

# Differential Effects of Pentoxifylline on Learning and Memory Impairment Induced by Hypoxic-ischemic Brain Injury in Rats

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**Objective:** Hypoxic-ischemic (HI) brain injury in the human perinatal period often leads to significant long-term neuro-behavioral dysfunction in the cognitive and sensory-motor domains. Using a neonatal HI injury model (unilateral carotid ligation followed by hypoxia) in postnatal day seven rats, the present study investigated the long-term effects of HI and potential behavioral protective effect of pentoxifylline.

**Methods:** Seven-day-old rats underwent right carotid ligation, followed by hypoxia ( $F_iO_2 = 0.08$ ). Rats received pentoxifylline immediately after and again 2 hours after hypoxia (two doses, 60–100 mg/kg/dose), or serum physiologic. Another set of seven-day-old rats was included to sham group exposed to surgical stress but not ligated. These rats were tested for spatial learning and memory on the simple place task in the Morris water maze from postnatal days 77 to 85.

**Results:** HI rats displayed significant tissue loss in the right hippocampus, as well as severe spatial memory deficits. Low-dose treatment with pentoxifylline resulted in significant protection against both HI-induced hippocampus tissue losses and spatial memory impairments. Beneficial effects are, however, negated if pentoxifylline is administered at high dose.

**Conclusion:** These findings indicate that unilateral HI brain injury in a neonatal rodent model is associated with cognitive deficits, and that low dose pentoxifylline treatment is protective against spatial memory impairment.

**KEY WORDS:** Brain hypoxia-ischemia; Memory and Learning Tests; Pentoxifylline; Brain injuries.

## INTRODUCTION

Despite important progress in obstetric and neonatal care, hypoxic-ischemic encephalopathy (HIE) during gestation and perinatal period are common causes of neonatal brain damage which are frequently associated with neuron-developmental disabilities such as cerebral palsy, epilepsy and learning and memory,<sup>1</sup> and other cognitive impairments.<sup>2,3</sup> Unilateral carotid artery ligation on postnatal day 7 has been used as a rodent model of neonatal HIE.<sup>4,5</sup> Pyramidal CA1 neuronal death,<sup>6,7</sup> decrease of dendritic spine density<sup>8</sup> and neural atrophy in the hippocampus,<sup>6,7,9</sup> sensorimotor cortex and striatum<sup>10-12</sup> follow-

ing neonatal HIE have been already reported. These neural changes are frequently associated to behavioral findings, mostly to the hippocampus, a structure essential for spatial navigation, learning, and memory.<sup>13</sup> It is well established that neonatal HIE leads to significant long-term behavioral impairments of spatial memory in the water maze in adult rats,<sup>14-17</sup> in aversive inhibitory avoidance memory,<sup>15,18</sup> in working memory<sup>10,15,17,19</sup> and in sensorimotor tasks.<sup>11,20</sup>

HIE is still a major cause of mortality and morbidity with the incidence of 1 to 2 per 1,000 live births, more common in developing countries.<sup>21</sup> Despite some adverse effects such as sinus bradycardia and thrombocytopenia, mild hypothermia is a standard neuroprotective treatment in newborn infants following peripartum hypoxia-ischemia,<sup>22-28</sup> by preventing apoptosis,<sup>29,30</sup> and by attenuating epileptic discharges.<sup>31,32</sup> Therefore, new pharmacologic and therapeutic approaches preventing glutamate toxicity, intracellular calcium accumulation, lipid

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peroxidation, free radical generation and inflammation implicate in the progression of HIE continue to be investigated.

Today, it is well known that neuroinflammation plays an important role in brain injury induced by neonatal HIE.<sup>33-35</sup> Cytokines are important inflammatory mediators, and also plays role both in maintain brain tissue homeostasis and in the response of the brain to diverse forms of injury.<sup>33</sup> Recent evidences shown that the expression of tumor necrosis factor (TNF)  $\alpha$  and interleukin (IL) 1 $\beta$  increase within hours after a cerebral hypoxic-ischemic insult in rats.<sup>34-36</sup> Furthermore, recent data indicate that TNF- $\alpha$  can potentiates glutamate neurotoxicity by inhibiting glutamate uptake in induce neuronal cell death.<sup>37</sup> Systemic administration of either a pharmacologic antagonist of the IL-1 or TNF- $\alpha$  receptor attenuates apoptosis to caused hypoxic-ischemic injury in seven day old rats.<sup>38,39</sup>

Pentoxifylline, a methylxanthine derivative, is a non-selective phosphodiesterase inhibitor that is commonly used for the treatment of symptomatic vascular insufficiency because of its haemorrhological activity. In recent years, pentoxifylline (PTX) has been also found *in vivo* and *in vitro* to prevent or to attenuate the release of TNF- $\alpha$  and other proinflammatory cytokines.<sup>40-42</sup> These immunomodulatory effects are likely underlying the therapeutic effects of PTX in mature animal models of cerebral ischemia; however, results of these studies are inconsistent.<sup>43-47</sup>

One report addressed the efficacy of PTX in an immature animal model of cerebral ischemic injury shows that post-hypoxic-ischemic treatment with PTX resulted in only a modest reduction in cortical damage, without an overall reduction in incidence of infarction.<sup>48</sup> However, possible neuroprotective effect of PTX against memory deficits induced by HIE has remained unstudied. In this study, we used a rodent model of neonatal HIE with unilateral carotid artery ligation and subsequent exposure to 8% oxygen for 2 hours on postnatal day 7 (the day of birth was designated as P0). Learning and memory performance was evaluated in a task finding a hidden platform using the Morris water maze on early adult stage.

## METHODS

### Experimental Animals

The experiments were carried out on seven-day-old

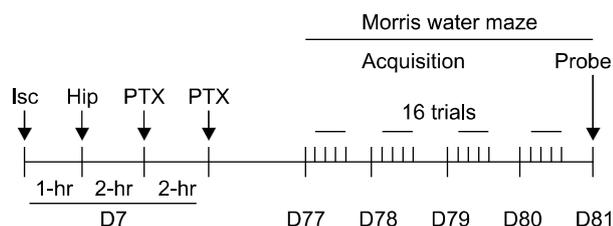
Wistar male rats with a mean body weight of  $11.8 \pm 1.2$  g in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the protection of animals used for experimental purposes, and with the guiding principles for the care and use of laboratory animals approved by Erciyes University. The rat pups were randomly divided into one of the five groups described below, breastfed by their own mothers from day 0 to day 30, and then with tap water and Purina rodent chow *ad lib*. The rat pups were housed under a 12-hour light/dark cycle.

### Surgical Procedure

Hypoxic-ischemia was performed according to the Levine-Rice model<sup>5</sup> on postnatal day 7 since the brain of this developmental stage is similar to the brain of a 32- to 34-week-old human fetus histologically.<sup>49,50</sup> Rat pups were anaesthetized by isoflurane inhalation and duration of surgery was less than 5 minutes. At supine position and under the microscopic magnification, the right common carotid artery was ligated with a 6-0 silk suture through a median cervical incision. After the ligation of carotid artery the animals were given to the dam for 1 hour to recover and feeding period. After recovery, except of the sham group, the animals were placed in a plastic chamber and exposed to hypoxia duration of 2 hours (8% O<sub>2</sub>-92% N<sub>2</sub>). The air temperature of chamber was maintained at  $37 \pm 0.5^\circ\text{C}$ . After from these procedures, animals were grown to 11 weeks.

### Treatments and Groups

Seventeen naïve rats were served as the control group. Study groups were treated as follows: Sham group (n = 13): median cervical incision was made. Right common carotid artery was found, but not ligated. HIE group (n = 16): Saline (0.5 ml) was injected intraperitoneally immediately after ligation and hypoxia. PTX60 group (n = 20): 60 mg/kg PTX administered immediately after ligation and hypoxia through intraperitoneally to the animals. PTX100 group (n = 11): 100 mg/kg PTX was administered immediately after ligation and hypoxia through intraperitoneally to the animals. Administration of saline and PTX was repeated after 2 hours. Pentoxifylline were purchased from Sanofi Aventis (Paris, France; Trental 100 mg/5 ml, intravenously) and was diluted in saline before usage. Experimental schedule was presented for details in Figure 1.



**Fig. 1.** Schedule of experimental design. The Levine-Rice model of 7-day-old Wistar rat was used to induce hypoxic-ischemia on post-natal day 7. The rat pups underwent surgery for the right common carotid artery ligation (ischemia, Isc) and then exposed to hypoxia (Hip). Pentoxifylline (PTX, 60 mg/kg or 100 mg/kg) was administered immediately after and 2 hours after hypoxia. When animals were grown to 77 days (D), spatial learning and memory performance was measured by using Morris water maze.

### Morris Water Maze

The cognitive function of rats was evaluated using Morris water maze test on day 77 to day 81. The background assessment used a well-established Morris water maze protocol as described in detail elsewhere.<sup>51</sup> Briefly, the rats were trained for 4 days (four trials per day) to locate a hidden escape platform that remained at the same location throughout training in a water maze (130 cm in diameter). Seventeenth trial consisted of a probe trial (free swim with no escape platform) that served to assess the development of a spatially localized search for the escape platform. An automated video-tracking system (Noldus) recorded the position of the rat in the tank. Escape latency, traveled distance, mean distance to the platform, swimming speed and time spent in target quadrant were measured. Each individual rat was released gently into the water at a randomly chosen quadrant except the target one that contained the platform for facing an extra maze cue. After reaching, the rat was allowed to stay on the platform for 15 seconds and was then taken back into the cage. The rats were placed on the platform by hand for 15 seconds if they could not escape to the platform within 120 seconds by themselves. During the inter-trial intervals, animals were kept in a dry home cage for 60 seconds. The day after the acquisition phase, a probe test was conducted by removing the platform. The rats were allowed to swim freely in the pool for 60 seconds. The time in seconds spent in the correct quadrant (target quadrant) containing the hidden platform, was recorded. The time spent in the target quadrant indicated the degree of memory consolidation taken place after learning.

### Hippampectomy

One day after probe trial, in deeply anesthetized rat, the brain was carefully dissected out from the skull, placed into ice-cold phosphate-buffered saline, cut along the longitudinal fissure of the cerebrum using a surgical knife, and cut off the regions posterior to lambda (midbrain, hindbrain, and cerebellum). Then medial side of the cerebral hemisphere was placed up and carefully removed the diencephalon (thalamus and hypothalamus) under a dissection microscope, allowing for visualization of the hippocampus. Using a forceps, right and left hippocampus separately dissected from surrounding area and weighed.

### Statistics

Data measured in different times (path length, escape latency, mean distance to the platform, and speed) were subjected to repeated measures of ANOVA, with group as between subjects factor, and day and trial as the “within subjects” factor. Data measured once (time spent in target quadrant and weight) were subjected to one-way ANOVA. Follow-up comparisons consisted of interaction contrasts or simple main effects as dictated by the outcome of the overall ANOVA, followed by individual means comparisons as appropriate (Tukey’s test). One sample *t* tests (one-tailed) were carried out in order to test the group means of probe performance against chance performance (i.e., 25%). Probabilities of less than 0.05 were regarded as statistically significant. Data were expressed as arithmetic means  $\pm$  standard error of mean for all experimental measurements.

## RESULTS

To verify that unilateral carotid artery ligation alone was sufficient to cause hippocampal atrophy, we present relative weight of right hippocampus to that of left one in all groups. As shown in Table 1, there was main effect of group on the weight of right hippocampus ( $F_{4,72} = 12.84$ ;  $p < 0.001$ ); however the weight of the left hippocampus was not affected by group ( $F_{4,72} = 1.11$ ,  $p > 0.05$ ). When the relative weight of right hippocampus to that of the left one were analyzed, the ratio was significantly lower in HIE group and PTX100 group than each of other groups ( $p < 0.028$ ). The difference between control and PTX60 group remained a trend level ( $p = 0.054$ ). These analyses

**Table 1.** The weight of hippocampus

Group	Right hippocampus (mg)	Left hippocampus (mg)	Ratio
Control	61.3 ± 3.0	63.6 ± 3.7	103.4 ± 9.0
Sham	59.3 ± 3.4	62.1 ± 3.3	98.6 ± 7.2
HIE	23.4 ± 2.8 <sup>csp</sup>	65.3 ± 4.4	41.8 ± 7.95 <sup>cs</sup>
PTX60	40.4 ± 5.3 <sup>cs</sup>	57.2 ± 1.0	71.9 ± 9.3
PTX100	36.9 ± 6.8 <sup>cs</sup>	65.6 ± 4.4	55.0 ± 7.9 <sup>cs</sup>

Values are presented as mean ± standard error. The right hippocampus atrophy resulted from unilateral carotid artery ligation on postnatal day 7 as a rodent model of neonatal hypoxic-ischemic encephalopathy (HIE). All rats were aged 77 to 85 days.

<sup>c</sup>Different from control, <sup>s</sup>different from sham, <sup>p</sup>different from pentoxifylline (PTX) 60 group, <sup>h</sup>different from HIE group.

suggest that hippocampal atrophy following unilateral carotid artery ligation could be partly conserved by PTX at dose of 60 mg, but not by dose of 100 mg.

### Behavioral Measurements, Acquisition Phase

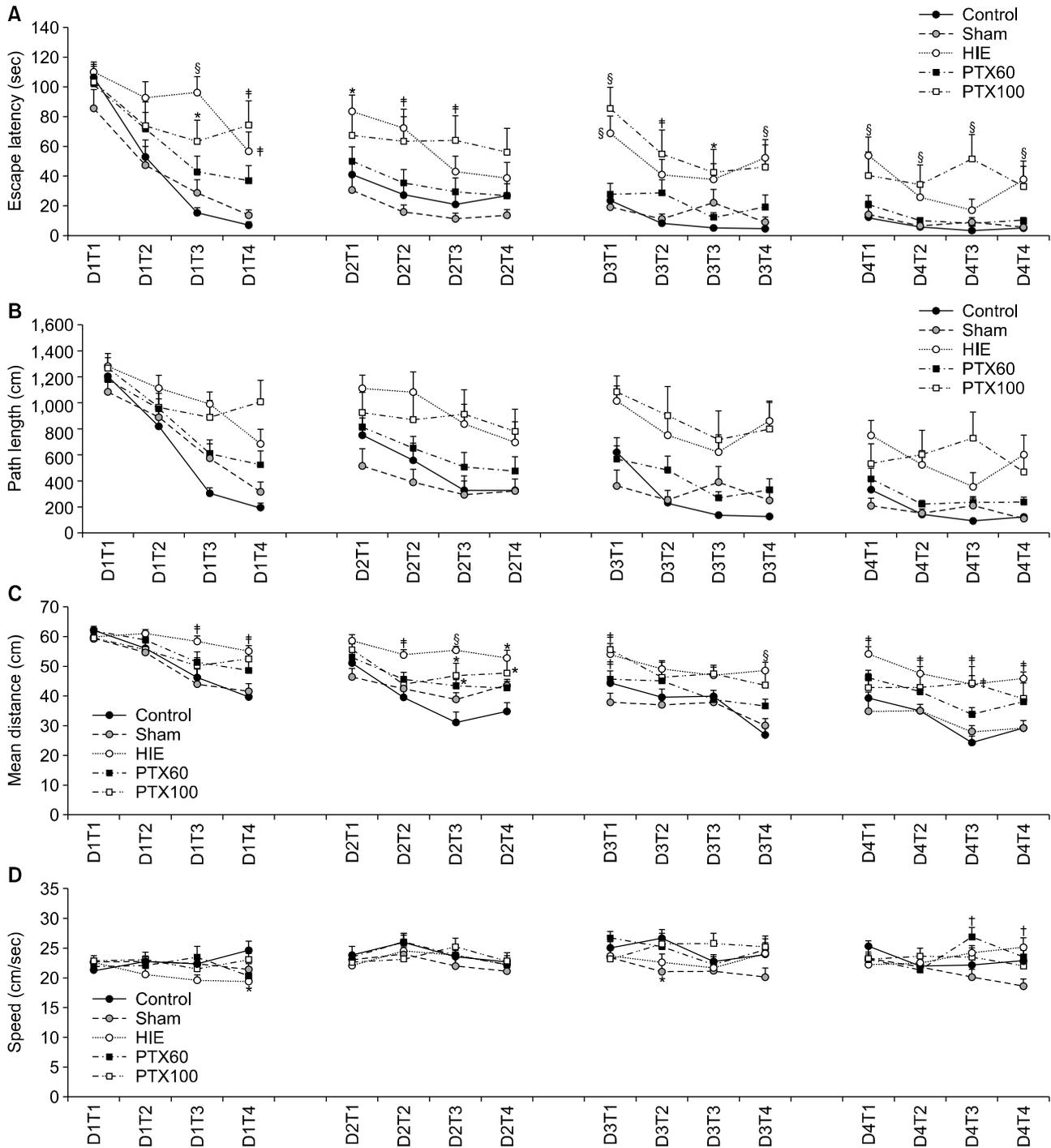
Results after four days training in hidden-platform task (each day consisted of one block, and each block included four trials) are summarized in Figure 2. Across days, there was an overall decrease in latency (Fig. 2A, main effect of days;  $F_{3,216} = 62.40$ ;  $p < 0.001$ ), path length (Fig. 2B, main effect of days;  $F_{3,216} = 62.44$ ;  $p < 0.001$ ) and the mean distance to the platform (Fig. 2C,  $F_{3,216} = 104.60$ ;  $p < 0.001$ ), and improved reductions in these parameters between trials (interaction day × trial:  $F_{59,648} = 6.09$ – $18.86$ ;  $p < 0.001$ ), indicating that all groups acquired the water maze. These performance of groups differed in terms of latency (interaction group × day × trial:  $F_{36,648} = 1.71$ ;  $p = 0.007$ ), probably confounding by differences in swim speed (group × day × trial interaction:  $F_{36,648} = 1.53$ ;  $p = 0.027$ ), and the mean distance to platform (group × day × trial interaction;  $F_{36,648} = 1.57$ ;  $p = 0.027$ ).

To determine whether groups differed in their escape latencies and the mean distance to platform in specific trials on specific days of the spatial learning task, significant group×day×trial interactions were further analyzed using unpaired one-way ANOVA followed by Tukey's test. These analyses showed that both the HIE group and PTX100 group require more time to find the platform (Fig. 2A,  $p < 0.002$ ) and moved more distance to the platform location (Fig. 2C,  $p < 0.05$ ) relative to the control, sham and PTX60 groups in most of the daily trials, whereas the PTX60 group performed equally with sham rats and con-

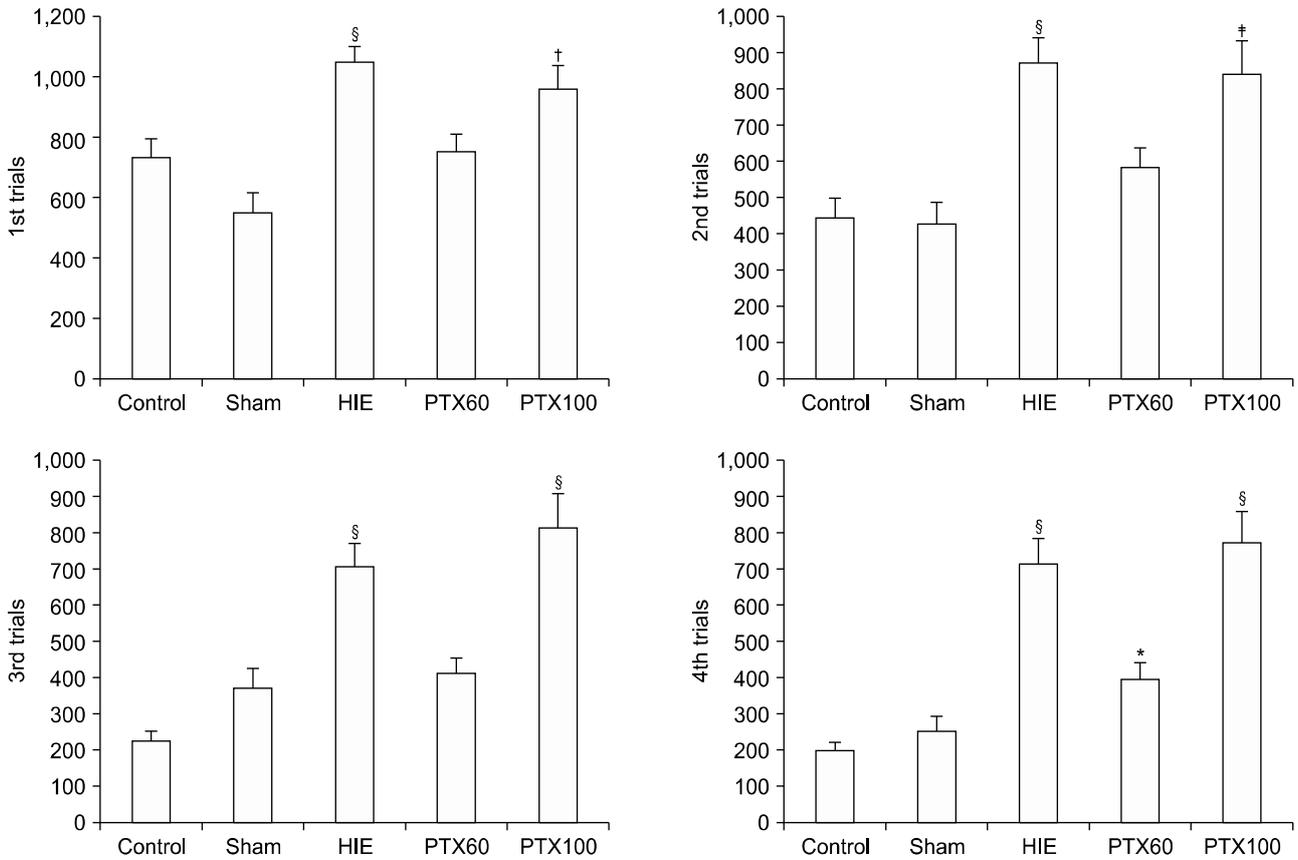
trol rats in the all training trials of each day. Although interactions between group and day and trial as well as group and day were not significant for the path length, a significant group × trial interaction (Fig. 2B,  $F_{12,216} = 1.87$ ;  $p = 0.039$ ) indicated that the five groups differed in mean distance score across trials but not across days. Therefore we further analyzed path length by taking the average across trials in each day (Fig. 3). Following an overall effect of group determined by a one-way ANOVA, Tukey's *post-hoc* testing revealed that hypoxic-ischemic rats had increased path length relative to the control and sham rats in the all training trials of each day (Fig. 3,  $p < 0.002$ ). Similar results were obtained for PTX100 group, except on the first trial from control group. PTX60 rats had similar path length to the control and sham rats in the first three training trials of each day, but at fourth trials they had increased path length relative to the control ( $p = 0.042$ ). There were also significant differences between the PTX60 group and the PTX100 group on 3rd and 4th trials of each day ( $p < 0.001$ ). These analyses clearly suggest that carotid artery ligation resulted in a prolongation of path length to reach the hidden platform and that PTX at low dose, but not at high dose, prevented this impairment.

### Retrieval phase (Fig. 4)

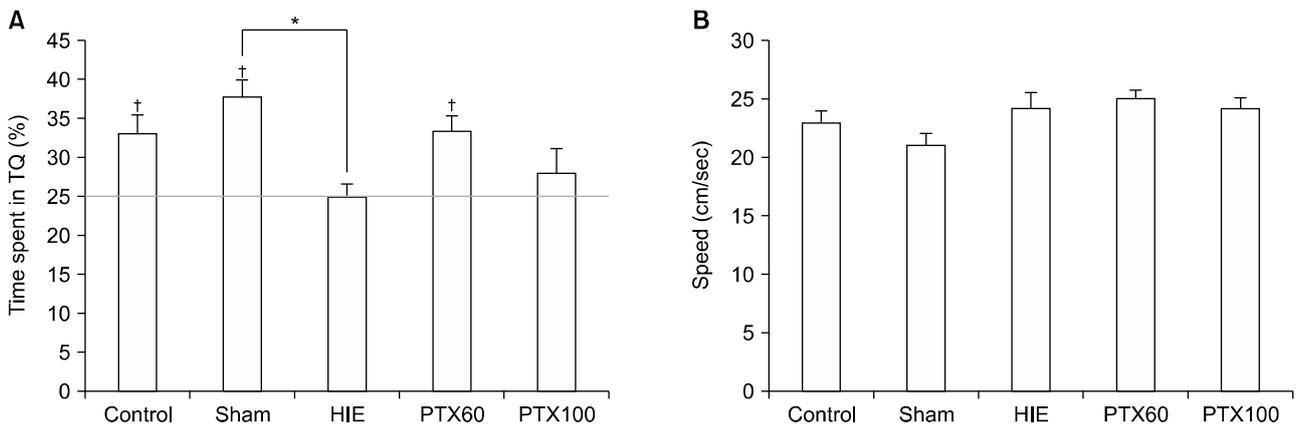
Memory for the hidden platform location was assessed in a probe trial without the platform present. A one sample *t* tests revealed that the proportion of time spent in the correct quadrant, compared to the other quadrants, by HIE group and PTX group were not significantly above chance ( $25.13 \pm 1.47\%$  and  $28.03 \pm 3.19\%$ , respectively;  $p > 0.05$ ). However, control group ( $33.16 \pm 2.36\%$ ), sham group ( $37.85 \pm 2.08\%$ ), and PTX60 group ( $33.47 \pm 1.94\%$ ) spent more time in target quadrant ( $p < 0.003$ ). A one way ANOVA test showed that there was significant difference between groups ( $F_{4,71} = 4.77$ ;  $p = 0.002$ ). *Post-hoc* Tukey test showed that the percentage of the time spent in the target quadrant by the HIE group was significantly lower when compared to sham group ( $p = 0.002$ ), but remained a trend level when compared to the control group ( $p = 0.065$ ) and PTX60 group ( $p = 0.058$ ). There was a "trend-level" significance ( $p = 0.050$ ) in comparison between sham group and PTX100 group. Control group was not significantly different from the sham group in the percentage of the time spent in the target quadrant through the 1-minute trial ( $p > 0.05$ ). All groups did not



**Fig. 2.** Low doses of pentoxifylline (PTX) partly prevent the impairment of memory induced by hypoxic-ischemic brain injury. Group means latencies (A), path length (B), and mean distance to the hidden platform location (C) for each of the 16 training trials across 4 consecutive days showed improvement for all groups with training. Group ( $n = 5$ )  $\times$  day ( $n = 4$ )  $\times$  trial ( $T, n = 4$ ) ANOVAs revealed significant interactions of group with day and trial for escape latency and the mean distance to the platform (A and C), but only with trial for path length (B). Note that both the hypoxic-ischemic encephalopathy (HIE) group and PTX100 group require more time to find the platform (A) and moved more distance to the platform location (C) relative to the control, sham and PTX60 groups in most of the daily trials. Further analyzed path lengths by taking the average across trials in each day are shown in Figure 3. (D) Mean swim speed of control, sham and experimental groups for each of the 16 training trials across 4 consecutive days. Circles for control ( $n = 17$ ), sham ( $n = 13$ ), HIE ( $n = 16$ ), and squares for PTX60 ( $n = 20$ ) and PTX100 ( $n = 11$ ) are mean  $\pm$  standard error. \* $p < 0.05$  versus control group;  $^{\dagger}p < 0.05$  versus sham group;  $^{\ddagger}p < 0.05$  versus control and sham groups;  $^{\S}p < 0.05$  versus control, sham and PTX100 groups.



**Fig. 3.** Group path lengths averaged across each trial of four days. Bars for control (n = 17), sham (n = 13), hypoxic-ischemic encephalopathy (HIE, n = 16), pentoxifylline (PTX) 60 (n = 20) and PTX100 (n = 11) are mean  $\pm$  standard error. \* $p < 0.05$  versus control group; <sup>†</sup> $p < 0.05$  versus sham group; <sup>‡</sup> $p < 0.05$  versus control and sham groups; <sup>§</sup> $p < 0.05$  versus control, sham and PTX100 groups.



**Fig. 4.** Carotid artery ligation results in impairment of memory retrieval and low dose of pentoxifylline (PTX) are more effective than its high dose to prevent this impairment. (A) The proportion of time spent in the correct quadrant, compared to the other quadrants, by the control group (n = 17), the sham group (n = 13), and the PTX60 group (n = 20) were significantly above chance (i.e., 25%; horizontal line), as indicated by “<sup>†</sup>”, but not by the hypoxic-ischemic encephalopathy (HIE) group (n = 16) and the PTX100 group (n = 11). In addition, the percentage of the time spent in the target quadrant (TQ) by the HIE group (n = 16) was significantly lower as compared to sham group (\* $p = 0.002$ ). (B) All groups did not differ significantly in speed on the probe trial ( $F_{4,71} = 1.96$ ;  $p > 0.05$ ). Bars are means  $\pm$  standard error of mean.

differ significantly in speed on the probe trial ( $F_{4,71} = 1.96$ ;  $p > 0.05$ ). These results indicate that carotid artery ligation results in impairment of memory retrieval and that low dose of PTX are more effective than its high dose to prevent this impairment.

## DISCUSSION

Herein, we used the Levine-Rice model to study neonatal hypoxic-ischemic brain damage in the present study. This model has been widely used for three decades for histological analysis as well as behavioral tests. It produces unilateral brain injury to the structures such as the hippocampus, the striatum and cortex of the hemisphere ipsilateral to arterial occlusion.<sup>52</sup> Unilateral hippocampal atrophy was detected following unilateral carotid artery ligation in the present study. This model of unilateral cerebral hypoxia-ischemia causes sensorimotor deficits<sup>20</sup> and delays in maturation of reflexes.<sup>53</sup> Behavioral studies also revealed cognitive disabilities, as for example short and long-term spatial and aversive memory impairments.<sup>15,17,54-60</sup> In agreement with these studies, we showed that HIE resulted in significantly impaired performance of spatial learning and memory in a hidden platform task. Because hypoxic-ischemia was performed on postnatal day 7 and the cognitive function was evaluated when these rats were grown up adult ages, we concluded that a compensatory redistribution of blood to the hippocampus following hypoxia/ischemia<sup>61</sup> failed to recover memory impairment.

The present results clearly demonstrate the neuroprotective efficacy of PTX in neonatal rats that underwent unilateral carotid ligation, as reported in brain injury induced by amyloid  $\beta$ -protein,<sup>62</sup> glutamate neurotoxicity<sup>63</sup> and closed head trauma.<sup>64</sup> A protective effect has been also reported against experimental models of epilepsy.<sup>65,66</sup> Pentoxifylline promotes learning and memory function in naturally aging rats and mice.<sup>67</sup> In contrast to these reports focusing on permanent hypoxia, spatial memory was not affected by transient global ischemia, but PTX treatment of both ischemic animals and control animals significantly improved spatial memory.<sup>68</sup> It must also be noted that the present study did not focus on the protective effects of PTX at cellular level but included hypoxic-ischemia (HI)-induced impairments at the behavioral level. Nevertheless, another study from our labo-

ratory examined neuronal apoptosis that was evaluated by the caspase-3 immunostaining, and found that PTX administration after hypoxia-ischemia reduces neuronal apoptosis and necrosis.<sup>69</sup> Using spectrophotometric assay, it has been recently reported that treatment of PTX 60 mg/kg/dose (a single dose) significantly decreased caspase-3 activity in the brain of ischemia group.<sup>70</sup> Although these two studies suggest that the caspase-3 activity probably mediates neuroprotective effect of PTX at behavioral level, other intracellular effects such as inhibition enzyme phosphodiesterase,<sup>48</sup> increased cyclic adenosine monophosphate (cAMP) levels,<sup>62,63,71,72</sup> a decline in TNF- $\alpha$  production and in platelet-activating factor levels<sup>41,42,68,73</sup> and inhibition of adenosine A1 receptors<sup>63</sup> cannot be excluded.

We observed in the present study that beneficial effects of PTX negated at 100 mg/kg dose. It is unclear as to why that the higher concentration of PTX did not improve learning and memory deficits after HI as compared with lower doses of PTX. Although speculative, we have thought that differential effects of PTX can contribute to explain the mechanism of protective action. cAMP plays a major role as a second messenger in biochemical processes regulating the cognitive and memory processes.<sup>74,75</sup> However, phosphodiesterase inhibition seems not to be associated with ineffectiveness of high dose PTX because its effect on cAMP levels is dose-dependent.<sup>76</sup> On the other hand, differential effects of PTX on the production of immune mediators have been reported.<sup>77-79</sup> There is some evidence for involvement of IL-1 $\beta$ ,<sup>80</sup> TNF- $\alpha$ ,<sup>81</sup> and IL-6<sup>82</sup> in specific memory processes including acquisition, consolidation, or retrieval. These immune proteins are not required for learning and memory, but contribute to impairments in cognitive function after an inflammatory event.<sup>83-85</sup> Therefore it can be expected that PTX differentially modulates the inflammatory environment depending on its dosage, and thereby produces different effects on memory impairment induced by HI.

Our result of Morris water maze test is in consistent with the data of the step-down avoidance task that is a kind of hippocampus-dependent test related with short-term memory from a recent study.<sup>86</sup> In that study, PTX treatment (50 mg/kg) improved short-term memory, in rat pups that were trained in a step-down avoidance task 28 days after induction of hypoxic-ischemia. Moreover, beneficial effect of PTX was associated with a decrease in in-

creased apoptotic cell death and an increase in cAMP levels in the hippocampus.<sup>86)</sup> In the light of these results, it can be concluded that both short-term and long-term memory impairments can be sensitive to low-doses of PTX treatment.

Conversely to the present study, Wu and co-workers recently reported that 100 mg/kg dose of PTX improved memory impairment in a transient focal cerebral ischemia-reperfusion model.<sup>87,88)</sup> Hypoxia-ischemia without reoxygenation inactivates oxidative phosphorylation only, leading to disrupt mitochondria,<sup>89)</sup> whereas, upon reperfusion, oxygen interacts with the damaged respiratory chain to produce a burst of reactive oxygen species that underlies reperfusion injury and secondary tissue damage.<sup>90)</sup> Pentoxifylline does not have significant antioxidant capacity at therapeutic concentrations, but increasing concentrations led to a significant scavenger effect.<sup>91)</sup> Therefore modulation of oxidative stress at higher doses can explain why protective effect of high-dose PTX was not observed in brain injury following ischemia alone, in contrast to following ischemia-reperfusion.

Although our study does not address, PTX can have an effect of its own, as shown by significantly decreased the time to find the platform, as compared to normal and sham-operated rats.<sup>68)</sup> In the study of Bruno Rde *et al.*,<sup>68)</sup> similar escape latency values (time to find the platform) were observed in normal, sham-operated, and ischemic rats. The escape latency is one of the most commonly used performance measures to measure spatial memory performance in a water maze task. On the other hand, the mean distance to platform is suggested to be a better measure than escape latency as it is not dependent on subject's swimming speed.<sup>92)</sup> The mean distance to platform parameter is the average of distances between the platform in the water maze and each sample of the animals' positions collected by the image analysis system. Thus, if an animal swims in the vicinity of the learned platform location, the mean distance will be short. This parameter was correlated with time spent in the platform quadrant but not with latency to and crossings of the platform location.<sup>93)</sup> Therefore, the escape latency parameter, as reported in the study of Bruno Rde *et al.*,<sup>68)</sup> was not able to characterize the behavior in the Morris water maze.

There are several investigations on the effective neuroprotective dose of PTX in the brain ischemia models. Some of studies showed that PTX had protective effect on

the brain trauma on the doses of 30 to 60 mg/kg, but others informed that PTX at the higher doses had neurotoxic effect on brain function.<sup>48)</sup> Based upon the results of previous studies, in the present study, to determine the effective dose of PTX in neonatal rat model of HIE, two different PTX dosage regimens (60 and 100 mg/kg) were used. The present results suggest that protective effects of PTX are mediated by its fine-tuning effects on the hippocampus tissue losses and spatial memory impairments and its beneficial influence is negated if PTX is administered at high dose.

In conclusion, PTX even administrated as a two doses two hour apart immediately after neonatal hypoxic-ischemic insult provides benefit over a prolonged period in still developing rat brain. The present data indicate that PTX is an effective neuroprotective agent in this particular animal model. Furthermore, combining of PTX with hypothermia may be more successful in treatment of HIE, since PTX has significant hemorheologic properties.<sup>48,94)</sup> For this reason, we have concluded that our results can provide a basis for combing therapy of HIE; however, detailed studies are needed.

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#### ■ Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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