA, and if the results were significant, the *post hoc* Tukey test was used. Univariate and multivariate regression analyses were performed to identify risk factors for IVH. For multivariate regression analysis, MAP, FBF, and LBF at each time point and demographic factors with *P* values < 0.15 in univariate regression analysis were selected. In addition, receiver operator characteristic analysis was used to calculate the area under the curve, sensitivity, specificity, and positive and negative predictive values of LBF, FBF, and MAP at 18 h for the infants without IVH at each time point. Optimal cutoff values were determined by correspondence to a point on the receiver operator curve nearest to the upper left corner. All statistical values are shown as mean (SD) unless otherwise indicated, and a two-tailed *P* value of <0.05 was considered statistically significant. Stat Flex (StatFlex; Artec, Osaka, Japan) was used for all statistical analyses.

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Original article

# Intraperitoneal and intravenous deliveries are not comparable in terms of drug efficacy and cell distribution in neonatal mice with hypoxia–ischemia

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**Abstract**

*Background and purpose:* Most therapeutic agents are administered intravenously (IV) in clinical settings and intraperitoneally (IP) in preclinical studies with neonatal rodents; however, it remains unclear whether intraperitoneal (IP) injection is truly an acceptable alternative for intravenous (IV) injection in preclinical studies. The objective of our study is to clarify the differences in the therapeutic effects of drugs and in the distribution of infused cells after an IP or IV injection in animals with brain injury.

*Methods:* Dexamethasone or MK-801, an N-methyl-D-aspartate receptor antagonist was administered either IP or IV in a mouse model of neonatal hypoxic–ischemic encephalopathy. Green fluorescent protein-expressing mesenchymal stem cells (MSCs) or mononuclear cells (MNCs) were injected IP or IV in the mouse model. Two hours and 24 h after the administration of the cells, we investigated the cell distributions by immunohistochemical staining. We also investigated distribution of IV administered MNCs labeled with 2-[$^{18}$F]fluoro-2-deoxy-D-glucose in a juvenile primate, a macaque with stroke 1 h after the administration.

*Results:* IP and IV administration of dexamethasone attenuated the brain injury to a similar degree. IP administration of MK-801 attenuated brain injury, whereas IV administration of MK-801 did not. The IV group showed a significantly greater number of infused cells in the lungs and brains in the MSC cohort and in the spleen, liver, and lung in the MNC cohort compared to the IP group. In the macaque, MNCs were detected in the spleen and liver in large amounts, but not in the brain and lungs.

*Conclusions:* This study demonstrated that the administration route influences the effects of drugs and cell distribution. Therefore, a preclinical study may need to be performed using the optimal administration route used in a clinical setting.


*Keywords:* Cell transfusion; Dexamethasone; MK-801; Mesenchymal stem cell; Mononuclear cell; Intraperitoneal injection; Intravenous injection; Neonatal hypoxic–ischemic encephalopathy; Primate

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## 1. Introduction

Children with severe neonatal hypoxic–ischemic encephalopathy (HIE) typically die or develop lifelong neurological impairments [1]. No therapeutic method – except for hypothermia – is available to treat neonatal HIE [2,3]. When treating newborns with HIE in a clinical setting, the administration route for a therapeutic medication is generally intravenous (IV). Postnatal day 7–12 (P7–12) mouse or rat pups are widely used for animal models of neonatal HIE [4]. Although there are several reports on the techniques of IV injection in neonatal rodents [5–7], such studies are difficult to perform accurately because of the small size of the rodent pups. Therefore, most researchers using neonatal rodents choose the intraperitoneal (IP) route as an alternative to the IV route to administer an agent [8–14]. Examining the therapeutic effect of a drug using a administration route other than the expected in clinical settings raises the question whether preclinical evaluations of the agent in neonatal rodents accurately simulate the clinical use of the agent; therefore, investigating whether and how different delivery routes influence the therapeutic effects of agents for neonatal brain injury is very important.

Cell therapies have recently attracted much attention as a novel therapeutic strategy for treating neonatal HIE [15]. Among the several administration methods for cell transfusion, IV administration appears to have the lowest risk for clinical use in HIE. Most studies on cell therapies use IP injection rather than IV injection in neonatal rodents for technical reasons; however, fewer transplanted cells may be distributed in the brain when using the IP route compared to the IV route. When translating neonatal rodent data into clinical trials, the difference between the administration routes is more of a crucial issue for cell therapies than for small chemical compounds.

In this report, we introduce a precise and simple technique of IV injection via the femoral vein in P7–8 mice. The objectives of the study are to clarify whether the IP route is an appropriate administration route for agents in comparison to the IV route. We examined the influence of administration route by injecting the following substances of different sizes: dexamethasone and MK-801 (i.e., small chemical compounds) and mesenchymal stem cells (MSCs) and mononuclear cells (MNCs) (i.e., large substances) in a mouse model of neonatal HIE. Dexamethasone is a steroid hormone, has anti-inflammatory effects, and exerts neuroprotective effects against hypoxic–ischemic (HI) brain damage [16–18]. MK-801 is an N-methyl-D-aspartate (NMDA) receptor antagonist and exerts neuroprotective effects by blocking NMDA type glutamate receptors expressed in neurons [19,20]. MSCs are adhesive cells derived from culturing mesenchymal tissue such as bone marrow and adipose tissue and have the potential to differentiate into several cell types such as muscle and bone [21–24]. The MNC fraction of bone marrow contains a variety of blood cells including hematopoietic stem cells [25]. Furthermore, to clarify whether different recipient animals show different distributions of infused cells, we examined the systemic distribution of intravenously (IV) transfused cells in a non-human primate, a macaque with ischemic brain injury.

## 2. Materials and methods

All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center.

### 2.1. Hypoxia–ischemia procedure

HI was induced in eight-days-old (i.e., P8) CB17 mouse pups (CLEA Japan Inc., Tokyo, Japan) as previously described [26]. In brief, P8 CB17 mouse pups (with a body weight of 4.5 ± 0.1 g) were anesthetized with isoflurane. The left carotid artery was permanently occluded, and after a one-hour recovery period, the pups were subjected to hypoxia (8% oxygen) for 30 min.

### 2.2. IV injection via the femoral vein

The materials included a 35 G needle (ReactSystem, Osaka, Japan), which has an outer diameter of 0.15 mm and an inner diameter of 0.1 mm, a 100-μl Hamilton syringe, scissors, and forceps. Each mouse pup was anesthetized and was laid on its back. The limbs were immobilized with tape pasted to an operating board. The skin over the left femoral vein was incised from the inguinal region to 5 mm distal from the incision. The adipose tissue over the vessel was removed, and the femoral vein was exposed. Using the 35 G needle, we manually injected solutions under a stereoscopic microscope (Fig. 1). Intravascular administration was easily confirmed by observing the infused solution, which was transparent fluid in the red blood, flowing from the tip of the needle into the bloodstream. Extremely slow withdrawal of the needle caused no bleeding in approximately 30% of the pups. To stop the bleeding after needle withdrawal, a cotton swab was pressed onto the injection site immediately after pulling the needle from the vein, and ligating the vessel was unnecessary.

### 2.3. Drug administration

We used dexamethasone and MK-801, which are neuroprotectants [19,20,27,28]. The mouse pups were randomly assigned to one of three groups in each drug cohort. *The dexamethasone cohort*: according to reports showing its neuroprotective effects [16,26], dexamethasone

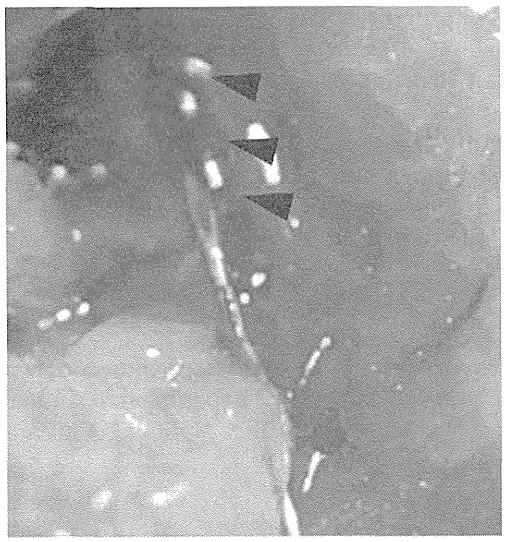

Fig. 1. Intravenous injection via the femoral vein. The skin of the inguinal region is incised, which reveals the left femoral vein. A 35 G needle is inserted into the femoral vein (arrowheads).

(0.5 mg/kg) (Banyu Co., Inc., Tokyo, Japan) in 40 μL of phosphate-buffered saline (PBS) was injected IV 24 h before the HI insult in the first group (i.e., Dex-IV group; $n = 9$) and injected intraperitoneally (IP) in the second group (i.e., Dex-IP group; $n = 8$). *MK-801 cohort*: according to reports showing its neuroprotective effects [28], MK-801 (0.5 mg/kg) (Sigma–Aldrich, St Louis, MO, USA) in 40 μL of PBS was injected IV immediately after the HI insult for the first group (i.e., MK-801-IV group; $n = 9$) and injected IP for the second group (i.e., MK-801-IP group; $n = 9$). The vehicle (PBS) was injected IV (i.e., PBS-IV group) 24 h before the HI insult for the control group ($n = 8$) in the dexamethasone cohort and at 0 h after the HI insult for the control group ($n = 12$) in the MK-801 cohort.

### 2.4. Evaluations of drug efficacy

In the dexamethasone and MK-801 cohorts, animals were deeply anesthetized, perfused intracardially, and fixed 7 days after the HI insult. In this model, 7 days post-insult is the most common time-point for the evaluation of brain injuries, because the majority of the injury-associated processes have been completed [29,30]. The brain of each animal was removed and coronally sectioned into 1-mm slices. The hemispheric brain volume of each pup was estimated by summing the hemispheric area of the brain slices and multiplying the sum by the section interval thickness as previously described [26,31]. Neuropathological injury was evaluated using hematoxylin–eosin-stained sections from four brain regions: the cortex, striatum, hippocampus, and thalamus. We used a previously established system to evaluate neuropathological injury [32]. Neuropathological injury in the cerebral cortex was scored on a scale ranging from 0 to 4 points (0 = no injury; 4 = extensive confluent infarction), whereas neuropathological injury in the hippocampus, striatum, and thalamus was scored on a scale ranging from 0 to 6 points. The total injury score is the sum of the ratings from all four brain regions (ranging from 0 to 22 points). The investigators evaluating the injuries were blinded as to the experimental group. Immunohistochemical analyses were performed to evaluate neuronal survival or apoptotic cell death using paraffin-embedded brain slices cut into 5-μm sections. Surviving neurons and apoptotic cells were stained with anti-mouse antibodies to NeuN (1:500, Millipore, Bedford, MA, USA) and to cleaved caspase-3 (1:800, Cell Signaling Technology, Inc., Beverly, MA, USA), respectively. After treatment with secondary antibodies, the cells were visualized with 0.5% diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) and counterstained with hematoxylin. The total NeuN-positive area was measured automatically using image processing software (WinROOF, Mitani Co. Ltd., Tokyo, Japan), and the number of cleaved caspase-3 positive cells was manually counted in three slices per animal. The average density of NeuN positive cells, and of cleaved caspase-3 positive cells, was calculated for the whole hemisphere.

### 2.5. Administration of green fluorescent protein-expressing MSCs and MNCs

Bone marrow cells were isolated by flushing out the femoral and tibial cavities of green fluorescent protein (GFP)-expressing Lewis rats (Institute of Laboratory Animals, Kyoto University, Kyoto, Japan) with PBS. *MSC cohort*: the MSCs were prepared as previously described [33]. In brief, bone marrow cells were plated onto 10-cm dishes in a complete culture medium: α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Five days after plating, the nonadherent cells were removed, and the adherent cells (i.e., MSCs) were further propagated for 4–5 passages. *MNC cohort*: the MNCs were isolated by density gradient centrifugation using Ficoll (GE Healthcare UK Ltd., Amersham Place, England) at 400*g* for 30 min in accordance with the manufacturer's protocol as previously described [34].

Forty-eight hours after the HI insult, we injected GFP-expressing MSCs or MNCs ($1 \times 10^5$ cells/pup) into the neonatal HIE mice IV (i.e., the IV group) or IP (i.e., the IP group).

### 2.6. Evaluation of the infused cells

Two or 24 h after the administration of the cells, the pups were euthanized. Their brain, lungs, liver, and

spleen were removed and fixed with 4% paraformaldehyde. The organs were embedded in paraffin blocks and were cut into 2-μm sections. The GFP-positive cells in the organ sections were stained with rabbit anti-GFP polyclonal antibody (1:100, Invitrogen) and anti-rabbit Envision+ system-HRP labeled polymer (Dako Cytomation, Glostrup, Denmark). The cells were visualized with 0.5% diaminobenzidine (Wako Pure Chemical Industries).

### 2.7. Administration and evaluation of the infused cells in a non-human primate

A 6-years-old male *Macaca fascicularis* (macaque) (body weight, 5 kg) (Keari Co., Ltd. Osaka, Japan) was deeply anesthetized, and the left femoral artery was isolated. Three autothrombins were plugged in the M1 region of the left MCA via a 1.2 French microcatheter. The left MCA was chosen for consistency with the laterality in the mouse HI model. Three days after the occlusion, the macaque was scanned via MRI and an infarct in the left hemisphere was confirmed (Fig. 6A). Seven days after occlusion, bone marrow derived-MNCs were isolated from the bilateral iliac bone of the macaque and were incubated with 100 MBq 2-[$^{18}$F] fluoro-2-deoxy-D-glucose ($^{18}$F-FDG) (specific activity, 10 MBq/mL) for 30 min at 37 °C under gentle rolling in serum-free PBS (pH 7.2) and 10 U/mL heparin. To remove excess unbound $^{18}$F-FDG, the cells were subjected to a three-step centrifugation and washing process in PBS (7*g*, 27*g*, and 60*g*; each for 120 s). Radioactivity in the supernatant and in the cell pellet was measured with a dose calibrator [35], and $5 \times 10^6$ cells radio-labeled with $^{18}$F-FDG at 4.5 MBq were injected via the femoral vein of the macaque. Sixty minutes after the cell injection, the radioactivity of the whole body of the macaque was visualized using a three-dimensional positron emission tomography (PET) imaging system (ECAT HR scanner; Siemens-CTI, Knoxville, TN, USA).

### 2.8. Statistics

All data were expressed as the mean ± the standard error of the mean (SEM). As for the hemispheric volume, comparisons between two parameters were analyzed by Student's *t*-test, and comparisons between three or more groups were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey's test. As for the neuropathological scores and cell distributions, the comparisons between the two parameters were analyzed by the Mann–Whitney *U* test, and the comparisons between three or more groups were analyzed by the Kraskal–Wallis test, followed by the Steel–Dwass test. A *p*-value less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. The IV injection via the femoral vein

The success rate of this IV injection procedure was greater than 90% across different operators (data not shown).

### 3.2. The neuroprotective effects of dexamethasone treatment

Both Dex-IP and Dex-IV significantly ameliorated hemispheric volume loss as compared to PBS-IV (Dex-IP, 3.5 ± 0.9% and Dex-IV, 7.7 ± 1.7% vs. PBS-IV, 16.9 ± 3.2%) (Fig. 2A). Injury to the cortex and hippocampus was also significantly ameliorated by either Dex-IP or Dex-IV, leading to a reduced total injury score compared to PBS-IV (Fig. 2B). Dex-IP or Dex IV also reduced damage to the striatum, although the injury score decrease was only significant for Dex-IP compared to PBS-IV. Despite differences when compared to PBS-IV, there was no significant difference between the injury scores of Dex-IP compared to Dex-IV (Fig. 2B). Neither the ipsilateral/contralateral ratio of the NeuN positive area nor the ratio of cleaved caspase-3 positive cells showed a difference between Dex-IP and Dex-IV (NeuN, Dex-IP, 0.9 ± 0.1 and Dex-IV, 0.8 ± 0.1; cleaved caspase-3, Dex-IP, 1.4 ± 0.2 and Dex-IV, 1.9 ± 1.0, $n = 4$–5) (Fig. 2C and D). Collectively, these results reveal that Dex-IP and Dex-IV treatments result in nearly equal neuroprotective effects.

### 3.3. The neuroprotective effects of MK-801 treatment

Cerebral hemispheric volume loss was significantly ameliorated by MK-801-IP but not by MK-801-IV compared to PBS-IV (MK-801-IP, 10.2 ± 2.9% and MK-801-IV, 28.6 ± 7.6% vs. PBS-IV, 32.8 ± 5.5%) (Fig. 3A). Similarly, neuropathological injury scores for the striatum were significantly reduced by MK-801-IP but not MK-801-IV compared to PBS-IV. Neither MK-801-IP nor MK-801-IV produced statistically significant neuroprotection in the other regions of the brain or in the total score (Fig. 3B). The ipsilateral/contralateral ratio of the NeuN positive area, which is indicative of surviving neurons, was significantly higher in MK-801-IP (1.0 ± 0.1) than in MK-801-IV (0.7 ± 0.1) ($n = 9$ in each group) (Fig. 3C). The ipsilateral/contralateral ratio of the cleaved caspase-3 positive cells, which is indicative of apoptotic cell death, was significantly lower in MK-801-IP (1.2 ± 1.1) than in MK-801-IV (3.2 ± 0.9) ($n = 9$ in each group) (Fig. 3D). Collectively, MK-801-IP exerted neuroprotective effects whereas MK-801-IV did not.

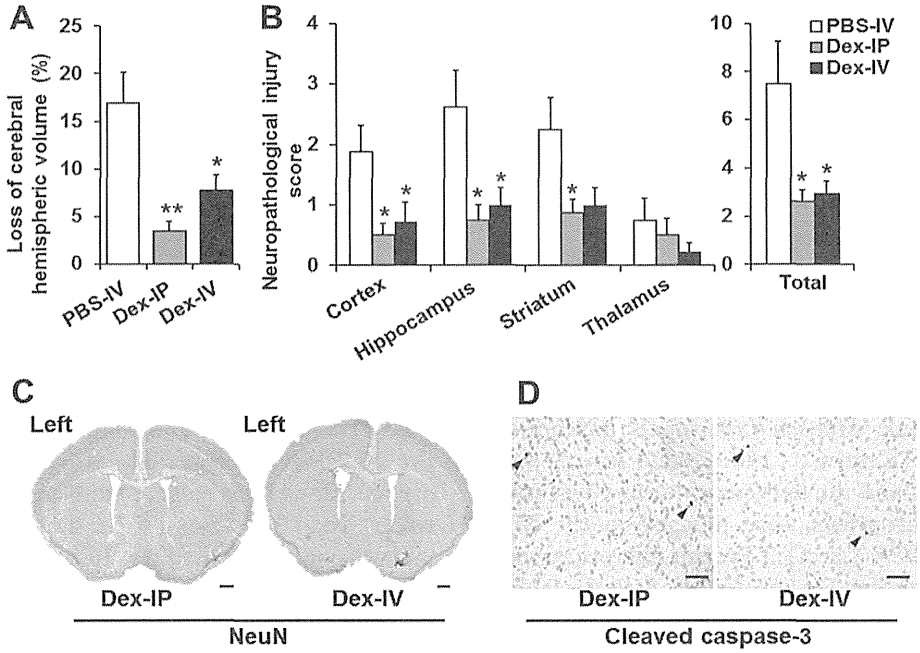


Fig. 2. The neuroprotective effects of intravenously or intraperitoneally injected dexamethasone in neonatal mice with hypoxic–ischemic encephalopathy. (A) The loss of ipsilateral cerebral hemispheric volume is calculated as follows: ((contralateral volume − ipsilateral volume)/contralateral volume × 100%). (B) The neuropathological injury scores of the cortex, hippocampus, striatum, and thalamus (0 indicates no injury; the cortex is scored 0–4, and the hippocampus, striatum, and thalamus are each scored 0–6). The total injury score is the sum of the scores for the four brain regions. Dex-IP and PBS-IV groups, $n = 8$; Dex-IV group, $n = 9$. $^{*}p < 0.05$ vs. PBS-IV. $^{**}p < 0.01$ vs. PBS-IV. (C) Representative images of the coronal brain sections stained with NeuN. The scale bars represent 500 μm. (D) Representative images of the cleaved caspase-3 expressing cells in the ipsilateral cerebral hemisphere. The scale bars represent 50 μm. *Abbreviations:* Dex-IP, mouse pups intraperitoneally injected with dexamethasone; Dex-IV, mouse pups intravenously injected with dexamethasone; PBS-IV, mouse pups intravenously injected with phosphate-buffered saline.

### 3.4. The distribution of administered MSCs

The mean diameter of the MSCs was 21.5 ± 0.6 μm. At 2 h after administration, a remarkable accumulation of infused MSCs was observed in the lungs among the four organs examined in the MSC-IV group, whereas the cells were evenly detected in the four organs in the MSC-IP group (Fig. 4A and B). The MSC-IV resulted in a significantly higher number of cells in the lungs and the brain than the MSC-IP at 2 h after administration. From 2 h to 24 h after the administration of MSCs, the mean number of infused cells tended to decrease in the IV group but tended to increase in the IP group across each organ, although these temporal changes were not statistically significant, except the significant decrease in the brain in the IV group. At 24 h after administration, no significant differences were observed between the MSC-IV and the MSC-IP groups. A comparison between the ipsilateral cerebral hemisphere and the contralateral cerebral hemisphere revealed no significant difference in the number of transfused cells in either the IP or IV group at either time point (at 2 h after transplant; ipsilateral 10.7 ± 4.7 cells/cm$^2$ vs. contralateral 8.2 ± 2.2 cells/cm$^2$ in the IV group, ipsilateral, 3.8 ± 0.7 cells/cm$^2$ vs. contralateral, 3.7 ± 1.4 cells/cm$^2$ in the IP group, at 24 h after transfusion; data not shown). We also analyzed the localization of the cells within the brain (e.g., cortex, hippocampus, striatum, thalamus, and white matter) and found no difference between the IV and IP groups in any of the five brain regions (data not shown).

### 3.5. The distribution of administered MNCs

The mean diameter of MNCs was 7.1 ± 0.6 μm. At 2 h after administration, the MNC-IV resulted in a significantly higher number of cells in the liver, the lungs, and the spleen but not in the brain than the MNC-IP (Fig. 5). From 2 h to 24 h after the administration of MNCs, the mean number of infused cells significantly decreased in the liver and the lungs but remained at the same level in the spleen in the IV group. Only a few cells were detected in any of the four regions in the IP cohort at either time point, and no obvious temporal changes were observed in the number of cells across the organs. At 24 h after the administration, in the spleen, MNC-IV resulted in a significantly higher number of cells compared to the MNC-IP. A comparison between the ipsilateral cerebral hemisphere and the contralateral cerebral hemisphere revealed no significant difference in the number of transfused cells in either the IP or IV group at either time point (data not shown).

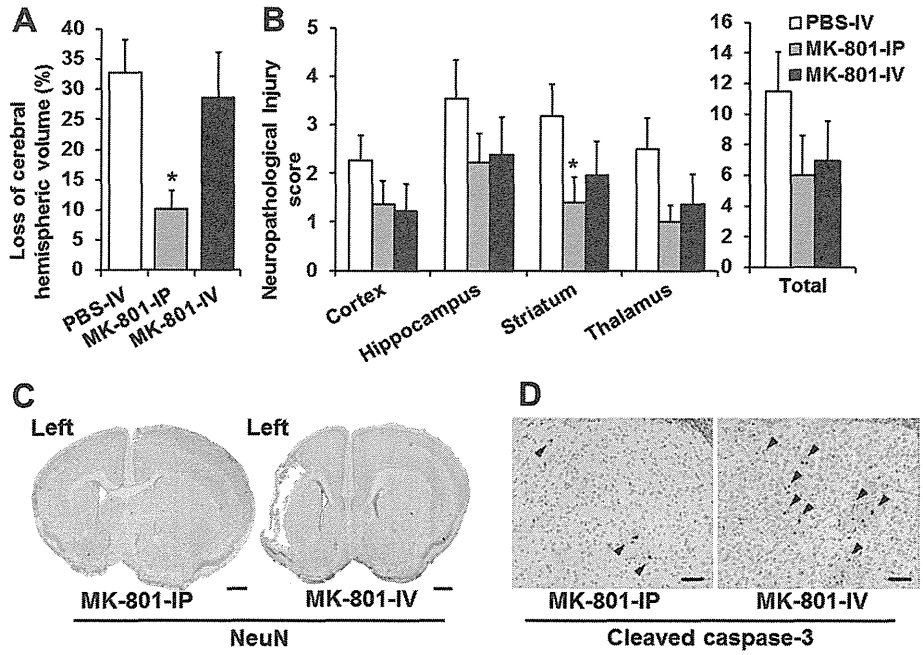


Fig. 3. The neuroprotective effects of intravenously or intraperitoneally injected MK-801 in neonatal mice with hypoxic–ischemic encephalopathy. (A) The loss of ipsilateral cerebral hemispheric volume is calculated as follows: ((contralateral volume − ipsilateral volume)/contralateral volume × 100%). (B) The neuropathological injury scores of the cortex, hippocampus, striatum, and thalamus (0 indicates no injury; the cortex is scored 0–4, and the hippocampus, striatum, and thalamus are each scored 0–6). The total injury score is the sum of the scores for the four brain regions. MK-801-IP and MK-801-IV groups, $n = 9$; PBS-IV group, $n = 12$. $^{*}p < 0.05$ vs. PBS-IV. (C) Representative images of coronal brain sections stained with NeuN. The scale bars represent 500 μm. (D) Representative images of cleaved caspase-3 expressing cells in the ipsilateral cerebral hemisphere. The scale bars represent 50 μm. *Abbreviations:* MK-801-IP, mouse pups intraperitoneally injected with MK-801; MK-801-IV, mouse pups intravenously injected with MK-801; PBS-IV, mouse pups intravenously injected with phosphate-buffered saline.

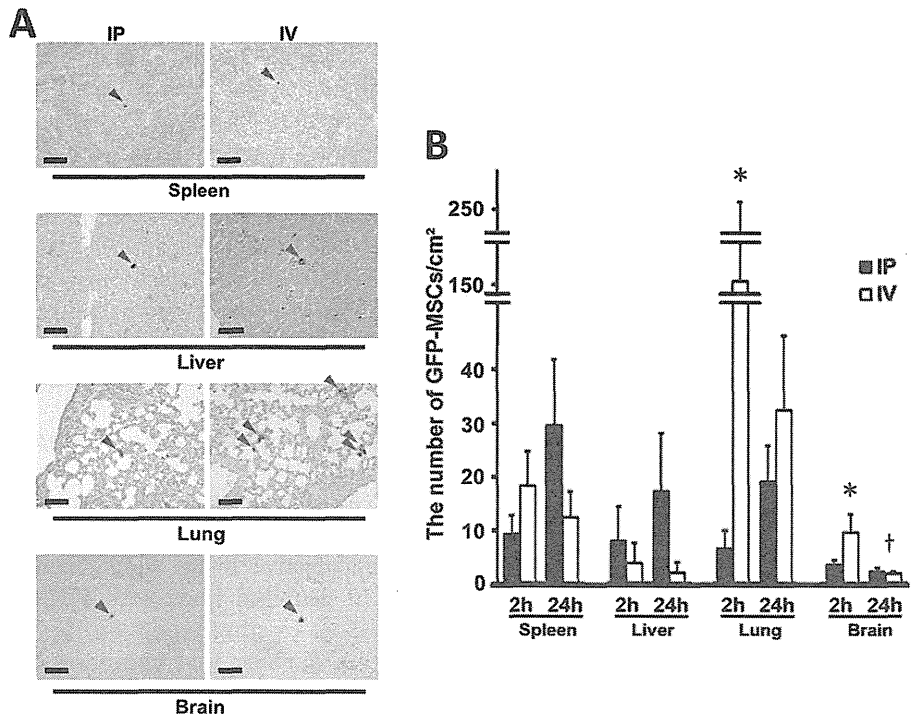


Fig. 4. The distribution of green fluorescent protein (GFP)–positive mesenchymal stem cells (MSCs) intraperitoneally or intravenously injected into neonatal mice with hypoxic-ischemic encephalopathy at 2 h and 24 h after the cell injection. (A) Representative immunohistological staining (i.e., anti-GFP antibody with diaminobenzidine) of the MSCs (arrowheads) in the spleen, liver, lung and brain at 2 h after the cell injection. The scale bar represents 50 μm. (B) Quantitative analysis of MSC distribution at 2 h and 24 h after intraperitoneal (solid bars) and intravenous (open bars) administrations. $n = 10$–13; $^{*}p < 0.05$, vs. IP; $^{\dagger}p < 0.05$, vs. 2 h. *Abbreviations:* IP, intraperitoneal; IV, intravenous.

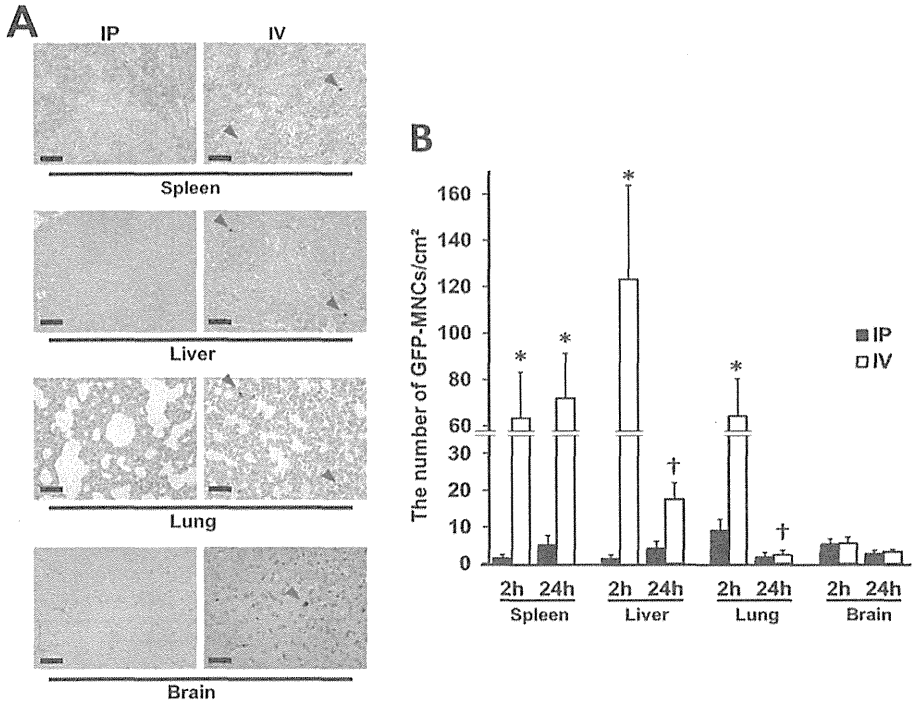


Fig. 5. The distribution of green fluorescent protein (GFP)–positive bone marrow mononuclear cells (MNCs) intraperitoneally or intravenously injected into neonatal mice with hypoxic-ischemic encephalopathy at 2 h and 24 h after the cell injection. (A) Representative immunohistological staining (i.e., anti-GFP antibody with diaminobenzidine) of the MNCs (arrowheads) in the spleen, liver, lung and brain. The scale bar represents 50 μm. (B) Quantitative analysis of MNC distribution at 2 h and 24 h after intraperitoneal (solid bars) and intravenous (open bars) administrations. $n = 8$–9; $^{*}p < 0.05$, vs. IP; $^{\dagger}p < 0.05$, vs. 2 h. *Abbreviations:* IP, intraperitoneal; IV, intravenous.

With regard to the localization of the cells within the brain, no difference was observed between the IV and IP groups (data not shown).

### 3.6. Distribution of MNCs in a macaque model of stroke

Following permanent MCA occlusion in a macaque, an infarct in the left hemisphere was confirmed at 3 days by MRI (Fig. 6A). At 1 h after the IV administration of MNCs, a PET scan demonstrated that a large number of infused MNCs accumulated in the spleen (9.1 kBq/mL), followed by the liver (2.7 kBq/mL) and the lung (1.4 kBq/mL) (Fig. 6B and C). The accumulation in the brain was weak (0.7 kBq/mL), and no laterality was observed (Fig. 6B and C).

## 4. Discussion

### 4.1. A simple and precise IV injection technique in neonatal mice

In the present study, we demonstrated a simple and precise injection technique into the femoral vein in neonatal mice. Three papers have recently described an IV injection technique in neonatal mice [5–7]. In these papers, the superficial temporal vein or jugular vein was the injection site, and the procedure can require two people [6]. Although IV injection in P1–3 mice has been documented in several experiments [36,37], the technique has not been commonly used in experiments with neonatal mice and rats. For example, among the 11 reports on the systemic administration of umbilical cord blood cells in neonatal rodents with HI brain injury in literature, 4 reports used IV injection [38–41] and 7 reports used IP injection [8–14]. All of these reports were on P7 rat pups, and mice pups were not studied in the field. The benefits of our technique are that one person can execute the whole procedure alone and can also confirm that a solution is flowing within a blood vessel.

### 4.2. Different effects of drug therapies

To examine whether IP injection is comparable to IV injection, we examined the therapeutic effects of two neuroprotective drugs (i.e., dexamethasone and MK-801) in neonatal mice with HIE. To our knowledge, there is no existing report on the use of animal models of brain damage that compares the differences in the therapeutic potency or in the plasma and brain levels after the IV or IP administration of either dexamethasone or MK-801. We demonstrated that the neuroprotective effects of MK-801 were dependent on the administration route, whereas the effects of dexamethasone were not. Dexamethasone (molecular weight,

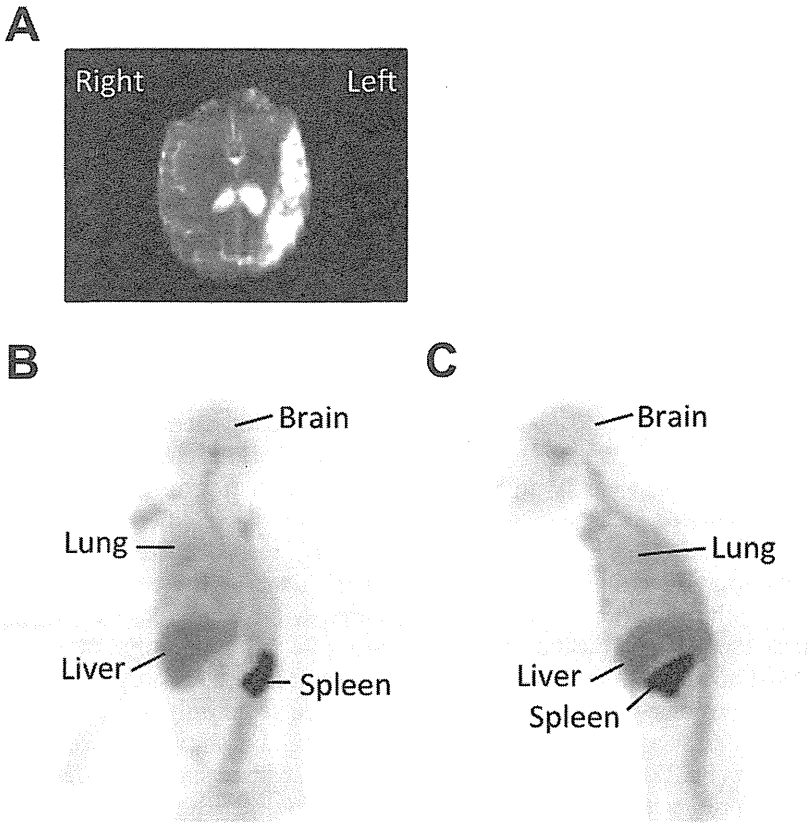


Fig. 6. The distribution of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose ($^{18}$F-FDG)-labeled bone marrow derived-mononuclear cells (MNCs) intravenously injected in a macaque model of stroke. (A) Axial T2-weighted image of MRI at 3 days after middle cerebral occlusion (MCAO). The image shows a large infarct in the left hemisphere. (B, C) Positron emission tomography (PET) images at 60 min after the intravenous injection of $^{18}$F-FDG-labeled bone marrow derived-MNCs. The black color shows the radioactivity of $^{18}$F-FDG-labeled cells. (B) Front elevation image of the macaque. (C) Side elevation image of the macaque.

392.461 g/mol) has a long period of bioactivity, although the plasma level of dexamethasone rapidly decreased after administration [42,43]. The plasma half-life of dexamethasone after IV injection in adult rats is 2.3 h [44]. After IV injection of dexamethasone, rapid accumulation occurs in the adult rat brain, followed by rapid washout. After 10 min and 30 min, 74% and 13% of the concentration observed at 2.5 min remains, respectively [45]. The therapeutic time window of its pretreatment for brain injury is long (e.g., single IP injection at either 6 h or 24 h before HI insult prevents infarction) [27]. Single IP injection at 0 h or 3 h before HI insult does not exert neuroprotection; therefore, the effects of dexamethasone may involve the modification of gene expression [27,43]. Ultimately, differences in pharmacokinetics caused by a different administration route may not have been critical when animals were treated 24 h before the insult with dexamethasone.

MK-801 (molecular weight, 337.37 g/mol) is a non-competitive antagonist of the NMDA-type of excitatory amino acid receptors and enters the brain with no diffusion restriction because of its lipophilic nature [46]. We subsequently used MK-801 to eliminate the local (i.e., intraperitoneal and/or intravascular) and systemic effects that dexamethasone may exert after IV or IP injection. MK-801 reaches maximal concentrations in the adult rat brain within 10–30 min after IP administration with an elimination half-life of 2.05 h [46]. A recent study showed a somewhat delayed time to the maximal concentration in the adult rat brain 40–60 min after IP administration [47]. The MK-801 concentration at 60 min after IV administration in the non-ischemic brain regions of adult rats with permanent MCA occlusion decreases by more than 50% from the level at 15 min [48]. No study has directly compared the pharmacokinetics of MK-801 after IV and IP, but the above-mentioned studies indicate that the brain level of MK-801 may drop faster after IV than after IP administration, although the difference may not be large. Brain ischemia causes a rapid and drastic increase in excitatory amino acids, specifically glutamate, and activation of NMDA receptors [19]. The duration of glutamate accumulation and activation of NMDA receptors is short in models with reperfusion [19], which results in a narrow therapeutic time window of MK-801 activity [19,49,50]. Therefore, a small difference in the duration in which a drug maintains its therapeutic concentration in the brain may make a big difference. Correctively, the

different therapeutic effects of MK-801 observed in the present study may be due to the subtle difference in the pharmacokinetics caused by the different administration routes.

In using a therapeutic agent that has a long bioactivity time such as dexamethasone, the IP route can be used in preclinical studies as an alternative to IV, which is the expected administration route in the clinical setting. By contrast, when using a neuroprotectant that has a short therapeutic time window such as MK-801, it is important to choose the optimal administration route in the preclinical study.

### 4.3. Different cell distributions

The present study demonstrated that the IV administration of MSCs or MNCs resulted in a significantly higher number of cells accumulating in several organs compared to IP administration. The difference in cell accumulation according to administration route was dependent on the time after the administration, the cell type, and the organ. To our knowledge, only one report has compared the cell distributions after IV and IP administrations in immature animals. The study using the polymerase chain reaction (PCR) for a human-specific gene fragment in neonatal rats with excitatory brain injury showed that human MNCs were detectable at 5 min and up to 2 h after IV administration primarily in the blood, lung, and liver, and only in small amounts in the brain; however, no human MNCs were detected in either the blood or the lungs at any experimental time point from 5 min to 5 days after IP administration [51]. Additionally, that report [51] is the only study that examined the systemic cell distribution in animals with neonatal brain injury. A few studies have examined the differences in the systemic cell distribution after different infusion methods in adult rodents. Gao et al. used the γ camera to detect $^{111}$In-oxine-labeled MSCs and demonstrated that the radioactivity accumulated in the lung immediately after IV infusion and primarily in the liver 48 h after IV infusion. After IP infusion, radioactivity was observed in the liver, spleen, kidneys, and lungs in small amounts [52].

In the short term, IV administration appears to be more advantageous for conveying donor cells to the brain compared to IP administration; however, studies have shown that the amount of donor cells that reach the brain is limited even after IV administration [41,53], which is supported by our observations. Collectively, although the data are limited, they indicate that donor cell distributions differ depending on the administration route.

In contrast to the aforementioned study [51], several studies on neonatal rats with HI showed that human MNCs were detectable in the cerebral hemisphere at 1 day [11], 2 days [10], 2 weeks [9], and 40 days [8] after IP administration using immunohistochemical staining and 13 days after IP administration using PCR [11]. These studies showed the exclusive distribution of donor cells (MNCs) in the injured hemisphere. By contrast, the present study showed an even distribution of donor cells (MSCs and MNCs) across the ipsilateral and contralateral hemispheres either after IV or IP administration, and this observation is in accordance with a study that used IV administration of MNCs [40] and one that used intracardiac administration of MSCs [54]. In addition to the time after the administration, the cell type, and the organ, detecting the transfused cells may depend on various factors such as the type and intensity of brain injury, the transfused cell dose, species and strain, capillary size, and detection method. With these numerous factors and the temporally changing nature of cell distribution, it may not be appropriate to compare different sets of data obtained under different experimental paradigms.

With respect to the influence that different cell types may cause in the cell distribution, 4% of IV-administered MNCs entered the common carotid arterial, and the rate was markedly lower for MSCs (1/30 of MNCs) in adult rats [55]. Administered cells (MNCs and MSCs) were detected only in the lungs and not in other organs. Studies have shown that the vast majority of IV-administered cells, especially MSCs, are trapped within the lungs at least during the acute phase after administration and that the entrapment depends on the size of the infused cells [55,56]. The mean diameter of adult mouse pulmonary capillaries is approximately 15 μm, and larger cells are consequently trapped within the lung [56]. The mean diameter of MSCs is approximately 20 μm, and the mean diameter of bone marrow derived-MNCs is approximately 7 μm [55,56]. In our observation, in a larger animal such as a macaque with a stroke, IV administration of MNCs resulted in high accumulation of the cells in the liver and spleen, and the distribution in the lung was low, unlike in neonatal mice. Clinical studies with intra-arterial administration of MNCs in patients with ischemic stroke or myocardial infarction demonstrate that MNCs accumulate in the liver and spleen rather than the targeted organ [35,57]. Collectively, the evidence shows that infused cells tend to accumulate in the liver and spleen regardless of the administration route and recipient species. In addition to the donor cell size and host capillary size, other factors may influence the entrapment. For example, MSCs are adhesive cells, whereas MNCs are not adhesive. Blocking cell adhesive molecules reportedly increases pulmonary passage [55].

### 4.4. Conclusion

The route of administration may influence the effects of drugs. IP injection differs from IV injection with respect to cell distribution after cell transplantation. Therefore, caution should be exercised when translating

data obtained in animal studies using IP administration for use in clinical trials with IV administration. Preclinical studies using IV administration may be necessary before clinical trials when the anticipated administration route is IV, especially for cell-based therapies.

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# Administration of Umbilical Cord Blood Cells Transiently Decreased Hypoxic-Ischemic Brain Injury in Neonatal Rats

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**Key Words**
Neuroprotection · Oxidative stress · Cell therapy · Asphyxia

**Abstract**
This study aimed to investigate whether the administration of mononuclear cells derived from human umbilical cord blood cells (UCBCs) could ameliorate hypoxic-ischemic brain injury in a neonatal rat model. The left carotid arteries of 7-day-old rats were ligated, and the rats were then exposed to 8% oxygen for 60 min. Mononuclear cells derived from UCBCs using the Ficoll-Hypaque technique were injected intraperitoneally 6 h after the insult ($1.0 \times 10^7$ cells). Twenty-four hours after the insult, the number of cells positive for the oxidative stress markers 4-hydroxy-2-nonenal and nitrotyrosine, in the dentate gyrus of the hippocampus in the UCBC-treated group, decreased by 36 and 42%, respectively, compared with those in the control group. In addition, the number of cells positive for the apoptosis markers active caspase-3 and apoptosis-inducing factor decreased by 53 and 58%, respectively. The number of activated microglia (ED1-positive cells) was 51% lower in the UCBC group compared with the control group. In a gait analysis performed 2 weeks after the insult, there were no significant differences among the sham-operated, control and UCBC groups. An active avoidance test using a shuttle box the following week also revealed no significant differences among the groups. Neither the volumes of the hippocampi, corpus callosum and cortices nor the numbers of neurons in the hippocampus were different between the UCBC and control groups. In summary, a single intraperitoneal injection of UCBC-derived mononuclear cells 6 h after an ischemic insult was associated with a transient reduction in numbers of apoptosis and oxidative stress marker-positive cells, but it did not induce long-term morphological or functional protection. Repeated administration or a combination treatment may be required to achieve sustained protection.


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## Introduction

Perinatal hypoxia-ischemia (HI) remains an important cause of neonatal death and permanent neurological deficits [1]. Notwithstanding the developments made in perinatal medicine, perinatal HI occurs in 1.3–1.7/1,000 live births; its incidence is high even in developed countries [2]. Many survivors of perinatal HI experience long-term neurological disabilities and impairments resulting in major socioeconomic burdens. At present, there are no effective treatments for HI-induced brain damage, except for brain hypothermia [3], which is not effective in severe cases [4, 5]. Therefore, it is of the utmost importance to develop a novel and effective therapy against perinatal HI-induced brain injury.

Stem cell therapy is expected to be used in the treatment of many central nervous system diseases in the future. Various kinds of stem cells are possible sources of cell therapy for future clinical applications [6, 7]. We recently demonstrated that intracerebroventricular injection of neural stem/progenitor cells together with chondroitinase ABC – which digests glycosaminoglycan chains on chondroitin sulfate proteoglycans – significantly decreased the degree of cerebral infarction after perinatal HI injury in a rat model [8]. However, ethical concerns hinder the use of postmortem human brains as a source of neural stem/progenitor cells in future clinical applications. Furthermore, intracerebral administration is an invasive procedure, and the injected cells themselves may lead to gliotic changes in the host brain [9], thereby necessitating more detailed examinations to ensure the safety of the procedure.

Umbilical cord blood cells (UCBCs) are a promising source of stem cell therapy. They are readily available and can be used for autologous transplantations. Thus, many ethical considerations can be avoided. Furthermore, UCBCs can be administered intravenously [10] and cross the blood-brain barrier [11]. Meier et al. [12] first reported the treatment effects of UCBCs in the amelioration of HI-induced brain damage in a neonatal rat model; moreover, several recent studies reported favorable effects of UCBCs [13–18]. However, the mechanisms underlying the favorable effects remain to be fully elucidated. In the present study, we administered UCBCs to HI rats to investigate their effects and the underlying mechanisms.

## Materials and Methods

*Animals*

All animal experimental protocols in the present study were approved by the Institutional Review Board of Nagoya University School of Medicine (Nagoya, Aichi Prefecture, Japan; permit No.: 23181-2011 and 24337-2012). Wistar/ST rat pups were obtained from Japan SLC Inc. (Shizuoka, Japan) and maintained under a 12-hour light/dark cycle (8.00 a.m. to 8.00 p.m.) with ad libitum access to food and water. The animal room and experimental space were always maintained at 23°C.

*UCBC Preparation*

Human UCBCs were donated by women who delivered at Nagoya University Hospital. Written, informed consent was obtained from the donors and their spouses, and this experimental protocol using human cells was reviewed and approved by the local ethics committee of our hospital (permit No.: 794). The donors underwent normal delivery or elective cesarean section because of previous cesarean section, breech position or cephalopelvic disproportion. The donors and infants had no major perinatal complications; all were singleton pregnancies of more than 36 weeks of gestational age.

Umbilical cord blood was collected immediately after placental delivery in bags containing citrate phosphate dextrose (CBC-20; Nipro Corporation, Osaka, Japan). Mononuclear cells were isolated using the Ficoll-Hypaque technique, suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Carlsbad, Calif., USA) at a concentration of $1 \times 10^7$ cells/ml, and cryopreserved in liquid nitrogen with an equal amount of a cryoprotectant (CP-1; Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). CP-1 is a mixture of dimethylsulfoxide and hydroxyethyl starch, which makes it possible to preserve stem cells in a frozen state. Immediately before administration, the cells were thawed to 37°C, washed 3 times with phosphate-buffered saline (PBS) and resuspended in 0.3 ml of RPMI 1640 medium.

*HI Insult and UCBC Administration*

HI brain damage was produced using postnatal day 7 (P7) rats according to the method of Rice et al. [19] with slight modifications. Each pup was anesthetized using isoflurane inhalation and the left carotid artery was subsequently doubly ligated and incised between the ligatures. After a 1-hour rest with a dam, the pups were exposed to a hypoxic environment of 8% $O_2$ at 37°C for 60 min, after which they were returned to the dam in the animal room maintained at 23°C. Six hours later, the pups in the treatment group (UCBC group) were injected intraperitoneally with mononuclear cells derived from UCBCs ($1 \times 10^7$ cells/0.3 ml). A control group underwent ligation of the left carotid artery and hypoxia in the same manner, but received an equivalent volume of RPMI medium alone. The sham group underwent neither left carotid artery ligation nor hypoxia.

*Histological and Immunohistochemical Procedures*

Histological and immunohistochemical procedures were performed as previously described [20] with minor modifications. Briefly, rats were deeply anesthetized and intracardially perfusion-fixed with 0.9% NaCl followed by 4% paraformaldehyde in PBS. The brains were removed and immersion-fixed in the same solution at 4°C for 24 h, dehydrated with a graded series of ethanol and xylene, embedded in paraffin and cut into 5-μm-thick coronal sections. After deparaffinization and rehydration, antigen retrieval was performed by heating the sections for 10 min in 10 mM citrate buffer (pH 6.0). Nonspecific binding was blocked with 3% donkey serum in PBS. Then, sections were incubated overnight at 4°C with rabbit anti-active caspase-3 (product No. 559565; dilution 1:200; BD Pharmingen, Franklin Lakes, N.J.,

USA), goat anti-apoptosis-inducing factor (AIF; product No. SC-9416; dilution 1:100; Santa Cruz Biotechnology, Dallas, Tex., USA), rabbit anti-4-hydroxy-2-nonenal (4-HNE; product No. HNE11-S; dilution 1:400; Alpha Diagnostic International, San Antonio, Tex., USA), rabbit antinitrotyrosine (product No. A-21285; dilution 1:200; Life Technologies; control samples are shown in the online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000368396), mouse anti-ED1 (activated microglia marker; product No. MAB1435; dilution 1:300; Merck Millipore), goat anti-neuronal nuclei (NeuN) antibody (product No. MAB377; dilution 1:400; Merck Millipore) or rat anti-myelin basic protein antibody (product No. MAB386; dilution 1:200; Merck Millipore) in PBS with 0.1% Triton. The sections were subsequently incubated with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, Calif., USA) for 1 h at room temperature. Endogenous peroxidase activity was blocked with 3% $H_2O_2$ in PBS for 10 min and then avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories), followed by peroxidase detection for 10 min (0.12 mg/ml 3,3′-diaminobenzidine, 0.01% $H_2O_2$ and 0.04% $NiCl_2$).

*Cell Counting for Acute Injury Markers*

Cells positive for active caspase-3, nuclear AIF, 4-HNE, nitrotyrosine and ED1 were counted throughout the hippocampal granule cell layer (GCL). In the section at the hippocampal level, which is approximately −3.3 to −3.6 mm posterior to the bregma in the adult rat brain [21], the GCL was outlined under low magnification (×100), and the area was measured. Further, the positive cells were counted under high magnification (×400) using Stereo Investigator version 10 stereology software (MicroBrightField Europe EK, Magdeburg, Germany) and expressed as densities.

*Behavioral Tests*

Gait Analysis

To evaluate hemiparesis, the CatWalk® quantitative gait analysis system (Noldus Information Technology, Wageningen, the Netherlands) was used for gait assessment following experimental stroke [22]. Briefly, we had the experimental rats run across a glass walkway transversely and recorded the runs using a camera positioned below. If an animal failed to complete a run within 5 s, walked backwards or reared during the run, it was made to repeat the process. Each rat ran 3 times, and the average was calculated. The experiment was performed in the dark; the glass walkway was illuminated with beams of light, thereby allowing the animals' paws to reflect light as they touched the glass floor. An observer labeled each paw on the recorded video to calculate paw-related parameters. The gait-related parameters measured using the CatWalk system were the following: run duration, time duration of entire run; print area, area of paw print; stride length, distance the paw traveled from one step to the next; swing speed, the stride length divided by the duration of one stride length; mean intensity, the mean intensity of each paw in the run (the intensity is proportional to the load on the paw); duty cycle, percentage of time the paw accounted for the total step cycle of that paw.

Active Avoidance Test

The active avoidance test was performed according to the methods of Ichinohashi et al. [23] using the same equipment. For 4 consecutive days, each rat underwent 20 sessions of the active avoidance test each day. The test was conducted in an automated shuttle box (Med Associates Inc., St. Albans, Vt., USA) subdivided into 2 compartments with independently electrified stainless steel bars as a floor. One session consisted of a buzzer tone and stimulation with light (conditioning stimulus), and an electric shock (unconditioned stimulus). The conditioning stimulus was continued for 5 s, as was the subsequent unconditioned stimulus with a positive half-wave constant current of 0.5 mA. When the conditioning stimulus occurred, the rat had to escape to the other side of the shuttle box to turn it off and avoid the unconditioned one. The average interval time between each trial was 30 s (range, 10–90 s). The parameters were analyzed using the MED-PC IV program (Med Associates Inc.). Each day, we evaluated the avoidance proportion, i.e. the number of sessions in which the rat successfully avoided the unconditioned stimulus.

*Volume Measurement and Stereological Cell Counting*

After the behavior tests, the rats were deeply anesthetized, intracardially perfusion fixed, and had their brains removed. To evaluate the whole cortex, corpus callosum and hippocampus, every 150th section from the whole cerebrum and corpus callosum (typically 17 sections) and every 75th section from the hippocampus level (typically 6 sections) were stained using goat anti-NeuN antibody for cortex and hippocampus, or anti-myelin basic protein antibody for the corpus callosum. The bilateral cortex, hippocampus (NeuN-positive areas) and corpus callosum of each section were outlined, and the areas of each were measured using Stereo Investigator. The volumes of the bilateral cortex and hippocampus were calculated from the NeuN-positive areas according to the Cavalieri principle using the following formula: $V = \Sigma A \times P \times T$, where V = the total volume, $\Sigma A$ = the sum of area measurements, P = the inverse of the sampling fraction, and T = the section thickness [20]. The numbers of NeuN-positive cells were counted in the whole GCL, including the subgranular zone, of the hippocampus using unbiased stereological counting techniques. After outlining the borders of the GCL, the computer program overlays the outlined area with a grid system of counting frames. Cells within these frames as well as those touching 2 out of 4 predetermined sides of the frames were counted.

*Statistical Analysis*

All data are presented as mean ± standard error of the mean. A 2-sample Student's t test was used to compare the two groups in figure 2. The Steel-Dwass test as nonparametric multiple comparison procedure was used to compare the three groups in figures 3 and 4. A p value <0.05 was considered statistically significant.

## Results

### *Impact of UCBCs on Expression of Acute Injury Markers after HI*

P7 rats were subjected to HI, and 6 h later, they were administered cryopreserved, thawed and washed mononuclear cells isolated from the UCBCs or vehicle. We examined various acute injury biomarkers 24 h after HI: apoptosis markers (active caspase-3 and AIF), oxidative

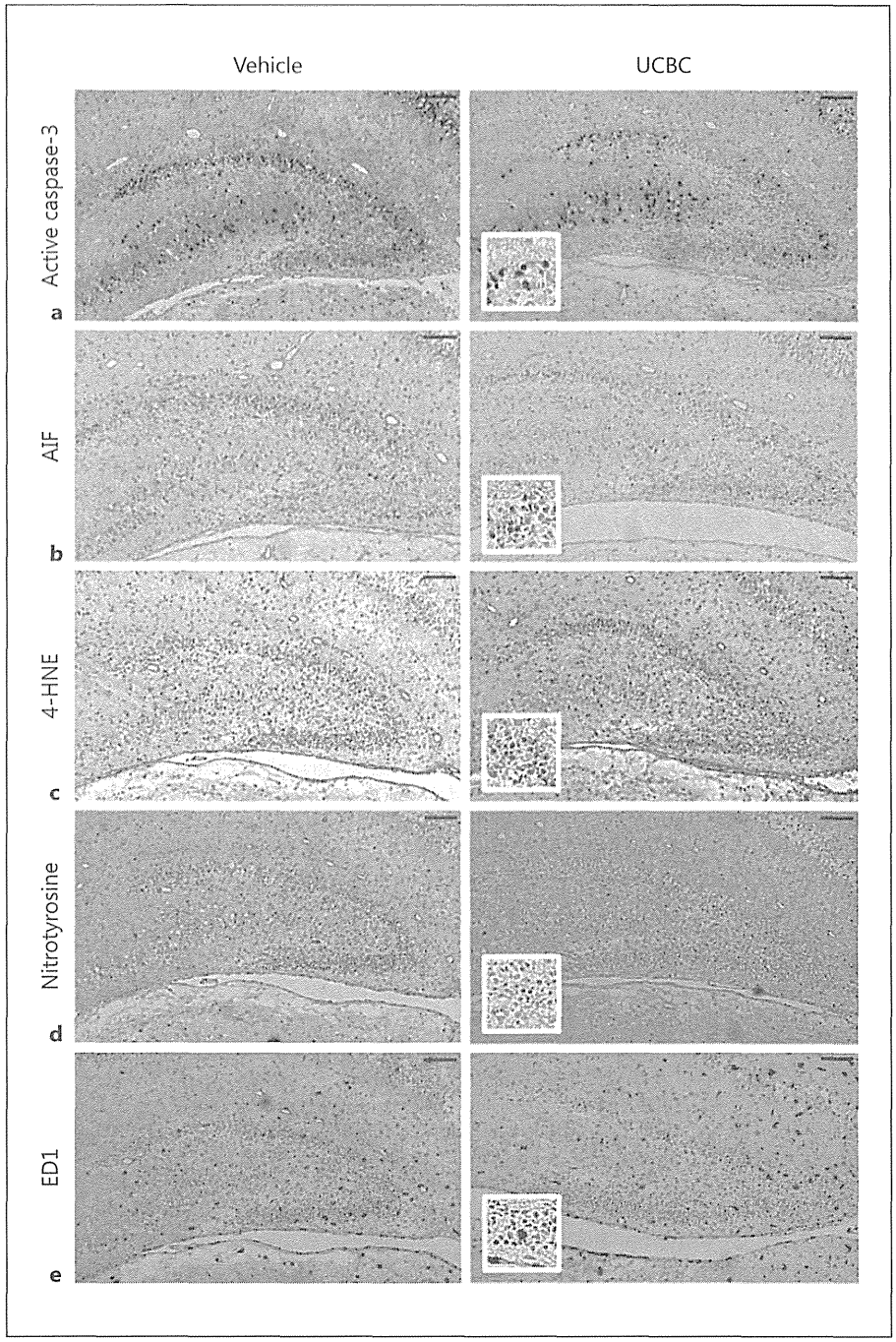


**Fig. 1.** Photomicrographs of the hippocampus stained for acute injury markers. Representative photomicrographs of the dentate gyrus of the hippocampus 24 h after HI insult. The sections from vehicle-treated rats (vehicle) and UCBC-treated rats (UCBC) were stained for active caspase-3 (**a**), AIF (**b**), 4-HNE (**c**), nitrotyrosine (**d**) and ED1 (**e**). Bar = 100 μm. Insets show higher magnifications.

stress markers (4-HNE and nitrotyrosine) and an activated microglia marker (ED1). Photomicrographs of representative hippocampi are shown in figure 1. The number of the apoptosis marker-positive cells in the ipsilateral GCL significantly decreased in the UCBC group compared with those in the control group (activated caspase-3, 53%, and AIF, 58%; fig. 2a, b; $p < 0.05$ and $p < 0.01$, respectively). The numbers of oxidative stress marker-positive cells also decreased in the UCBC group compared with the control group (4-HNE, 36%, and nitrotyrosine, 42%; fig. 2c, d; $p < 0.05$). Moreover, the number of ED1-positive cells was 51% lower in the UCBC group compared with the control group (fig. 2e; $p < 0.05$).

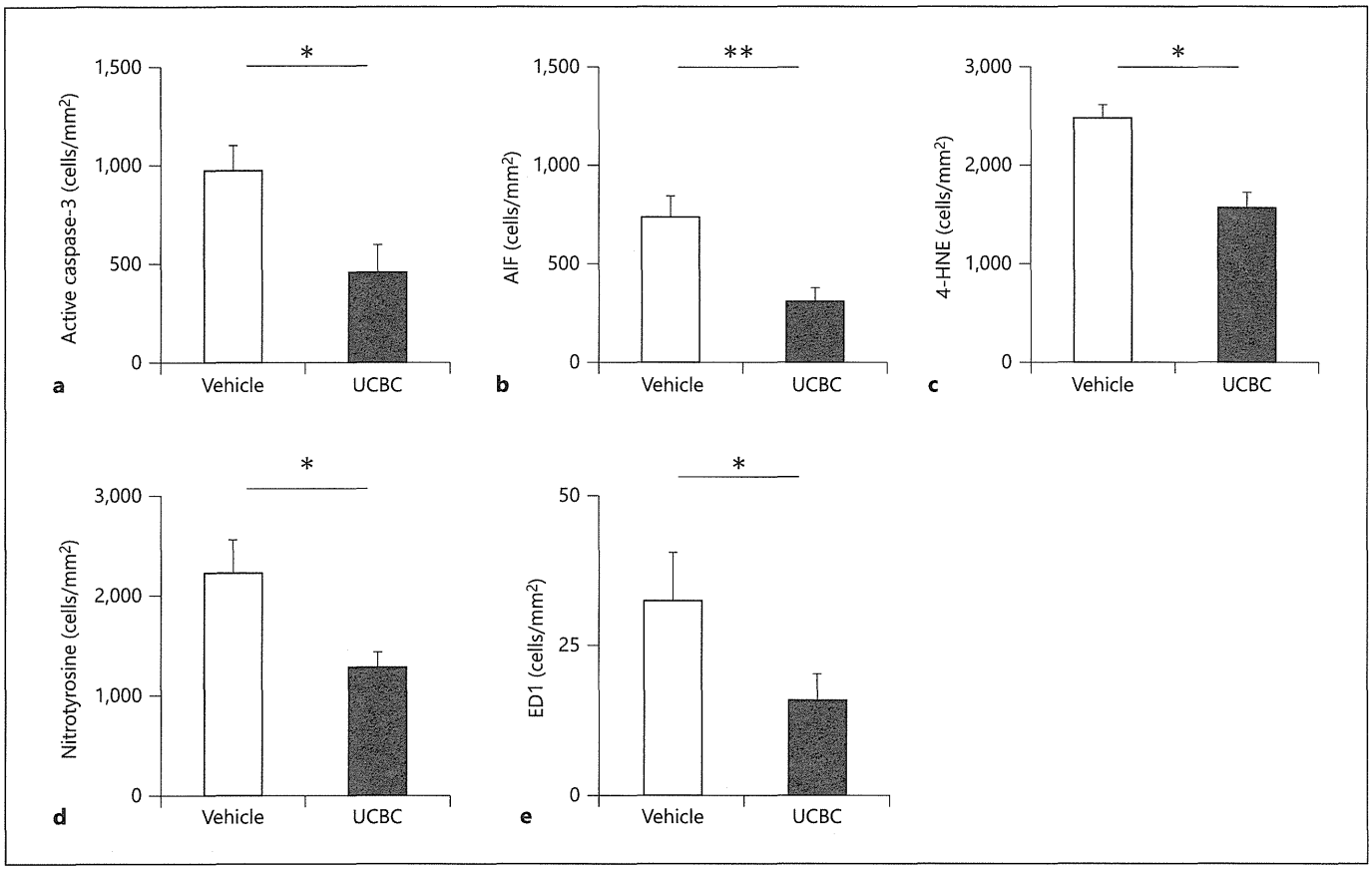


**Fig. 2.** Impact of UCBCs on apoptosis, oxidative stress and activation of microglia. The numbers of various marker-positive cells in figure 1 were counted. The numbers of active caspase-3-positive (**a**) and AIF-positive cells (**b**) in the ipsilateral GCL were significantly lower in the UCBC group (n = 11) than in the vehicle group (n = 12). The numbers of both 4-HNE- (**c**) and nitrotyrosine-positive cells (**d**) were also significantly lower in the UCBC group. The number of ED1-positive cells (**e**) was significantly decreased in the UCBC group. Data are presented as means ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$.

*Impact of UCBCs on Behavior after HI*

Gait Analysis

To evaluate the effect of HI on motor function and of UCBC administration on HI-induced motor deficits, gait analysis was performed 14 days after the insult (P21) using the CatWalk system. There were no significant differences in run duration, right front paw (RF) print area, RF swing speed, RF/left front paw (LF) ratio of mean intensity, RF duty cycle or RF stride length among the three groups (sham-operated, control and UCBC groups; fig. 3).

Active Avoidance Test

Further, to evaluate the effects of UCBCs on HI-induced learning impairments, an active avoidance test was performed 21–24 days after the insult (P28–31). The mean avoidance proportion of each group was calculated for each consecutive day (fig. 4). The avoidance rates increased with time in all groups; however, there were no significant differences among the three groups for each day.

*Impact of UCBCs on Histological Changes after HI*

Finally, to assess the absolute tissue loss after HI, the volumes of the cortex, corpus callosum and hippocampus of both hemispheres were evaluated. After the behavioral tests, sections throughout the whole cerebrum were evaluated.

Representative photomicrographs stained for NeuN are shown in figure 5a and b. In both groups, there was apparent unilateral atrophy with partial collapse. We evaluated the volumes of the cortex (fig. 5c), corpus callosum (fig. 5d) or hippocampus (fig. 5e), but found no significant differences in the volumes between the groups.

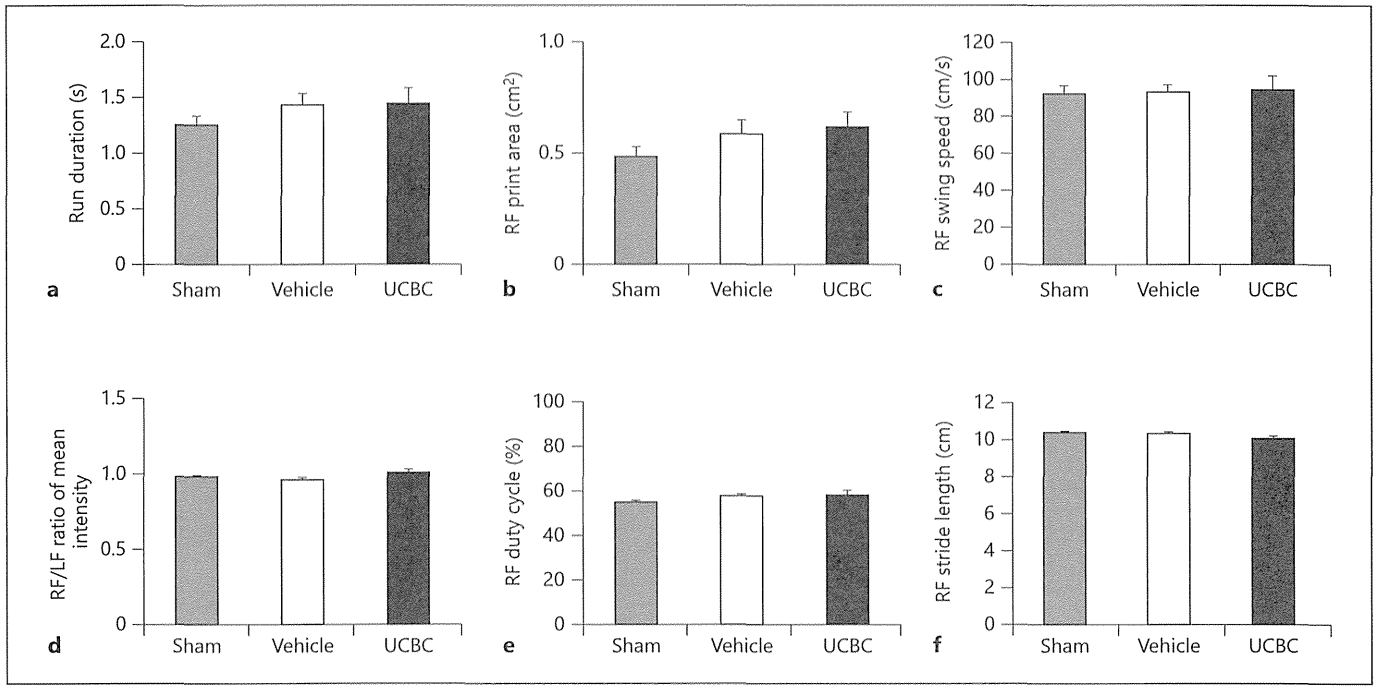


**Fig. 3.** Gait analysis. Gait analysis was performed 2 weeks after the insult. Each parameter was compared among the groups. There was no significant difference in run duration (**a**), RF print area (**b**), RF swing speed (**c**), RF/LF ratio of mean intensity (**d**), RF duty cycle (**e**) or RF stride length (**f**) among the three groups (sham-operated, n = 6; vehicle, n = 8; UCBC, n = 6).

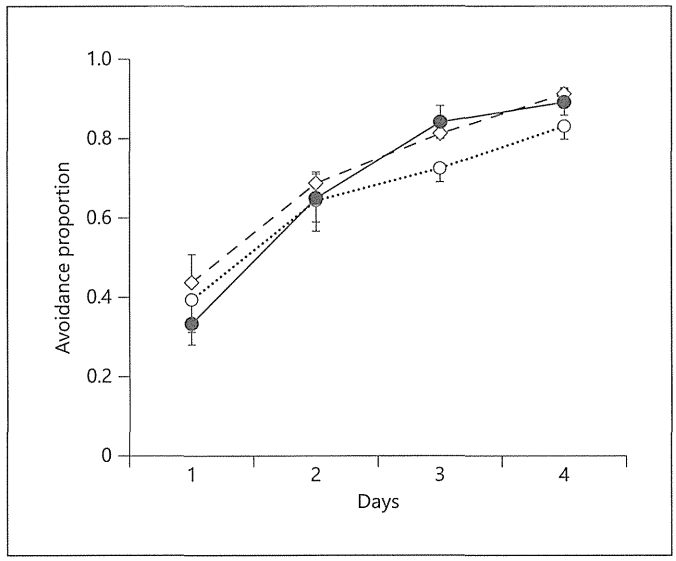


**Fig. 4.** Active avoidance test. The active avoidance was performed 21–24 days after insult (P28–31). Each rat underwent 20 sessions every day, and the mean avoidance proportion was calculated for each group. The avoidance proportions increased with time in all groups. There was no significant difference in avoidance proportions among the groups on any day. Open squares and dashed line: sham-operated, n = 4; open circles and dotted line: vehicle, n = 8; closed circles and solid line: UCBC, n = 6.

Therefore, we examined whether there was a difference in the number of neurons between the groups, even though the volume reductions were equivalent. The numbers of NeuN-positive neurons in the hippocampus were counted using stereological principles (Stereo Investigator, MicroBrightField) but they were not significantly different between the groups in the hippocampus of either the ipsilateral or contralateral hemisphere (fig. 5f).

## Discussion

In the present study, we demonstrated that intraperitoneal UCBC administration caused antiapoptotic and antioxidative effects 24 h after the insults; however, we failed to demonstrate a prolonged reduction of neurological damage. We administered UCBCs in the early phase (6 h after HI). The optimal timing of administration is one of the most critical points to establish a new cell therapy. In regenerative medicine using stem cells, grafting in the early postinjury phase is generally not recommended. In neural stem/progenitor cell transplantation, early grafting in the acute phase (i.e. 24 h after insult), during which inflammatory chemical mediator and

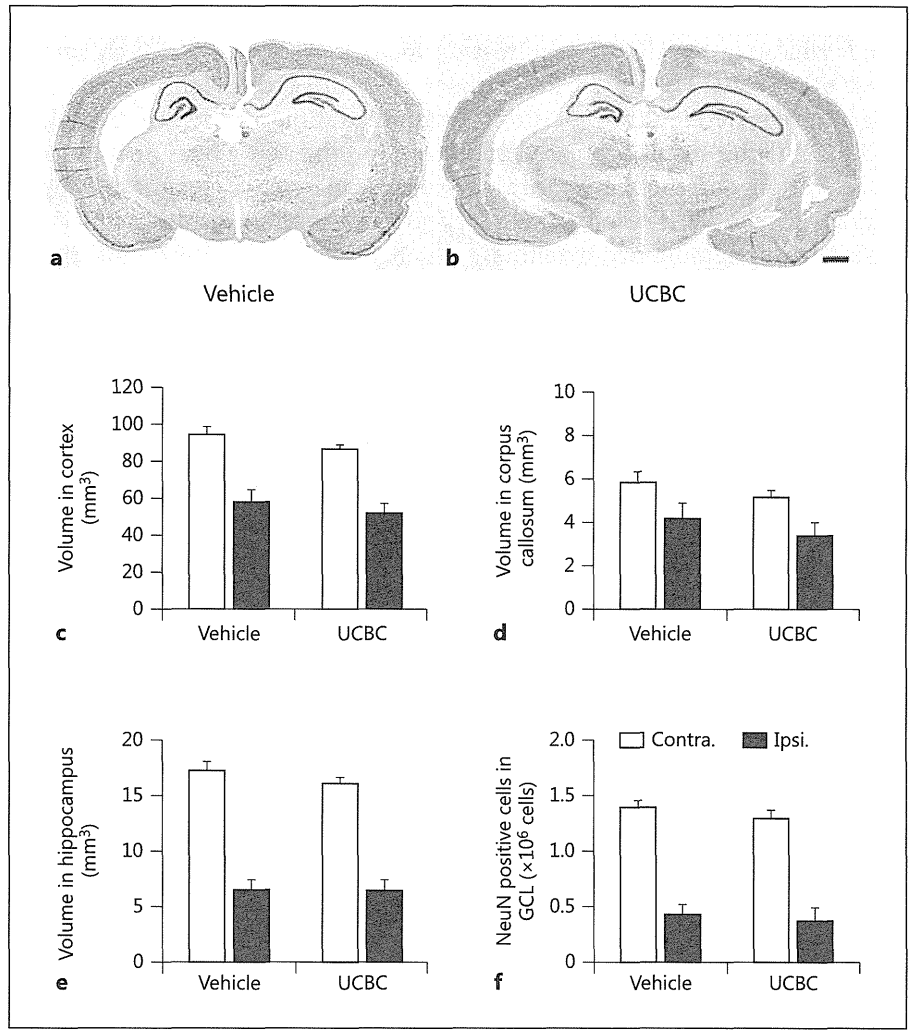


**Fig. 5.** Impact of the UCBCs on the histological change after HI. The volumes of whole cortex, corpus callosum and hippocampus, and the number of NeuN-positive neurons in whole hippocampus were evaluated using Stereo Investigator version 10 (stereology software) after behavioral tests. **a**, **b** Representative photomicrographs of the brain stained for NeuN from vehicle (**a**) and UCBC-treated rats (**b**) 2 months after HI. Bar = 1,000 μm. There was no significant difference in the volumes of the cortex (**c**), corpus callosum (**d**) or hippocampus (**e**) between the groups. The numbers of NeuN-positive neurons in the GCL were not significantly different between the vehicle and UCBC groups in the hippocampus of ipsilateral hemispheres (**f**). Contra. = Contralateral; Ipsi. = ipsilateral.

cytokine concentrations are increased [24], can induce greater differentiation of grafted cells to astrocytes, decrease the survival rates and/or decrease the beneficial treatment effects in various models such as spinal cord injury [25] and stroke [26] in adult rodents, and irradiation-induced brain injury in young mice [9]. In addition, early administration of mononuclear cells from bone marrow showed fewer treatment effects in a model of adult stroke [27]. However, in neonatal HI, there are two phases of pathological events: primary and secondary energy failure [28, 29]. Primary energy failure occurs within minutes after initial cerebral ischemia; in this phase, acidosis and depletion of oxygen, glucose and adenosine triphosphate lead to acute derangement of intracellular metabolism, resulting in necrosis and cell death. The subsequent secondary energy failure occurs after a variable period following the initial insult. Inflammation, excitatory amino acid release, intracellular calcium inflow, and production of nitric oxide and reactive oxidative species occur in this stage. Therefore, it is reasonable that most therapies, including hypothermia therapy, should be commenced at least before the second phase. The treatment effect induced by UCBC administration for brain injury is considered to be involved in secretion of neurotrophic factors that promote endogenous neurogenesis, prevent loss of neuronal cells and regulate immunity [28], which was shown also in the effect of bone marrow-derived mesenchymal stem cells [30]. Expression levels of various pro-inflammatory cytokines, including interleukin-1 and tumor necrosis factor, are elevated in the early phase of perinatal HI; UCBC administration can decrease these levels, which are also accompanied by decreased expression of cluster of differentiation 68, a biomarker of activated microglia/macrophages in the brain