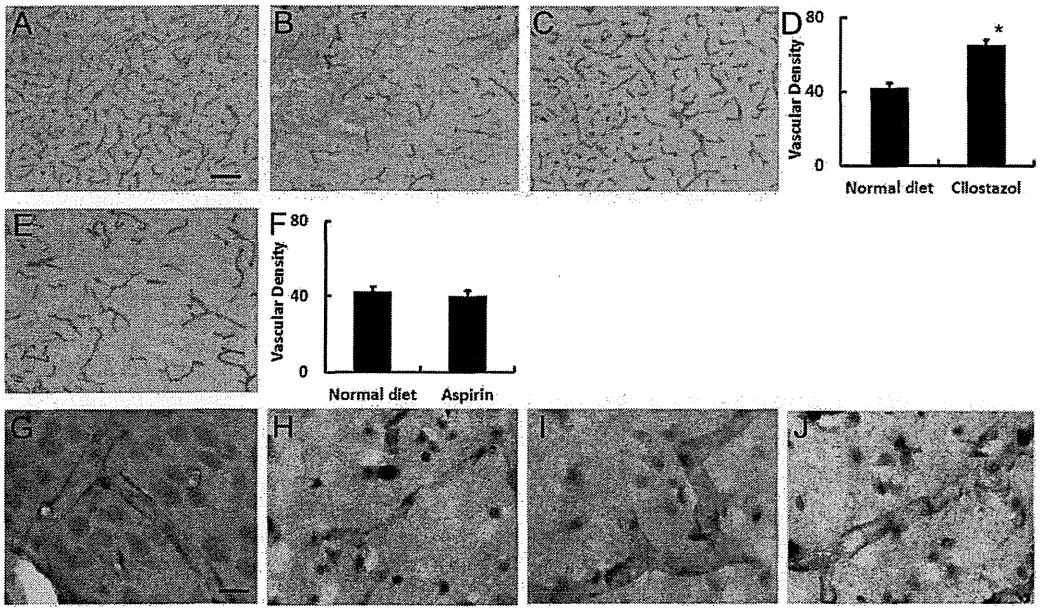

Figure 4. Pretreatment with cilostazol prevented cerebrovascular disruption after transient ischemia with tPA injection. **A–D**, Representative micrographs of cerebral cortex from the stroke-affected area in mice fed a normal diet (**A**, contralateral; **B**, ispilateral) or cilostazol (**B**) at 24 hours after ischemia. Quantitative analysis confirmed significant preservation of microvasculature by pretreatment with cilostazol compared with a normal diet alone (**D**). **E–F**, Representative micrographs of the stroke-affected cortex in mice pretreated with aspirin (**E**). No significant change in vascular density was observed between normal diet and aspirin-treated groups (**F**). **G–J**, Immunohistochemical staining with lectin at 24 hours after induction of ischemia. In contrast to the contralateral cortex (**G**), disruption of cerebral vascular structures was observed in stroke-affected cortex in mice fed a normal diet (**H**). Treatment with cilostazol had a protective effect on the cerebral vasculature (**I**), although no such effect was observed by treatment with aspirin (**J**). *$P<0.05$ vs normal diet control (**D**). N=4, in each group. Scale bars, 100 μm (**A**) and 20 μm (**G**). tPA indicates tissue-type plasminogen activator.

fed a normal diet (Figure 4G, contralateral; Figure 4H, ipsilateral). In contrast, preservation of vascular structure in the stroke area was observed in mice pretreated with cilostazol (Figure 4I). Similar to the results obtained with anti-PECAM-1 antibody, the pretreatment with aspirin had no protective effect on degradation of cerebrovasculature at the poststroke area (Figure 4J).

Cilostazol Prevented Activation of MMP-9 in the Poststroke Cortex

Activation of MMP-9 is well known to cause the deterioration of tight junctions and basement membranes.[32–34] To investigate activation of MMP-9 in the vasculature in the poststroke cortex, brain sections were costained with anti-PECAM-1 and anti-MMP-9 antibodies. Although no MMP-9-positive vascular structures were observed in the contralateral cortex (Figure 5A–C), MMP-9-positive vasculature was observed in the poststroke cortex in control mice (Figure 5D–F). In contrast, no MMP-9-positive vasculature was observed in mice pretreated with cilostazol (Figure 5G–I). In contrast, pretreatment with aspirin did not prevent activation of MMP-9 in the poststroke cortex (Figure 5J–L). To confirm these results, protein samples were extracted from each brain and MMP-9 activity was investigated by zymography. Consistent with results obtained by immunohistologic analysis, suppressed expression of MMP-9 activity was observed with pretreatment with cilostazol compared with pretreatment with aspirin (Figure 5M–N).

Discussion

In this study, we have demonstrated that treatment with cilostazol for 7 days before induction of cerebral ischemia significantly reduced the hemorrhagic risk accompanying tPA injection and was associated with suppressed MMP-9 activity in stroke vasculature (and its endothelium).

Thrombolysis with tPA after stroke is associated with an increased risk of hemorrhagic transformation.[33,35] In addition to endothelial cell injury caused by reperfusion after transient ischemia, tPA is known to induce disruption of the blood–brain barrier.[23,36,37] Consistent with these reports, administration of tPA after 90, 120, or 180 minutes of transient ischemia significantly increased the risk of cerebral hemorrhage, compared with PBS-injected mice, in our experimental model. Because of the homogeneity of cerebral vascular structure/organization between animals in CB-17 mice, the ischemia induced in this strain by transient occlusion of the MCA under direct visualization produced a highly reproducible ischemic area.[38] Although thrombolytic effects of tPA cannot be addressed in this model, these findings indicate the model in CB-17 mice is suitable to evaluate the effect of drugs on hemorrhagic transformation caused by tPA injection with high reproducibility.

Intracerebral hemorrhage is associated with worse clinical outcomes in the context of stroke.[39,40] Prior use of antiplatelet drugs remains a concern in terms of increasing the risk of hemorrhage after tPA treatment.[8,9] However, patients who received aspirin for prevention of stroke showed better

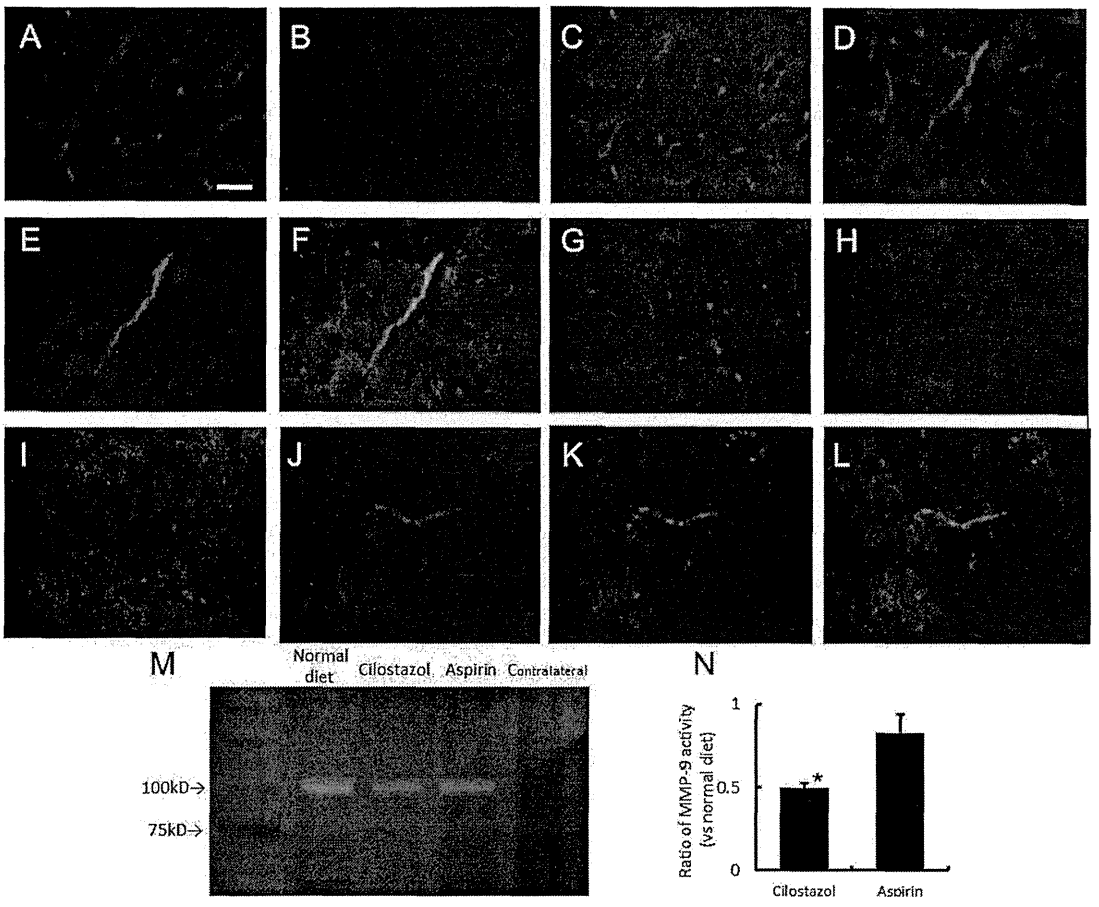

Figure 5. Pretreatment with cilostazol suppressed activation of MMP-9 in vasculature in the poststroke cortex. **A–L**, Representative micrographs of the contralateral (**A–C**) and ipsilateral cortex (**D–L**) at 24 hours after ischemia with tPA treatment (PECAM-1 [**A, D, G**, red]; MMP-9 [**B, E, K**, green]; and merged image [**C, F, L**, yellow]). Although no expression of MMP-9 was observed in the microvasculature of the contralateral cortex (**A–C**), expression of MMP-9 was observed in the ipsilateral cortex in mice pretreated with a normal diet (**D–F**). In contrast, reduced expression of MMP-9 was observed in the stroke-affected cortex in mice pretreated with cilostazol (**G–I**). Pretreatment with aspirin did not change the expression of MMP-9 in the microvasculature in the stroke-affected cortex compared with the normal diet (**J–L**). **M–N**, Representative photograph of zymogram. Suppressed activity of MMP-9 (105 kDa) was observed with pretreatment with cilostazol, although no change was observed with aspirin (**M**). Reverse images were obtained and the ratio of activity between cilostazol or aspirin vs the normal diet was quantified. Significant reduction of MMP-9 activity was observed in mice pretreated with cilostazol compared with mice pretreated with aspirin (**N**). *$P<0.05$ vs aspirin. N=3, in each group. Scale bar, 80 μm (**A**). MMP indicates matrix metalloproteinase; tPA, tissue-type plasminogen activator; PECAM-1, platelet endothelial cell adhesion molecule 1.

clinical outcomes after treatment with tPA, although some studies reported increased risk of cerebral hemorrhage in patients with aspirin compared with patients who did not receive it.[5,9,41,42] This discrepancy can be attributed to reocclusion of the artery after initial successful recanalization by tPA,[10,11] which can be suppressed by antiplatelet drugs, thereby improving outcome.[14] Cilostazol is an antiplatelet drug with additional effects, including improvement in function of vascular endothelium.[43] It is known to be superior to aspirin in terms of reduction of the risk of cerebral hemorrhage.[16] Consistent with these previous reports, pretreatment with cilostazol for 7 days before ischemia and subsequent tPA administration significantly suppressed the occurrence/extent of cerebral hemorrhage. In contrast, pretreatment with aspirin had no effect on the risk of bleeding compared with nontreated control mice. These findings suggest that patients treated with cilostazol for prevention of ischemic diseases would have a lower risk of hemorrhagic transformation after thrombolytic therapy compared with nontreated or aspirin-treated patients. Cilostazol-treated patients might be expected to have a reduced risk of reoccluding the recanalized cerebral artery compared with nontreated patients.

To extend the therapeutic time window for effective thrombolytic therapy, the risk of cerebral hemorrhage must be evaluated in individual cases. Our current study demonstrates that the risk of cerebral hemorrhage can be significantly modified by treatments administered before the onset of stroke. However, the effects of other commonly used drugs for patients with a high risk of stroke such as calcium channel blockers, angiotensin receptor blockers, and statins are still controversial.[44,45] We believe that analysis of the effects of multiple drugs on tPA-induced cerebral hemorrhage in animal models is essential for extending safe and effective thrombolytic therapy to a wider group of patients, especially for those beyond the current 3-hour window for treatment.

Activation of MMP-9 in injured endothelial cells has been suggested as a mechanism for tPA-induced cerebral hemorrhage[23,37,46] in addition to direct injury due to ischemia-reperfusion. MMP-9 activation enhances the permeability and decreases structural integrity of the blood–brain barrier in postischemic brain.[32,47] Our studies have shown that pretreatment with cilostazol markedly reduced the expression of MMP-9 in endothelial cells after injection of tPA and suppressed degradation of cerebral vasculature in the ischemic brain. Our findings are consistent with a previous study demonstrating that cilostazol decreased MMP-9 expression in balloon-injured vasculature.[48] Cilostazol is known to raise the intracellular cAMP concentration in endothelial cells. In this context, cAMP promotes functional integrity of tight junctions between endothelial cells in the blood–brain barrier.[49,50] The vasculoprotective effect of cilostazol was also shown in other studies in which cilostazol suppressed endothelial hyperpermeability by inhibiting redistribution of the actin-based cytoskeleton[51] and protected endothelial cells against lipopolysaccharide-induced apoptosis by the activation of MAP kinase.[52] These findings indicate that the beneficial effect of cilostazol on cerebral hemorrhage might be achieved, at least in part, through suppression of endothelial injury after thrombolysis with tPA injection. Consistent with these findings, cilostazol-treated mice displayed retention of vascular density in ischemic cerebral cortex after tPA treatment, whereas aspirin did not prevent reduction in the number of cerebral microvessels. In the current study, we used 1 dose of cilostazol. Because both antiplatelet and vasculoprotective activity of cilostazol are known to be dose-dependent,[52–54] further study will be necessary to determine the optimal dose of cilostazol to suppress cerebral hemorrhage after tPA treatment.

In conclusion, our results suggest that treatment of patients with cilostazol for prevention of stroke may have significant merit with regard to suppressing the risk of hemorrhagic transformation after thrombolytic therapy as well as reducing the risk of cerebral hemorrhage[15] compared with treatment with aspirin. Furthermore, our data suggest that the therapeutic time window of thrombolytic therapy using tPA might be extended in patients treated with cilostazol. Furthermore, antithrombotic treatment might be safely started with cilostazol soon after injection of tPA to reduce the incidence of reocclusion of the artery after initial successful recanalization.

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Disclosures

None.

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Abstract

シロスタゾールはマウス脳卒中モデルにおける組織プラスミノゲン活性化因子投与後の出血性梗塞リスクを低減する

Cilostazol Reduces the Risk of Hemorrhagic Infarction After Administration of Tissue-Type Plasminogen Activator in a Murine Stroke Model

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背景および目的：血栓溶解療法を行う患者に対し事前に抗血小板薬を投与すると，動脈再閉塞が減少し，患者の脳卒中転帰が改善するが，その一方で脳出血リスクが上昇する可能性がある。

方法：シロスタゾールは，細胞内cAMPのアップレギュレーションによって内皮機能を改善する抗血小板薬である。本研究では，再現性の高い一過性虚血モデルを用いて，血栓溶解療法後の脳出血に対するシロスタゾールの効果を検討した。

結果：虚血前7日間のシロスタゾール投与により，組織プラスミノゲン活性化因子注入後の脳出血リスクおよび重症度が有意に抑制された。一方，アスピリンを投与した場合には，非投与マウスに比べてこうした保護作用は認められなかった。免疫組織学的分析では，シロスタゾール投与によって，マトリックスメタロプロテアーゼ-9の活性低下に伴う虚血領域の微小血管系の破綻が抑制されることが示された。

結論：本研究結果が示唆するように，脳卒中発症前にシロスタゾールを投与した患者は，血栓溶解療法後の脳出血リスクが低くなり，血栓溶解の治療適応時間も延長する可能性がある。さらに，脳卒中前の治療によって脳出血リスクは大きく変化すると考えられる。より幅広い患者群に安全かつ有効な血栓溶解療法を行うには，動物モデルを用い，組織プラスミノゲン活性化因子誘発性の脳出血に対する各種薬剤の効果を分析することが不可欠である。

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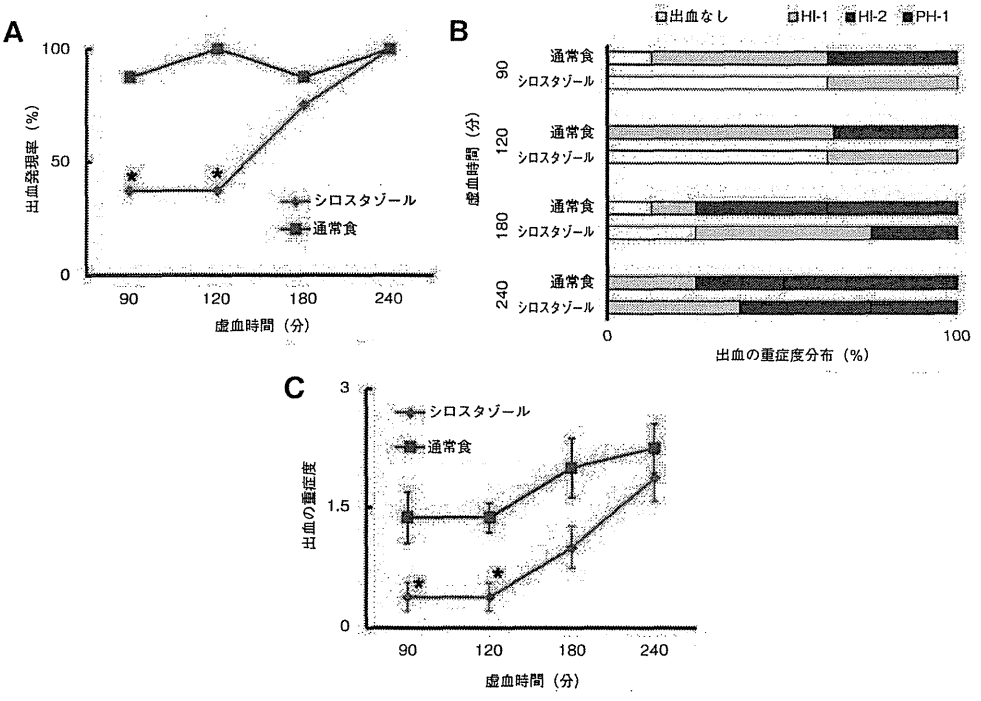

図2 シロスタゾール投与により，t-PA投与後の脳出血リスクが低下した。A：通常食を与えたマウスに比べ，虚血作製前7日間にわたってシロスタゾール含有食を与えたマウスでは，90分間および120分間の虚血後のt-PA注入に伴う脳出血リスクが有意に低下した。B-C：各出血の重症度分布をBに示す。定量的分析の結果，通常食を与えたマウスに比べ，シロスタゾール含有食を与えたマウスでは，90分間および120分間の虚血後のt-PA注入に伴う脳出血の重症度が低下したことが示された（C）。*$p < 0.05$，通常食対照群との比較（A，C）。各群8匹。t-PA：組織プラスミノゲン活性化因子，HI-1：出血性梗塞1型，HI-2：出血性梗塞2型，PH-1：脳実質内出血1型。

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Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats

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Immature rat

ABSTRACT

Progesterone and its metabolite, allopregnanolone, are neurosteroids that are present at high concentrations in fetal brains that decrease right after birth. Allopregnanolone is a potent positive modulator of γ-aminobutyric acid A ($GABA_A$) receptor function. We examined the effect of exogenous administration of these steroids on hypoxic–ischemic encephalopathy in immature rats. Progesterone (10 mg/kg), allopregnanolone (10 mg/kg), or vehicle alone was intraperitoneally administered immediately before and then subcutaneously 6 h and 24 h after hypoxia–ischemia to postnatal day 7 (P7), day 14 (P14), and day 21 (P21) rats. The effects of the treatments were evaluated using histological analyses (hemispheric volumes and semi-quantitative scoring for neuropathologic injury). Both progesterone and allopregnanolone significantly exacerbated brain injury in P7 and P14 rats, but not in P21 rats. This detrimental effect was similar across the examined brain regions (the cortex, striatum, hippocampus, and thalamus) and showed no sex differences. Co-administration of the $GABA_A$ receptor antagonist, bicuculline, partially mitigated the exacerbating effect of allopregnanolone. Based on the similarity of the effects of these neurosteroids, we speculate that progesterone accentuates neuronal injury mainly via the activity of allopregnanolone. The present study indicates that the detrimental effects of allopregnanolone were, at least in part, mediated via GABAergic neuroexcitability. This is in line with the notion that GABA is excitatory for immature neurons, while it is inhibitory for mature neurons.

Introduction

Fetuses physiologically experience hypoxic conditions because they have a relatively low oxyhemoglobin saturation (65%) in their cerebral circulation (du Plessis, 2009). Hence, we assumed that fetuses might have innate mechanisms for coping with hypoxia and possibly protect themselves from hypoxia–ischemia (HI) better than children and adults. We hypothesized that certain compounds present at higher concentrations in the brain during the fetal period compared with other periods of life might have neuroprotective properties against hypoxia. Neonatal HI encephalopathy is caused by respiratory and/or circulatory insufficiency, and many survivors have long-term cognitive dysfunctions, as well as cerebral palsy (Lindstrom et al., 2006).

Progesterone (PROG) and its metabolite, allopregnanolone (ALLO, 3α-hydroxy-5α-pregnan-20-one, 3α,5α-tetrahydroprogesterone), are neuroactive steroid hormones that are also known as neurosteroids because they are synthesized *de novo* in the nervous system (Belelli and Lambert, 2005). PROG and ALLO are present at high concentrations in the brains of fetal rats and sheep (Grobin et al., 2003, Nguyen et al., 2003). These two neurosteroids are both supplied from the maternal circulation and synthesized in the fetal brain. Serum PROG and ALLO levels in pregnant women continue to increase during pregnancy, with the highest levels at term, i.e., 10 to 100 times higher than during preconception (Luisi et al., 2000). The levels of ALLO in umbilical cord blood are almost the same as those in maternal blood (Hill et al., 2000), and these steroids easily penetrate the brain (Wang et al., 2010). The PROG and ALLO concentrations in the fetal brain decrease right after birth, mainly due to the loss of the maternal blood supply (Grobin et al., 2003, Nguyen et al., 2003). Given that the fetal brain is exposed to high levels of PROG and ALLO, we hypothesized that these neurosteroids might have some neuroprotective properties against hypoxia and that an exogenous supply of these steroids might alleviate HI-induced brain injury in immature subjects. Erythropoietin, for example, which is prominent in the fetal brain, has shown to be neuroprotective in rodents with HI injury when administered exogenously after birth, and is

Abbreviations: HI, hypoxic–ischemic, hypoxia–ischemia; PROG, Progesterone; ALLO, allopregnanolone; P, postnatal day; $GABA_A$, γ-aminobutyric acid A; ANOVA, analysis of variance.

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currently being tested for infants with HIE and for extremely-low birth weight infants (Juul, 2000; McPherson and Juul, 2010).

Despite the appeal of the hypothesis that compounds present at high concentrations in the fetal brain could have neuroprotective properties, there is a critical concern regarding the use of PROG and ALLO in immature animals and humans. ALLO acts as a potent positive modulator of γ-aminobutyric acid A ($GABA_A$) receptors (Belelli and Lambert, 2005). GABA depolarizes immature neurons and is excitatory, while it hyperpolarizes mature neurons and is inhibitory (Ben-Ari et al., 2007). Therefore, PROG and ALLO treatment could potentially exacerbate neonatal HI encephalopathy through a neuroexcitatory mechanism involving $GABA_A$ receptors.

To our knowledge, no study has examined the effect of an exogenous supply of PROG or ALLO on immature animals with brain injury. The purpose of this study was to examine the effects of PROG and ALLO on immature rats with HI-induced brain injury.

Materials and methods

Hypoxia–ischemia

Seven-day-old (P7; experimental paradigm), 14-day-old (P14), and 21-day-old (P21) Wistar rat (Japan SLC, Hamamatsu, Japan) pups were prepared for surgery. All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center. Rats were subjected to a modified Rice–Vannucci procedure to produce HI injury. The Rice–Vannucci model combines permanent ligation of the unilateral carotid artery with exposure to hypoxia for several hours in 7-day-old rat pups and has been widely used for numerous studies on the pathogenesis of HI injury (Rice et al., 1981; Johnston et al., 2005). The brain of newborn rats cannot be damaged by either anoxia alone or unilateral carotid artery ligation alone (Rice et al., 1981). Briefly, under isoflurane anesthesia (4.0% for induction and 1.5 to 2.0% for maintenance), the left carotid artery was permanently occluded. After a 1–2 h recovery period, the P7, P14, and P21 rats were subjected to hypoxia (8% oxygen and 92% nitrogen, at 33.0 °C) for 120, 80, and 50 min, respectively. The duration of the hypoxic exposure was optimized to obtain a similar degree of brain injury in each group as assessed by hemispheric volume and neuropathological scores. After 1 h recovery in a temperature-controlled incubator, rats were returned to the dams until sacrifice.

Drug administration

PROG (Sigma-Aldrich, St. Louis, MO) and ALLO (Calbiochem/EMD Biosciences, San Diego, CA) were dissolved in 22.5% (2-hydroxypropyl)-β-cyclodextrin. Bicuculline (Sigma-Aldrich, St. Louis, MO) was dissolved in hydrochloric acid and then titrated to pH 5.2 by adding sodium hydroxide and phosphate-buffered saline (PBS). A total of five different experimental groups were used: regular dose paradigm in P7, P14, and P21 rats, low dose paradigm in P7 rats, and bicuculline paradigm in P7 rats. Ten to fourteen littermates, both males and females, were randomly assigned to one of three or four different treatment groups. As sex differences were designed to be assessed in Experiment 1 (P7), double the number of littermates was assigned to each treatment group, so that each sex group consisted of approximately 10 pups. As four different treatment groups were assessed in Experiment 3 (P7), 14–20 pups were used.

Experiment 1 (P7): To produce physiological prenatal levels of the two steroids in P7 rats (the level of brain maturation in P7 rats is generally considered comparable to that of P0 human neonates (Dobbing and Sands, 1979), although other authors have suggested that P12–13 rats fulfill this criterion (Romijn et al., 1991) (Clancy et al., 2007)), PROG and ALLO were each administered at a dose of 10 mg/kg body weight (5 mg/ml) immediately before the start of the hypoxic exposure. To simulate the clinical situation of treating newborn babies in the P7 rats, the steroids were administered 6 and 24 h after the start of the hypoxic exposure. The first injections (immediately before hypoxia) were given intraperitoneally to ensure rapid absorption, and the subsequent injections were given subcutaneously for more gradual absorption. The vehicle was administered in the same manner. This protocol is based on the one reported for neuroprotective effects in adult rats with stroke (Jiang et al., 1996, Sayeed et al., 2006), with minor modifications.

Experiment 2 (P7): In this protocol, ALLO was administered at a dose of either 3 mg/kg or 1 mg/kg. Other than the dosage, the protocol was same as that used in experiment 1. The vehicle was also administered in the same manner.

Experiment 3 (P7): Littermates were randomly assigned to one of four groups: vehicle (PBS) + vehicle (β-cyclodextrin), bicuculline + vehicle (β-cyclodextrin), vehicle (PBS) + ALLO, or bicuculline + ALLO. ALLO and the vehicle (β-cyclodextrin) were both administered in the same manner as that described in experiment 1. The $GABA_A$ receptor antagonist, bicuculline (2 mg/kg), and its vehicle (PBS adjusted to pH 5.2) were each administered intraperitoneally just before and subcutaneously 6 h after each ALLO injection, for a total of 5 injections (Fig. 1A). This protocol is based on one used previously to study the effects of $GABA_A$ blockade in immature rats (Galanopoulou, 2008).

Experiments 4 (P14) and 5 (P21): The same protocol used in experiment 1 was used for P14 and P21 rats.

Quantitative histological analysis

Seven days after the HI insult, the rats were deeply anesthetized with an overdose of pentobarbital and perfused with saline followed by 4% formaldehyde via the left ventricle. After perfusion, the brains were removed and sectioned coronally into 2-mm slices using a rat brain slicer (Neuroscience Inc., Tokyo, Japan). The area (mm^2) of the contralateral and ipsilateral hemispheres in each brain section was measured using NIH Image software (ImageJ, 1.43r). The hemispheric volume of each brain was estimated by summing the hemispheric area of the brain slices and multiplying by the section interval thickness. The injury was evaluated in hematoxylin-eosin-stained sections from four brain regions (cortex, striatum, hippocampus, and thalamus). The system we previously developed for evaluating neuropathologic injury (Tsuji et al., 2004) was used in the present study. Neuropathologic injury in the cerebral cortex was scored from 0 to 4 (0: no injury, 4: extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored from 0 to 6. The total score (0–22) was the sum of these ratings. Both hemispheric volume measurement and neuropathological scoring were assessed blindly.

Statistics

The effects of the neurosteroid treatment on the cerebral hemispheric volumes were assessed using a two-way analysis of variance (ANOVA) followed by Bonferroni's test. The injury scores were not distributed normally, so differences in injury scores were assessed using a Kruskal–Wallis test, followed by Dunn's multiple comparison. Sex differences in the injury scores were assessed using Mann–Whitney *U* test with Bonferroni's correction for multiple comparisons. The death rate of the animals was analyzed using Fisher's exact test with Bonferroni's correction for multiple comparisons. The differences in body weight and in rectal temperature were analyzed using a one-way ANOVA, followed by Bonferroni's test. Differences were considered significant at $P<0.05$. The results are presented as the mean ± standard error of the mean (SEM).

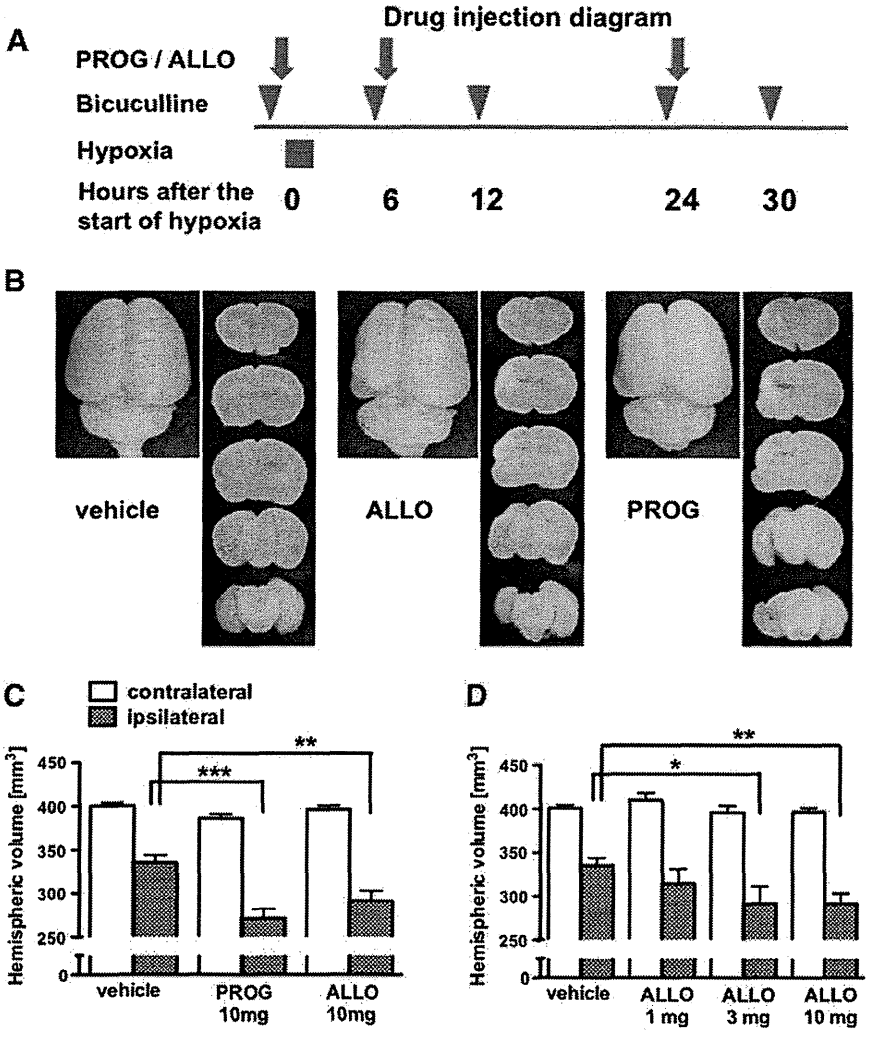

Fig. 1. [Exps. 1–3] Diagram of drug injections (A). Data from three experiments (Exps. 1–3) were pooled and analyzed together. Effects of progesterone (PROG; 10 mg/kg) and allopregnanolone (ALLO; 10 mg/kg) administration in postnatal day 7 (P7) rats. Representative photographs of rat brains at 7 days after hypoxia–ischemia (HI) (B). PROG and ALLO reduced the ipsilateral hemispheric volume (C). A lower dose of ALLO (1 mg/kg) did not reduce the hemispheric volume (D). ** $P<0.01$, *** $P<0.001$. (vehicle $n=42$; PROG $n=22$; ALLO, 10 mg $n=28$; ALLO, 3 mg $n=10$; ALLO, 1 mg $n=10$).

Results

PROG and ALLO exacerbate brain injury in P7 rats

First, the effect of PROG (10 mg/kg×3) and ALLO (10 mg/kg×3) administration on P7 rats with HI-induced brain injury was examined [Exp. 1]. With respect to hemispheric volumes, two-way ANOVA revealed a hemispheric difference and a treatment group difference. Ipsilateral hemispheric volume was significantly reduced in the PROG-treated group (271 ± 11 mm^3) and the ALLO-treated group (293 ± 15 mm^3) compared with the vehicle-treated group (345 ± 14 mm^3) (Fig. 1B). Second, the effect of the dose of ALLO administration on P7 rats with HI-induced brain injury was examined [Exp. 2]. Two-way ANOVA did not reveal a dose difference. Because the vehicle-control groups in three experiments with P7 rats [Exps. 1–3] did not differ from each other (two-way ANOVA) with respect to hemispheric volumes, these data were pooled together into a single control group. The exacerbating effects of PROG and ALLO were the same as the original analysis (Fig. 1C), and the effect of the dose of ALLO became significant. The administration of 10 mg/kg×3 ALLO or 3 mg/kg×3 ALLO, but not 1 mg/kg×3 ALLO, significantly reduced the ipsilateral hemispheric volume compared with that of the vehicle-treated group (Fig. 1D).

Based on the neuropathological scores, PROG (10 mg/kg×3) significantly exacerbated injury in all four regions examined, the cortex, striatum, hippocampus, and thalamus (Fig. 2A). Although ALLO (10 mg/kg×3) increased the injury scores, its effect was not statistically significant in any of the four regions. Given that the mortality rate was significantly higher in the ALLO-treated, but not the PROG-treated group, than in the vehicle group (Table 1), ALLO may be detrimental in HI-induced brain injury. The number of pups that died or were severely injured with a neuropathological score greater than 10 was significantly higher in both the PROG- and ALLO-treated groups compared with that in the vehicle-treated group ($P<0.05$, Fisher's exact test with Bonferroni's correction) (Fig. 2B).

There were no sex differences in the effects of PROG or ALLO on either evaluation of brain damage, i.e., hemispheric volume (data not shown) or the neuropathological injury score (Fig. 2C).

$GABA_A$ receptor antagonism abolishes the exacerbating effect

To better understand the mechanism behind the exacerbation caused by ALLO, bicuculline, a $GABA_A$ receptor antagonist, was co-administered with ALLO to the rats [Exp. 3]. After pooling data from the three P7 experiments [Exps. 1–3], two-way ANOVA revealed a

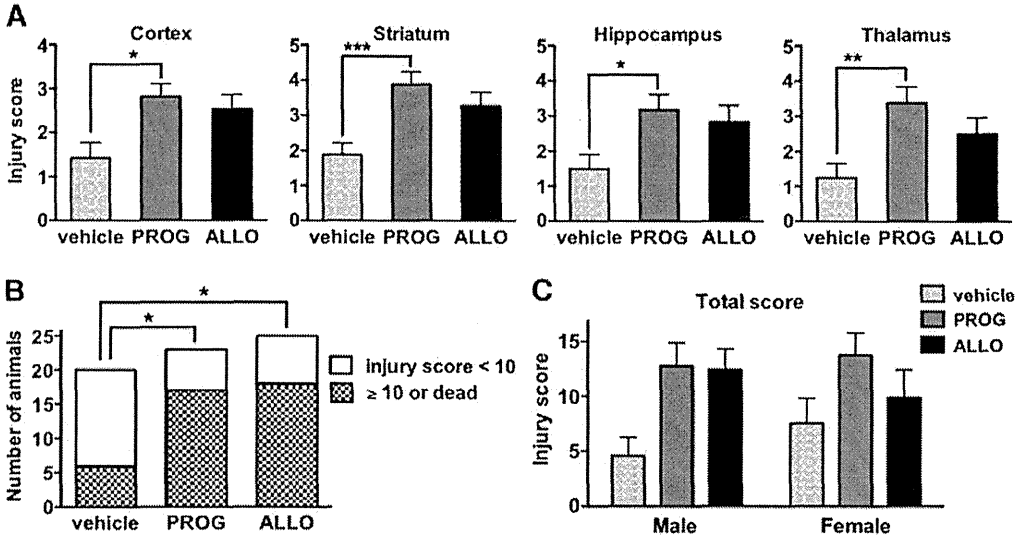

Fig. 2. [Exp. 1] Effects of PROG (10 mg/kg) and ALLO (10 mg/kg) administration in P7 rats. The neuropathological injury score 7 days after HI in each region (A). PROG administration exacerbated HI-induced brain injury in all four regions examined. The number of pups that died or were severely injured (i.e., an injury score greater than 10) was significantly higher in both the PROG and the ALLO groups when compared with the vehicle group (B). There was no sex difference in the effects of PROG or ALLO on total injury score (C). * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (vehicle $n=20$; PROG $n=22$; ALLO $n=18$).

treatment difference in hemispheric volumes. Post hoc tests showed statistical significance only for the comparison of ipsilateral hemispheric volumes between the vehicle- and the ALLO-treated groups and between the bicuculline- and the ALLO-treated groups (Fig. 3). Ipsilateral hemispheric volumes in the bicuculline + ALLO-treated group (312 ± 13 mm^3) were not significantly different from those of the vehicle-treated group. In other words, ALLO treatment exacerbated ipsilateral hemispheric volume loss from 16.3% in control to 26.6%, and bicuculline co-administration partially mitigated it to 18.6%. Ipsilateral hemispheric volume loss was calculated as follow: (1 − (ipsilateral volume/contralateral volume)) × 100%. The lack of any statistically significant difference between the ALLO- and the bicuculline + ALLO-treated groups may result from the relatively higher mortality rate in the ALLO-treated group (11/42) compared with that in the bicuculline + ALLO-treated group (1/20) ($P=0.08$). Detrimental effects of ALLO may be underestimated, as only the surviving pups were analyzed. Likewise, the mitigating effects of bicuculline may be underestimated in comparison with ALLO. The number of pups that died or were severely injured, with a neuropathological score greater than 10, was significantly lower in the bicuculline + ALLO-treated groups (8/20) compared with that in the ALLO-treated group (27/39); because a few P7 pups were killed by their stressed dams, only the number of pups that died during the hypoxia and the 1 h recovery period was analyzed.

PROG and ALLO are less detrimental in P14 and P21 rats

The effects of PROG (10 mg/kg × 3) and ALLO (10 mg/kg × 3) on HI-induced brain injury in more mature rats, i.e., those at P14 [Exp. 4] and P21 [Exp. 5], were examined. In rats subjected to HI-induced injury at P14, ipsilateral hemispheric volume was significantly reduced in the PROG- (346 ± 8 mm^3) and ALLO-treated (348 ± 10 mm^3) groups

Table 1
Mortality rates and the numbers of rats analyzed. The number of rats that died/the number of rats subjected to hypoxia are indicated in the upper rows. The mortality rate differed significantly between the allopregnanolone (ALLO; 10 mg/kg)-treated group and other two groups in experiment 1 [Exp. 1] using postnatal day 7 (P7) rats. Two males and five females out of 28 pups died in ALLO-10 mg group in Exp.1. Because a few P7 pups were killed by their stressed dams, only the number of pups that died during the hypoxia and the 1 h recovery period was analyzed. The number of males:females analyzed are indicated in the lower rows.

	Exp. 1		Exp. 2		Exp. 3		Exp. 4	Exp. 5
	P7		P7		P7		P14	P21
Vehicle	0/22	Vehicle	0/9	Vehicle +	1/15	Vehicle	1/14	0/12
	10:10		7:2	vehicle	7:6		6:7	6:6
PROG	1/25	ALLO	0/10	Bicuc +	0/19	PROG	1/12	3/14
10 mg	11:11	3 mg	5:5	vehicle	10:9	10 mg	6:5	5.6
ALLO	7/28*	ALLO	0/10	Vehicle +	4/14	ALLO	1/13	3/13
10 mg	9:9	1 mg	5:5	ALLO	5:5	10 mg	5:7	6:4
				Bicuc +	1/20			
				ALLO				
					10:9			

PROG: progesterone, Bicuc: bicuculline.
* $P<0.05$.

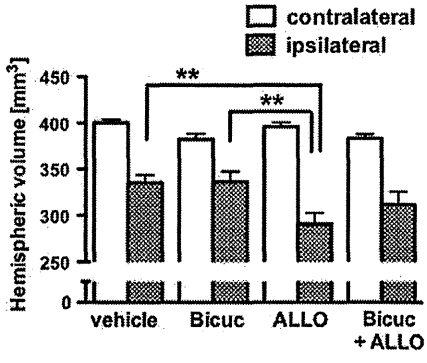

Fig. 3. [Exps. 1–3] Effects of the co-administration of a $GABA_A$ receptor antagonist, bicuculline (Bicuc; 2 mg/kg), with ALLO in P7 rats. Data from three experiments (Exps. 1–3) were pooled and analyzed together. Co-administration of the $GABA_A$ receptor antagonist partially mitigated the effect of ALLO. Two-way analysis of variance and post hoc tests showed statistical significance only for the comparison of ipsilateral hemispheric volumes between the vehicle- and the ALLO-treated groups and between the bicuculline- and the ALLO-treated groups. The ipsilateral hemispheric volumes in the bicuculline + ALLO-treated group were not significantly different from those of the vehicle-treated group. ** $P<0.01$. (vehicle $n=42$; Bicuc $n=19$; ALLO $n=28$; Bicuc + ALLO $n=19$).

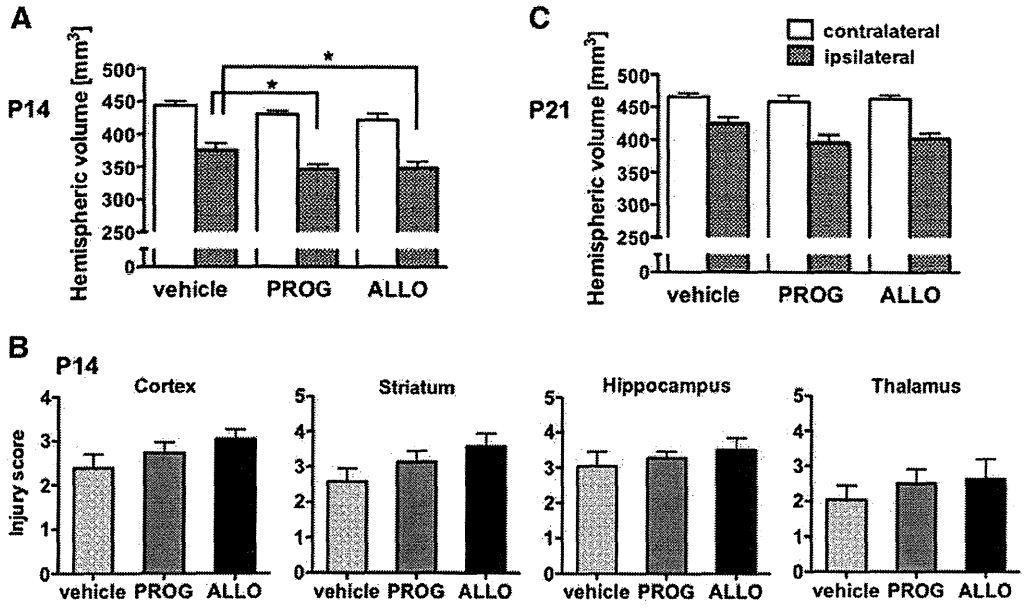

Fig. 4. [Exps. 4, 5] Effect of PROG (10 mg/kg) and ALLO (10 mg/kg) administration in more mature rats. PROG and ALLO reduced the ipsilateral hemispheric volume in P14 rats (A) but did not significantly alter the neuropathological injury score in any regions examined (B). * $P<0.05$. (vehicle $n=13$; PROG $n=11$; ALLO $n=12$). PROG and ALLO did not significantly alter the hemispheric volume in P21 rats (C). (vehicle $n=12$; PROG $n=11$; ALLO $n=10$).

compared with that of the vehicle-treated group (375 ± 10 mm^3) (Fig. 4A). There was no difference in the neuropathological scores among the three groups in any of the four regions examined (Fig. 4B).

In rats subjected to HI-induced injury at P21, two-way ANOVA showed no difference in hemispheric volumes between treatment groups ($P=0.109$) (Fig. 4C).

Body temperature and weight

Rectal temperatures did not differ between the groups at any time point before or up to 7 h after HI in any age group (data not shown).

Body weights on the day of HI did not differ significantly between the treatment groups for each age group. The weight gain until the day of perfusion (7 days after HI) was significantly smaller in the P7 PROG-treated group than in the other P7 groups (table 2). The weight gain did not differ among treatment groups in the P14 or P21 age groups.

Discussion

Contrary to our initial hypothesis, our results clearly showed that PROG and its metabolite, ALLO, can worsen HI-induced brain injury in immature rats. To our knowledge, no study in brain injury models has shown a consistent detrimental effect of either PROG or ALLO using either immature or mature animals, with the exception of one study using ovariectomized rats (Murphy et al., 2000). The exacerbating effects in the present study were demonstrated by a reduction of hemispheric volume and determined using the neuropathological evaluation of four brain regions, i.e., the cortex, striatum, hippocampus, and thalamus, in P7 rats. We previously reported that ipsilateral hemispheric areas correspond well with the results of behavioral tests (Mishima et al., 2005). The effects of these neurosteroids were both dose- and age-dependent. In particular, PROG and ALLO were detrimental in P14 rats to a lesser degree than in P7 rats and were not detrimental in P21 rats. A $GABA_A$ receptor antagonist, bicuculline, partially mitigated the effects of ALLO. PROG is also a sex steroid, and sex differences in neonatal HI-induced brain injury have recently been reported on the effects of the treatment (Tsuji et al., 2010), and of the brain injury itself (Hill et al., 2011). However, in the present study, we did not find any statistically significant differences according to gender.

Exacerbating mechanisms: progesterone

The effects of PROG and ALLO were quite similar in the present study. In particular, each neurosteroid demonstrated the same age-dependent exacerbating effects on HI-induced brain injury. Based on this similarity, we speculate that PROG accentuated neuronal injury mainly via the activity of its metabolite, ALLO. PROG exerts its action via both its classical receptor and the activity of its metabolite, ALLO, and it has multiple effects on neurons and the brain (Gibson et al., 2009). We cannot exclude other mechanisms of action that

Table 2
Body weights. The body weight gain (grams) over a 7-day period, from the day of hypoxia–ischemia (HI) to the day of perfusion, was significantly smaller in the PROG (10 mg/kg)-treated group than in the other four groups using postnatal day 7 (P7) rats. Data from three experiments [Exps. 1–3] were pooled and analyzed together. There were no other statistical differences in either the weight before HI or the weight gain until the day of perfusion among the treatment groups for each age group.

	P7 [Exps. 1–3]		P14 [Exp. 4]		P21 [Exp. 5]	
	Before HI	Gain	Before HI	Gain	Before HI	Gain
Vehicle	9.7±0.1	7.7±0.4	18.2±0.4	8.5±0.7	29.8±0.7	25.1±0.5
PROG 10 mg	9.4±0.2	5.5±0.4***	18.3±0.4	7.0±0.5	29.5±0.7	20.9±2.2
ALLO 10 mg	9.3±0.2	6.4±0.4	18.6±0.4	5.7±0.6	29.9±0.9	22.9±0.7
Bicuc	9.6±0.2	6.7±0.4				
Bicuc + ALLO	9.8±0.1	5.9±0.4				

*** $P<0.001$.

may have contributed to the detrimental effects observed in the present experiments. A few studies, however, have reported that PROG has negative effects in injured nervous systems. These negative results are mostly minimal and are inconsistent with those from other studies. Only one previous report demonstrated a detrimental effect of PROG in an animal model of stroke: Murphy et al. (2000) demonstrated that the daily administration of PROG for a week before ischemia exacerbated brain injury in ovariectomized rats. The authors speculated that this detrimental effect was due to modulation of the GABA system by a sharp decline in PROG levels after the pre-stroke treatment (Murphy et al., 2000), and they later reported that PROG administration both before ischemia and during reperfusion decreased brain injury (Murphy et al., 2002). The treatment of ovariectomized rats with PROG exacerbated the cerebrovascular inflammatory response (inducible nitric oxide synthase and cyclooxygenase-2) to lipopolysaccharide (Sunday et al., 2006). PROG suppressed the proliferation of progenitor cells but enhanced the survival of new neurons in adult male rats with ischemia (Zhang et al., 2010). These actions of PROG are unlikely to be the main mechanism responsible for the detrimental effect of PROG in the present study. Studies have shown that PROG is beneficial for transient middle cerebral artery occlusion in adult male rats (Jiang et al., 1996), in spontaneously hypertensive adult male rats (Kumon et al., 2000), in reproductively senescent female rats (Alkayed et al., 2000), for permanent middle cerebral artery occlusion in adult male rats (Sayeed et al., 2007), for four vessel occlusion in adult male rats (Morali et al., 2005), and for traumatic brain injury in adult male rats (VanLandingham et al., 2008). Therefore, PROG may be detrimental only in immature subjects with HI, probably due to the effects of its metabolite, ALLO.

Exacerbating mechanisms: allopregnanolone

We suggest that the detrimental effect of ALLO was, at least in part, mediated via GABAergic neurotransmission because a $GABA_A$ receptor antagonist, bicuculline, partially mitigated this effect. Another result supporting this idea is the age-dependent effect of ALLO, in which it became less detrimental with development. The effects of $GABA_A$ receptor activation are also age-dependent. Neurons have a higher intracellular chloride concentration at an early stage, leading to an efflux of chloride and the excitatory actions of GABA in immature neurons. The progressive reduction of the intracellular Cl^- concentration during early development causes the developmental switch from the excitatory (depolarizing) to inhibitory (hyperpolarizing) action of GABA (Ben-Ari et al., 2007). The timing of this shift depends on the species, sex, brain structure, and neuronal type. It occurs at around P8–12 in the Wistar rat hippocampus (Ben-Ari et al., 2007). Although ALLO became less detrimental with age in the present study, it was not neuroprotective in P21 rats, by which age GABA action reportedly becomes inhibitory, i.e., neuroprotective against excitotoxicity. We speculate that HI-induced injury may alter and delay the developmental switch or that exacerbating mechanisms in addition to the GABA-mediated action may occur in immature animals.

Given that the reversal effect of bicuculline on the ALLO-induced brain injury was not complete, additional mechanisms may be in play to worsen ischemic brain injury in immature animals. ALLO has been understudied in ischemic brain injury, and to our knowledge, no *in vivo* study has shown or suggested that ALLO has negative effects other than those on GABA-mediated neurotransmission in nervous system disease models. There are only three studies examining the effects of the administration of ALLO in stroke, all of which demonstrated beneficial results (Sayeed et al., 2006, 2009; Ishrat et al., 2010). ALLO has been shown to be beneficial in other neurological disease models, such as traumatic brain injury (VanLandingham et al., 2008), Niemann–Pick type C disease (Griffin et al., 2004), and Alzheimer's disease (Wang et al., 2010).

Evidence from *in vitro* studies has suggested that a high concentration of ALLO may be harmful. High concentrations of ALLO (>100 μmol/L) significantly repressed proliferation of neural progenitor cells (Wang et al., 2005). In addition, relatively high concentrations of ALLO (1–3 μmol/L, approximately 320–960 μg/L) induced the regression of neurite outgrowth (Brinton, 1994) and caused the death of rat hippocampal neurons via a $GABA_A$ receptor-dependent mechanism (Xu et al., 2000). Our protocol is not only based on protocols reported to be neuroprotective in adult rats (Sayeed et al., 2006), but was also chosen to reproduce the steroid levels found in the fetal brain in the brains of immature rats. Previous studies demonstrated that the administration of PROG and ALLO (8–10 mg/kg) at doses similar to those used in this study increases the plasma or cortical levels to the ranges observed in rodent fetuses (Jiang et al., 1996; Grobin et al., 2003). Cortical ALLO levels in a rat fetus at late gestation are almost 20 ng/g (Grobin et al., 2003), and a 10-mg/kg dose of ALLO results in a mean cortical level of 22 ng/g in mice (Wang et al., 2010). We do not consider the ALLO levels in our study to be as high as those shown to be harmful in the *in vitro* studies.

GABA is involved in the mechanism of thermoregulation in the preoptic area of the hypothalamus (Osaka, 2004). As neither ALLO nor PROG treatment altered the body temperature, we do not consider that GABA-mediated thermoregulation plays a role in the detrimental effects of these neurosteroids.

Translating the results into practice

PROG and ALLO are not always detrimental to immature animals and human neonates. A single injection of 25 mg/kg of ALLO at P7, P10, or P17 increased survival and delayed neurological impairments in a mouse model of Niemann–Pick type C disease (Griffin et al., 2004). Pilot studies on postnatal estradiol and PROG replacement for extremely preterm infants demonstrated no adverse effects on growth and psychomotor development (Trotter et al., 2001). Because HI increases excitability in neurons, PROG and ALLO may enhance HI-induced hyperexcitability and the associated excitotoxicity in immature brains. Caution should be exercised when interpreting our results and translating them into practice when dealing with neonates and infants. It was previously mentioned that in long-gestation species such as humans and sheep, as opposed to altricial species, such as rodents, GABAergic currents become inhibitory by the last third of gestation (Hirst et al., 2009). In contrast, other authors mentioned that Cl^- transport in the perinatal human cortex is as immature as that seen in the rat, suggesting GABA-mediated excitation (Dzhala et al., 2005).

The fact that the treatments turned out to be detrimental, contrary to our initial hypothesis and to data obtained in adult models, is meaningful. Thus, it is clear that speculations that a certain treatment could be beneficial to a similar but different group of subjects just because the treatment was beneficial to a group of tested subjects must be carefully reexamined. This is especially true when applying a treatment to neonates; at a stage when the brain is going through dramatic developmental changes. Considering the dramatic transitions of the central nervous system from the fetal to neonatal period, even a treatment proven to be neuroprotective in term neonates, such as hypothermia, may be of no benefit or could even be detrimental in preterm babies or infants beyond the neonatal period.

Conclusions

Our study shows that both PROG and its metabolite, ALLO, have adverse effects on hypoxic–ischemic brain injury in immature rats. The results presented herein represent a prime example of the dramatic difference between immature and mature brains. Studies are still required to clarify the uncertainties of the effects of PROG and ALLO in immature subjects. For the future development of

neuroprotectants in neonatal brain injury, the maturity, or immaturity, of the brain should be carefully and fully taken into consideration.

Disclosure/conflict of interest

None.

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● 新評議員

脳梗塞患者に対する再生医療開発

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要　旨

脳梗塞予防に関しては，降圧薬，抗血小板薬，抗凝固薬などの多くの有効な薬剤が存在し，また脳梗塞超急性期における脳組織の壊死の防止に関しても，血栓溶解療法や血管内治療など有効な治療法の開発が進んでいる．しかし，超急性期以降の脳組織壊死が生じた後の脳機能の再生に関しては，現状でもリハビリテーション以外には確立された治療法がなく，新規治療法開発が切望されている．我々は基礎研究において，脳梗塞後に誘導/動員される神経幹細胞の生着・機能には血管再生が必要不可欠であり，造血幹細胞投与で血管再生・神経機能回復が促進することを世界に先駆けて報告し，さらにこれらの知見に基づき，脳梗塞患者に対する自己骨髄単核球細胞移植の臨床試験を実施してきた．本稿ではそれらの概要とともに，今後の研究の方向性についても言及する．

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キーワード：脳梗塞，再生医療，細胞治療，神経再生，血管再生

はじめに

脳梗塞治療を目的に神経幹細胞移植を中心とした様々な基礎研究・臨床試験が行われてきたが，単なる神経幹細胞移植では脳梗塞後の機能回復に対してはほとんど治療効果がないことが示されてきた．一方，脳梗塞後には神経幹細胞が障害部位に誘導/動員されるものの，それらのほとんどは生着できず機能回復にも寄与しないことも知られていた．我々は鳴き鳥(Songbird)において神経再生には血管再生が必須であることに注目し，脳梗塞後の血管再生と神経再生および神経機能再生の関連についての基礎的/臨床的研究を行っている．

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脳梗塞モデルマウスを用いた幹細胞治療効果の検証

我々は，四肢虚血患者に対する造血幹細胞を含む自己骨髄単核球移植の臨床試験を実施し，虚血性疾患患者に対する造血幹細胞移植が血管再生を促進し臓器機能も回復させることを示してきた[1]．しかし脳梗塞後の神経機能再生に関しては治療効果を正確に定量するための実験評価系が存在していなかったため，兵庫医科大学松山教授と共同で，長期生存率がほぼ100％で脳梗塞サイズ・領域の再現性が極めて高くかつ作成が比較的容易な非常に優れた脳梗塞モデルマウスを新たに開発し[2]，その実験系を活用した研究を行ってきた．その結果，①脳梗塞後の造血幹細胞静脈内投与は梗塞周囲における血管再生を促進すること，②脳梗塞後の血管再生は脳梗塞により誘導/動員された神経幹細胞の生着に必須であること，③造血幹細胞投与による脳梗塞後の血管再生は脳神経組織の再生をもたらすこと，④脳梗塞後の脳組織再生は脳機能の再生をもたらすこと，など血管再生と機能再生に関する重要な知見を明らかにした[3,4]．また，⑤造血幹細胞投与の治療時期に関する検討では，脳梗塞後2，4，7，10日およ

び14日後の骨髄単核球投与においては治療効果はあるものの，脳梗塞発症24時間後の急性期や21日後以降の慢性期においては治療効果が弱いことを明らかにし[5]．さらに，⑥造血幹細胞を末梢血中に動員する作用のあるG-CSFの投与では，予想に反し骨髄からの顆粒球動員に伴い脳萎縮や神経機能の低下が引き起こされることを明らかにした[6]．これらの基礎研究の成果は神経幹細胞の誘導/動員など，内因性の組織修復機構が活性化されている時期においては造血幹細胞移植/血管再生による効果が期待できるものの，脳梗塞発生直後や脳梗塞慢性期においては治療効果が低いこと，またG-CSFでは代用不可であることを示していると考えている．

心原性脳塞栓症患者に対する自己骨髄単核球移植の臨床試験

以上の成果を基に国立循環器病研究センター病院および先端医療センター病院において“急性期心原性脳塞栓症患者に対する自己骨髄単核球静脈内投与に関する臨床研究”のPhase1/2a臨床試験を実施してきた．主な適格基準は，①心原性脳塞栓症と診断されている．②年齢が20歳以上75歳以下である．③発症後7日目の時点でNIHSSが10点以上である．④来院時に比し，発症7日後のNIHSS改善度が5点以下である．であり，重症の心原性脳塞栓症症例で，かつ脳梗塞発症1週間後においても神経機能回復が十分でない患者群のみを対象としている．国立循環器病研究センター脳神経内科における過去のデータより，これらの適格基準に合致する患者群は，ほとんど内頸動脈閉塞や中大脳動脈起始部の閉塞による脳梗塞であり，その予後は極めて悪く，また脳梗塞に伴う合併症が高頻度に起こることが判っている．治療プロトコールの概略は，①脳梗塞発症7〜10日目に，局所麻酔下で骨髄液を採取，②低用量群6例は25 mlの骨髄液採取，高用量群6例は50 mlの骨髄液採取，③採取日にセルプロセッシングセンターにて，比重遠心法を用いて単核球分画の分離，④採取日に静脈内に5分間で全量投与，⑤プライマリエンドポイントとして，脳梗塞7日後と比し投与1カ月後におけるNIHSS悪化症例の頻度(安全性)および脳梗塞7日後と細胞投与1カ月後におけるNIHSSの改善度(有効性)を設定，⑥検査可能な症例では細胞治療1カ月後および6カ月後にPETを用いた脳循環代謝測定を行う，である．

臨床試験は既に終了し，安全性および有効性に関しても十分なデータが得られ，論文作成もほぼ終了している．高用量群は低用量群に比し良好な機能回復がみられており，また安全性に関しても特別な問題はないことが明らかになっており，それらの臨床試験結果の詳細は論文上で発表予定である．

脳梗塞患者に対する再生医療開発の課題

脳梗塞後の新規機能再生療法開発における最大の特徴は，“いまだかつて全世界で成功例が1例もない”，という点であり，その原因として下記の問題が指摘されている．

①脳梗塞モデル動物が不適当：脳梗塞患者とは関連のない一過性脳虚血モデルや，再現性がなく長期生存もできない脳梗塞モデルの使用をしたため，治療効果判定が著しく困難であった．

②治療ターゲットが脳梗塞患者病態から乖離：神経細胞をターゲットにした細胞死抑制やapoptosis防止が研究の主流であったが，脳梗塞では血流がなくなると脳組織そのものが壊死に陥るため，神経細胞の生存にのみ焦点を当てた治療法開発は，そもそも実際の病態から乖離している部分が大きいと考えられている．

③臨床試験設計の精度が低い：機能再生療法のための治療有効期間(therapeutic time window)は，急性期から亜急性期が最も有効性が期待できると考えられているが，その時期においては患者の予後予測が困難であり，また治療効果を反映するサロゲートマーカーが見つかっていないため，治療効果を正確かつ高感度に判定する臨床試験設計が困難であった．

我々はこれらの現状を打破するため，①再現性/長期生存率が高い脳梗塞モデル開発を行い，さらに，②微小血管再生の促進や脳虚血領域の炎症制御による神経機能再生促進をターゲットにした治療法開発を行っている．また，③臨床試験設計に関しても，国立循環器病研究センター等と共同でMRIトラクトグラフィーや経頭蓋磁気刺激を用いて解剖学的・電気生理的な損傷/再生の定量的評価法を開発しており，脳梗塞治療法開発に関する問題点を一つずつ解決することにより，新規機能再生療法の確立につなげていくことができると考えている．

脳梗塞患者に対する新規治療法開発には，これからも非常に多くの技術や要素が必要不可欠であると考えておりますので，共同研究のお誘いも是非よろしくお願いいたします．

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Abstract

Cell-based therapy for patients after stroke

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We had demonstrated that intravenous administration of bone marrow derived mononuclear cells or hematopoietic stem cells improves functional recovery through enhanced angiogenesis in experimental stroke model. Based on these observations, we started phase 1/2a clinical trial of cell-based therapy for patients with cardiogenic cerebral embolism (ClinicalTrials.gov ID: NCT01028794). The results of clinical trial indicated that autologous bone marrow cell transplantation at day 7–10 after onset of stroke is feasible and safe in patients with severe stroke, and patients with cell therapy had better neurological outcomes, compared with historical control group. Our results encouraged us next randomized clinical trials to confirm the effect of cell therapy for patients after stroke.

Key words: stroke, regenerative medicine, cell-based therapy, neurogenesis, angiogenesis

脳梗塞患者に対する機能再生治療法の開発

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SUMMARY

脳梗塞後には内因性神経幹細胞が梗塞周辺部に誘導されることが知られているが，著者らはその生着や機能には血管再生が必要不可欠であり，造血幹細胞や骨髄単核球投与で血管再生・神経機能回復が促進することを明らかにし，これらの知見を基に脳梗塞患者に対する自己骨髄単核球細胞移植治療の臨床試験を実施してきた．本稿では脳梗塞患者に対する機能再生治療法の開発に関する著者らの基礎研究および臨床試験の成果，さらにはその未来に関して概説する．

KEY WORDS

再生医療
神経再生
血管再生
細胞治療
脳梗塞

はじめに

現在日本では，急速な高齢化社会を迎えており，それに伴う要介護者の急激な増加はきわめて深刻な社会問題となっている．脳梗塞の予防に関しては降圧薬や抗血小板薬，抗凝固薬などの治療が広く行われ，また脳血管閉塞後超急性期の再開通治療に関しても血栓溶解療法や血管内治療の進歩がみられるものの，脳梗塞による神経細胞死／脳組織壊死が生じた後は，リハビリ以外には治療法は存在せず，そのため脳血管障害はいまだに要介護者および寝たきり者の発生原因の第一位である．脳梗塞後の機能再生に関する治療法開発において，現状では世界でも成功例が一例もないが，その原因として，①不適切なモデル動物，②不適切な治療ターゲット，③不適切な臨床試験プロトコール，があると著者らは考えており，これらの問題点を打破するための研究を行っている．

I. 脳梗塞モデルマウスを用いた骨髄単核球細胞の治療効果

脳梗塞の基礎研究においては，実際の脳梗塞患者病態とは関連の薄い一過性脳虚血再灌流障害モデルや再現性がなく長期生存もできない脳梗塞モデルが頻用されてきた．そこで著者らは，再現性および長期生存率が非常に高い脳梗塞モデル（永久閉塞モデル）を兵庫

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医大松山グループと確立し[1,2]，種々の治療法の効果を検証してきた．その結果，①脳梗塞後の造血幹細胞（あるいは造血幹細胞を含む骨髄単核球細胞）の静脈内投与は脳における血管再生を促進すること，②脳梗塞後の血管再生は内因性の神経再生を誘導し，かつその生着に必須であること，③造血幹細胞投与による脳梗塞後の血管再生は，脳神経組織の再生を誘導すること，④脳梗塞後の血管再生による脳組織再生は脳機能の再生をもたらすことなど，脳障害における血管再生が神経再生・神経機能再生に必要不可欠であることを世界に先駆けて明らかにした[3,4]．また，幹細胞を骨髄から動員する作用のあるG-CSFの投与では，骨髄からの顆粒球動員に伴い逆に脳萎縮促進作用や神経機能の低下がみられることを報告し[5]，さらに，造血幹細胞の最適な投与時期に関する検討では，脳梗塞後2，4，7，10日および14日後の細胞投与において，脳梗塞巣の減少および行動の改善を認めるが脳梗塞後1日および21日後においては有意な改善効果を認めないことを示してきた[6]．これらの結果は内因性の血管再生や神経幹細胞の動員など，組織修復機構が活性化されている時期においては細胞移植による効果が期待できるものの，脳梗塞発生直後や脳梗塞慢性期においては治療効果が低いことを示していると考えられている．

II. 脳梗塞患者に対する自己骨髄幹細胞を用いた細胞治療の臨床試験

以上の知見を基に，先端医療センター病院および国立循環器病研究センター病院において“急性期心原性脳塞栓症患者に対する自己骨髄単核球静脈内投与に関する臨床研究”を「厚生労働省ヒト幹細胞を用いる臨床研究に関する指針」による認可を経て，実施した（ClinicalTrials.gov Identifier: NCT01028794）．

臨床試験のプロトコールの概略を**図1**に示す．主な適格基準は，

①心原性脳塞栓症と診断

②年齢が20歳以上75歳以下である

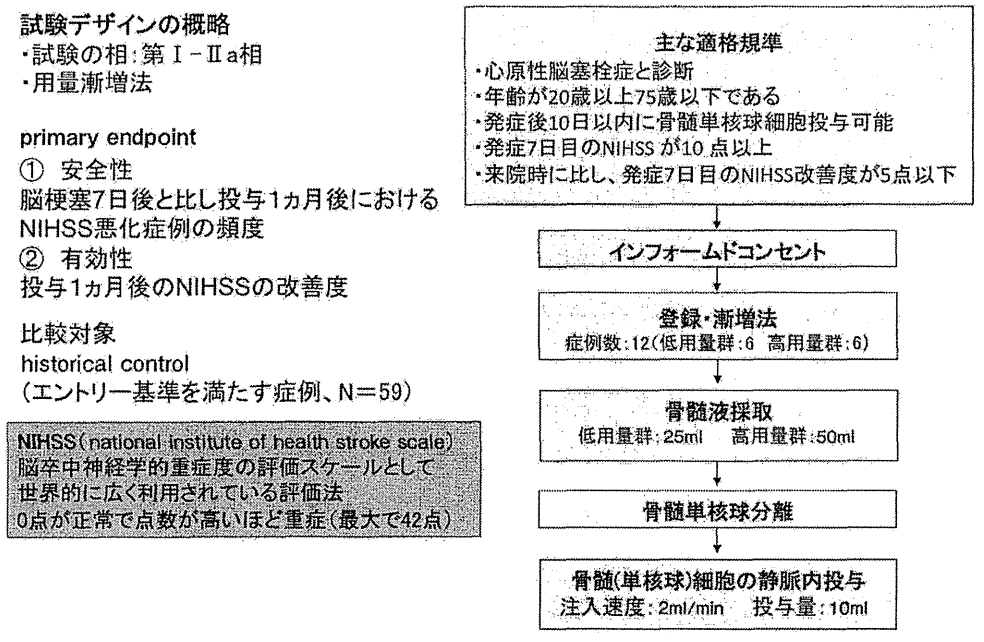

図1　心原性脳梗塞患者に対する細胞治療

臨床試験の概略を示す．予後不良が予想される重症心原性脳梗塞症患者を対象に，発症7～10日後に骨髄細胞を採取し，単核球分離後静脈内投与を行った．

③発症後10日以内に骨髄単核球細胞投与可能
④発症7日目のNIHSSが10点以上
⑤来院時に比し，発症7日目のNIHSS改善度が5点以下
であり，重症の心原性脳塞栓症症例で，かつ脳梗塞1週間後においても神経機能回復が十分でない患者群のみを対象としている．国立循環器病研究センターにおける過去のデータより，これらの適格基準に合致する患者群は，ほとんど内頸動脈閉塞や中大脳動脈起始部の閉塞による脳梗塞であり，その予後はきわめて悪く，また脳梗塞に伴う合併症が高頻度に起こることが知られている．

細胞治療の概略は，
①脳梗塞発症7～10日目に，局所麻酔下で骨髄細胞の採取（低用量群は骨髄25 ml，高用量群は骨髄50 ml）
②国立循環器病研究センターあるいは先端医療センターのセルプロセッシングセンターにて，比重遠心法を用いて単核球分画の分離
③静脈内に5分間で全量投与
と非常にシンプルな手技で構成されており（**図2**），本臨床試験において，その安全性およびある程度以上の有効性を示すことができれば，多くの病院・施設でも実施可能であると考えている．

プライマリエンドポイントは，
①脳梗塞7日後と比し投与1ヵ月後におけるNIHSS悪化症例の頻度（安全性）
②脳梗塞7日後と細胞投与1ヵ月後におけるNIHSSの改善度（有効性）
であり，比較対照群としては国立循環器病研究センター脳卒中内科データベースに含まれる症例で，本臨床試験の適格基準に合致する患者群（合計59症例）をヒストリカルコントロール群として用いた．

現在，予定していた12症例のすべてのfollow upが終了しており，安全性に関しては問題がなく，低用量群と高用量群の比較検討や，治療群とヒストリカルコントロール群との比較検討においても有望な結果が出ており，その詳細に関しては論文で発表予定である．

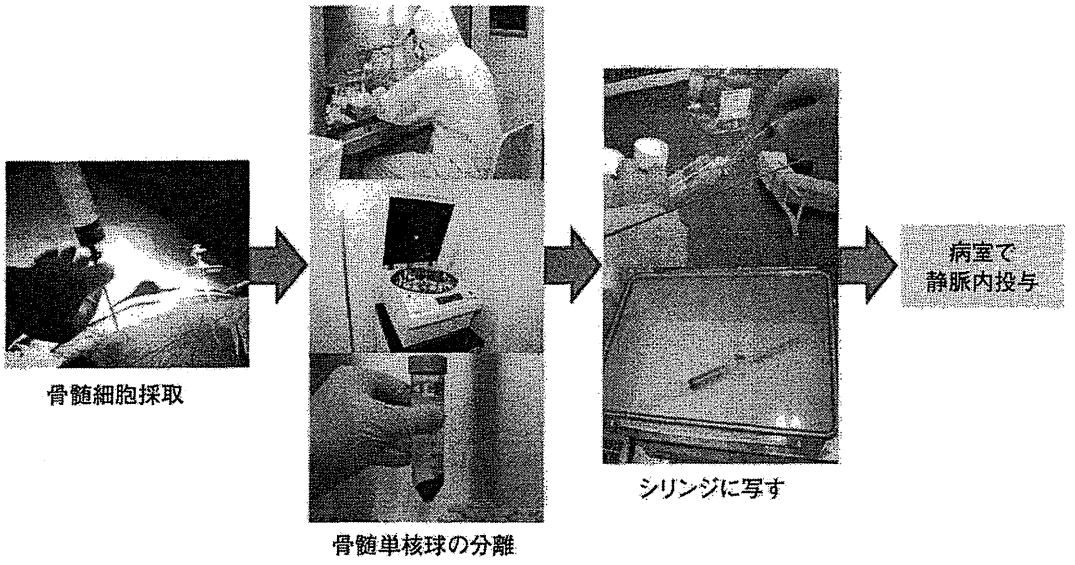

図2　細胞治療の実際
局所麻酔下で骨髄細胞を採取し，セルプロセッシングセンターで比重遠心法を用いて骨髄単核球の分離を行った．精製された骨髄幹細胞をシリンジに移し，病室で静脈内投与を行った．

III. 脳梗塞後の機能回復治療法開発の今後

過去の脳梗塞治療法開発に関する研究は，神経細胞死あるいは神経細胞の apoptosis 防止を中心に研究が進められてきたが，患者病態においては血流が遮断された部位においては神経細胞死だけでなく脳組織全体の壊死が生じるため，神経細胞にのみ照準を合わせた治療法はその効果が非常に限定されていた可能性があると考えている．著者らは脳梗塞後の微小血管再生による神経機能再生促進を中心に研究を進めてきたが，間葉系幹細胞などを用いた炎症制御などによっても神経機能再生が促進される可能性が示唆されており，今後はさまざまな手法を用いた治療法開発が進められると考えられている．

また，臨床試験の設計においても，現状では治療効果の客観的指標が存在しないが，その確立は感度の高い臨床試験の実施には必須である．脳梗塞後の機能回復療法の最適な治療時期は脳梗塞急性期から亜急性期であると著者らは考えており，その時点における解剖学的・電気生理的な損傷／再生の定量的評価法の確立に向け，MRI トラクトグラフィーや経頭蓋磁気刺激法を用いた研究を国立循環器病研究センターや神戸中央市民病院とともに実施している．

おわりに

脳梗塞患者に対する機能再生治療法の開発においては，非常に多くの要素が必要であるが，それらの課題を解決することにより，脳梗塞治療のパラダイムシフトを起こすことができると著者らは考えている．

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