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Weak brain damage under optimal controlled-constant blood flow in rat

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SUMMARY

How to protect brain damage during heart surgery with cardiopulmonary bypass (CPB) is getting important in along with a concomitant increase in complex aortic operations in the aged person. Effects of constant cerebral blood flow with CPB on protecting the brain have not been explored in detail at a cellular level. This study first developed a constant blood flow model rat, and then investigated whether optimal constant blood flow was associated with any neuronal damage in the hippocampus (HIP) and the somatosensory cortex (CTX). The model was made by blood infusion from the tail artery to the right carotid artery (CA) with subsequent left CA occlusion. Blood pressure was measured at various flow rates (0–25 mL/kg/min). To know optimal constant blood flow, 2,3,5-triphenyltetrazolium chloride (TTC) staining and immunohistochemical stainings for neuron marker and glial markers were carried out as well as Argyophilic III staining. Optimal blood flow was obtained in moderate-flow rate (10 mL/kg/min), showing no obvious tissue damage in TTC staining and immunohistochemical markers for neurons marker and glial cells. However, several argyrophilic

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cells, faintly damaged neurons, were seen in the HIP and CTX. Data suggest that slight damage was seen in the HIP and CTX at a cellular level during optimal constant blood flow.

Key words: CPB, cardiopulmonary bypass; CTX, somatosensory cortex; HIP, Hippocampus; CA: carotid artery; TTC, 2,3,5-triphenyltetrazolium chloride.

INTRODUCTION

Brain protection during heart surgery with cardiopulmonary bypass (CPB) is of major concern along with a concomitant increase in complex aortic operations in the aged. Postoperative delirium, short-term memory dysfunction and higher brain dysfunction are known side effects in heart surgery with CPB in the aged people [1].

Cerebral blood flow is kept constant by an autoregulatory mechanism under physiological conditions [2]. However, this system is disrupted in pathological conditions such as hypothermia, cerebrovascular disease, hypertension and aortic aneurysm [3-4] as well as during heart surgery with CPB [4-5]. Low cerebral blood flow may result in regional hypoperfusion and cerebral ischemia [5]. On the other hand, excessive blood flow may cause cerebral edema [6] and brain injury reflected by high post-CPB cerebral oxygen metabolism [8]. Thus, appropriate control of cerebral blood flow seems to be important for the prevention of brain damage, especially in heart surgery with CPB.

To investigate whether optimal constant cerebral blood flow has an effect on brain protection in heart surgery with CPB, we first tried to establish a constant-cerebral blood flow model rat. We then investigated the histological brain damage using argyrophil-III silver staining that can detect faint neuronal cellular damage [9-14].

MATERIAL AND METHOD

Constant cerebral blood flow model

Young male Sprague-Dawley (SD) rats (250–280 g) were housed under a 12-h light/dark cycle, with access to food and water *ad libitum*. Animal care was carried out according to the guidelines of the Institute for Experimental Animal Science, Nagoya City University Medical School, and experimental procedures were approved by the Committee of Animal Experiment in the university. Every effort was made to minimize the pain and discomfort of the animals.

SD rats were used to CPB under sterile conditions as follows. The CPB circuit consisted of a 100-cm long saline-filled tube connected to an infusion rollerpump (STC508, Terumo, Tokyo, Japan). For anesthesia, induction was carried out with 4% isoflurane (Mairan Corp,

Osaka, Japan) and mainteined with 1.5% isoflurane using a ventilator (683 Rodent Ventilator, Havard Apparatus Inc, Holliston, MA, USA). A 24-gauge catheter was inserted into the tail artery followed by a heparin (150 IU) injection and the opposite end of the catheter was then inserted into the right carotid artery (CA) in the direction to the brain, allowing arterial blood to be drained from the tail artery and returned to the animal via the right carotid artery (Figure 1).

To obtain constant cerebral blood flow, left CA was first clamped with Klemme and roller pump was then set to control blood flow (1～25 ml/kg/min) from the right CA. The animals were laid in a right decubitus position to try to keep $PaCO_2$ stable without intubation.

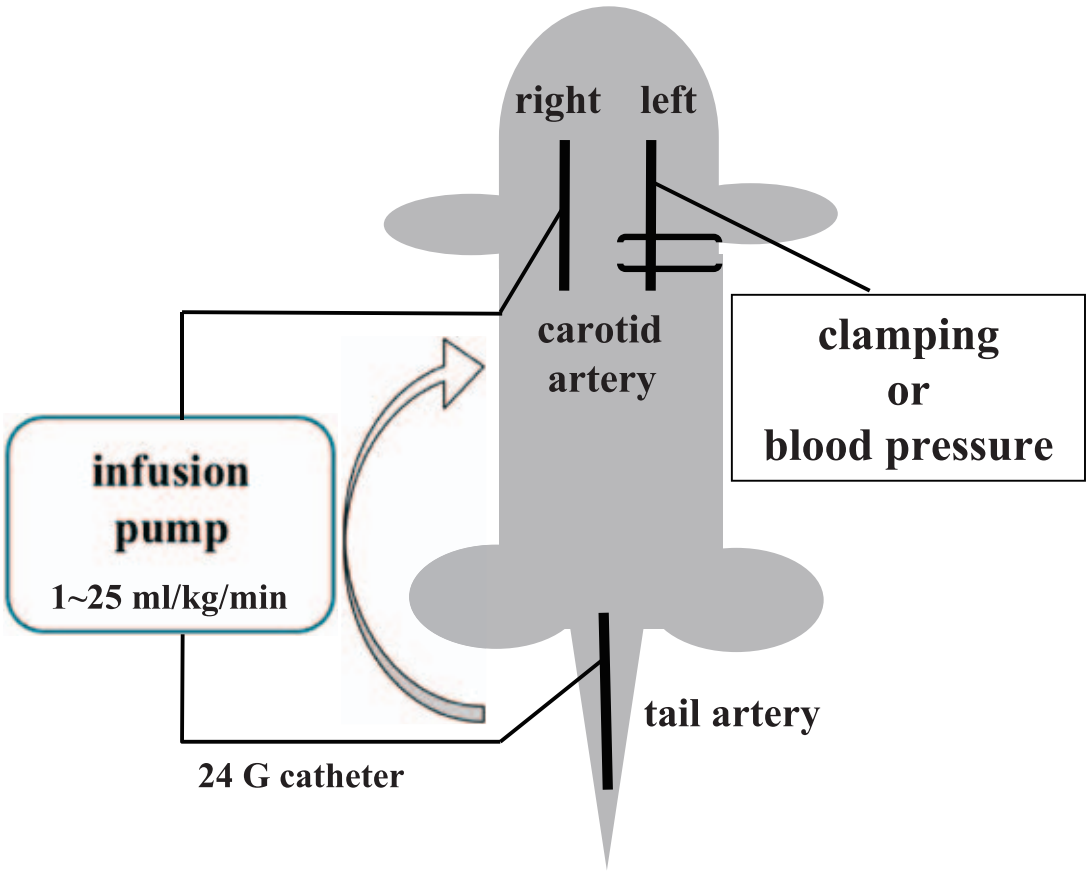

Figure 1 Constant blood flow model rats
A saline-filled tube was connected to an infusion pump to establish the cardiopulmonary bypass (CPB) circuit. Under anesthesia, arterial blood was drained from the tail artery and returned to the right carotid artery, followed by clamping the left carotid artery and changing the rate of blood flow (1–25 mL/kg/min). The hemoglobin level was maintained at 80 g/L. Following left CA clamp for 60 minutes, the tail artery and right CA were ligated after removal of the catheters. All the blood left in the CPB circuit was returned into the tail artery.

The rectal temperature was monitored and kept at 32℃ with a hot pad. The level of hemoglobin was maintained at 80 g/L during the experiment: all the blood left in the CPB circuit was collected, centrifuged at 2000 rpm for 5 minutes and the precipitates (mostly red blood cells) were returned into the tail artery. The clamp of left CA was released after 60 minutes and the tail artery and right CA were ligated after removal of the catheters. After recovery from anesthesia without the use of vasoactive agents, the animals were returned to their cages.

In sham-operated group, all surgical procedures were carried out: the roller pump was set to the animals but it was not used.

After instituting CPB, the experiments were carried out with various flow rates: no perfusion (0 mL/kg/min); low-flow mode (2 mL/kg/min); moderate-flow mode (10 mL/kg/min); and high-flow mode (25 mL/kg/min).

Measurement of arterial blood pressure

To measure arterial blood pressure in the constant-cerebral blood flow model, left CA was cannulated with a 22-gauge catheter instead of the clamp. The catheter was connected with a transducer (DS5100, Fukuda Electronics Co. Tokyo, Japan) to monitor blood pressure under different blood flow rates from 1 mL/kg/min to 25 mL/kg/min.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

At the end of the experiment, some rats were put under deep anesthesia with pentobarbital and transcardially perfused with saline. The brains were quickly removed and cut into 2-mm coronal sections. Sections were stained with 2% TTC (Sigma, ST. Louis, MO) for 20 min at 37℃. The sections were transferred into 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) to preserve them for later examination.

Immunohistochemistry

Seven days after the operation, rats were fixed with 4% PFA in 0.1 M PBS. The brains were removed and immersed in the same fixative for 24 h at 4℃. After cryoprotection with 20% sucrose, coronal brain sections (40 μm thick) were prepared. The sections were incubated in blocking solution for 30 min (3% bovine serum albumin and 0.25% Triton X-100 in 0.01 M PBS), followed by primary antibody in blocking solution for 16 h at 4℃. After rinsing, sections were incubated for 1 h at room temperature with biotinylated secondary antibody (1: 500, Vector Laboratories, Burlingame, CA), then reacted with avidin-peroxidase for 1 h (ABC-kit, Vector Laboratories) at room temperature, followed by detection solution (0.25 mg/ml diaminobenzidine, 0.03% H_2O_2 in PBS). The primary antibodies used were MAP-2

(MAP-2, mouse monoclonal antibody; 1: 200, Millipore, Billerica, MA), glial fibrillary acidic protein (GFAP, rabbit polyclonal IgG; 1: 500, Dako, Grostrup, Denmark), and OX-41 (mouse monoclonal antibody; 1: 800, Millipore) as previously reported [14-15].

Argyrophil-III silver staining

Argyrophil-III staining is used to detect histopathological changes associated with early neuronal damage, reflected as dark neurons (DNs) [9-11, 14] 24 hours after the operation. Following deep anesthesia with pentobarbital (>50 mg/kg), rats were transcardially perfused with saline followed by a fixative of 2% PFA and 2. 5% glutaraldehyde in cacodylate buffer as previously reported [12]. Briefly, the brain of each rat was removed, then immersed in the same fixative for at least 1 week at room temperature. After cryoprotection with 20% sucrose, 50-μm thick coronal sections were prepared on a cooled microtome. Sections were dehydrated and esterified in 1-propanol containing 1. 2% sulfuric acid and 2% distilled water at 56℃ for 16 h, then rehydrated and treated with 8% acetic acid for 10 min. These sections were processed in freshly made silicotungstate physical developer. The degree of argyrophilia was arbitrarily evaluated as "dark" (++) and "light dark or brown" (+) by 3 blinded investigators.

Statistics

All measured values are expressed as mean ± standard error of the mean. To analyze blood pressure measurements, we used univariate analysis of variance followed by Fisher's *post hoc* test. All statistical analyses were conducted using StatView-J 5. 0 (SAS Institute Inc, Middleton, Mass, USA). $P<0.05$ was considered significant.

RESULTS

Establishment of constant cerebral blood flow model rat

The CPB circuit first consisted of a saline-filled tube connected to an infusion pump. Under anesthesia using a ventilator, arterial blood drained from the tail artery was returned to the right CA. Constant blood flow was established by clamping the left CA (Figure 1). Various blood flows from 1 mL/kg/min to 25 mL/kg/min were given to the animals. The hemoglobin level was maintained at more than 80 g/L. No significant changes in arterial gases were found either in low flow or high flow modes (Table 1).

After left CA clamp for 60 minutes, the tail artery and right CA were ligated after removal of the catheters, allowing blood flow from the left CA and both vertebral arteries into the brain. All the blood left in the CPB circuit was collected, centrifuged at 2000 rpm for 5 min and the precipitates (mostly red blood cells) were returned into the artery. Arterial

Table 1. Arterial blood analysis

	Before constant flow	During constant flow		After constant flow	
		low-flow	high-flow	low-flow	high-flow
pH	7.37±0.07	7.36±0.03	7.35±0.03	7.37±0.05	7.31±0.01
pCO_2 (mmHg)	52.2±11.2	43.5±7.6	43.1±6.0	54.3±6.1	50.4±3.3
pO_2 (mmHg)	475.3±70.8	349.0±78.2	356.4±40.6	469.3±42.3	395.8±31.6
hemoglobin (g/L)	136±7.6	84±9.5	81±7.6	116±4.6	116±1.2

blood gases during either low-flow mode or high-flow SCP were unchanged after the surgery (Table 1).

Brain blood pressure

After instituting CPB, we investigated blood pressure in the brain under various flow rates (Figure 2). Blood pressure measured in the left CA increased in a flow-rate-dependent manner. It was 19.5 ± 1.2 mmHg ($n = 10$) with no perfusion (0 mL/kg/min); 27.2 ± 1.2 mmHg ($n = 10$) in low-flow (2 mL/kg/min) mode; 38.5 ± 3.0 mmHg ($n = 10$) in moderate-flow (10 mL/kg/min) mode; and 48.6 ± 4.1 mmHg ($n = 8$) in high-flow (25 mL/kg/min) mode.

Cellular brain damage in rats after SCP

To investigate tissue damage after constant blood flow, we performed TTC staining 24 h after the experiment (Figure 3A). At this time, functioning mitochondria (in living cells) changed TTC to a red substance. No macroscopic cell death was shown in the striatum and the hippocampus (HIP) in no-pump perfusion group. Constant low-blood flow and moderate-blood flow modes also showed no macroscopic cell death in the striatum and HIP (not shown).

To investigate microscopic cell damage, immunostaining for neuron marker (MAP-2), astrocytic marker (GFAP), and microglial marker (OX-41) was carried out in HIP and the sensorimotor cerebral cortex (CTX) (Figure 3B). No obvious cell loss in MAP-2-positive neurons was seen in HIP and CTX (upper panel). In addition, no apparent reactive response in GFAP-positive astrocytes was observed (middle panel). However, OX-41 positive microglia were detected in the CTX (lower panel).

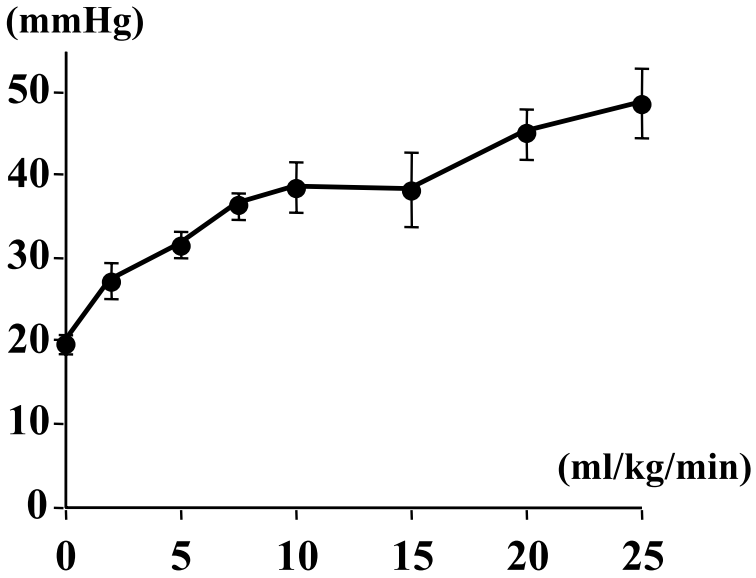

Figure 2. Increase of blood pressure in the brain by controlled blood flow
Blood pressure (vertical bar) was monitored at flow rates from 1 to 25 mL /kg/min (horizontal bar). At low-flow mode (＜10mL/kg/min), blood pressure increased in a flow-dependent manner. The blood pressure was unchanged during moderate-flow mode at 10–15 mL/kg/min. At high-flow mode (＞20mL/kg/min), blood pressure increased in a flow-dependent manner. For all statistical analyses, univariate analysis of variance followed by Fisher's *post hoc* test was conducted using StatView-J5. 0.

Argyrophil-III staining after constant blood flow

We used argyrophil-III staining to show very early histopathological neuronal damage as argyrophilic dark neurons (DNs) (Figure 4). Although DNs were seen in HIP and CTX by both low-blood flow group (left panels) and moderate-blood flow group (Figure 4, middle panels), the staining pattern of DNs was different between HIP and CTX: the pattern in HIP appeared to be a stress-related pattern [14], while typical DNs with corkscrew-like dendrites were seen in CTX (right panels). Table 2 presents a summary of DNs in HIP, CTX and brainstem. Fewer DNs were present in HIP and CTX with moderate-flow mode compared with low- or high-flow modes. DNs in the brainstem were only detected in high-flow mode.

DISCUSSION

To determine whether constant blood flow is advantageous for neuroprotection at the neuronal cellular level, we first developed a constant blood flow model. We found that a moderate flow rate at 10–15 mL/kg/min was optimal in that it caused minimal cellular damage in HIP and CTX. In these areas there were no apparent changes in TTC staining and MAP-2

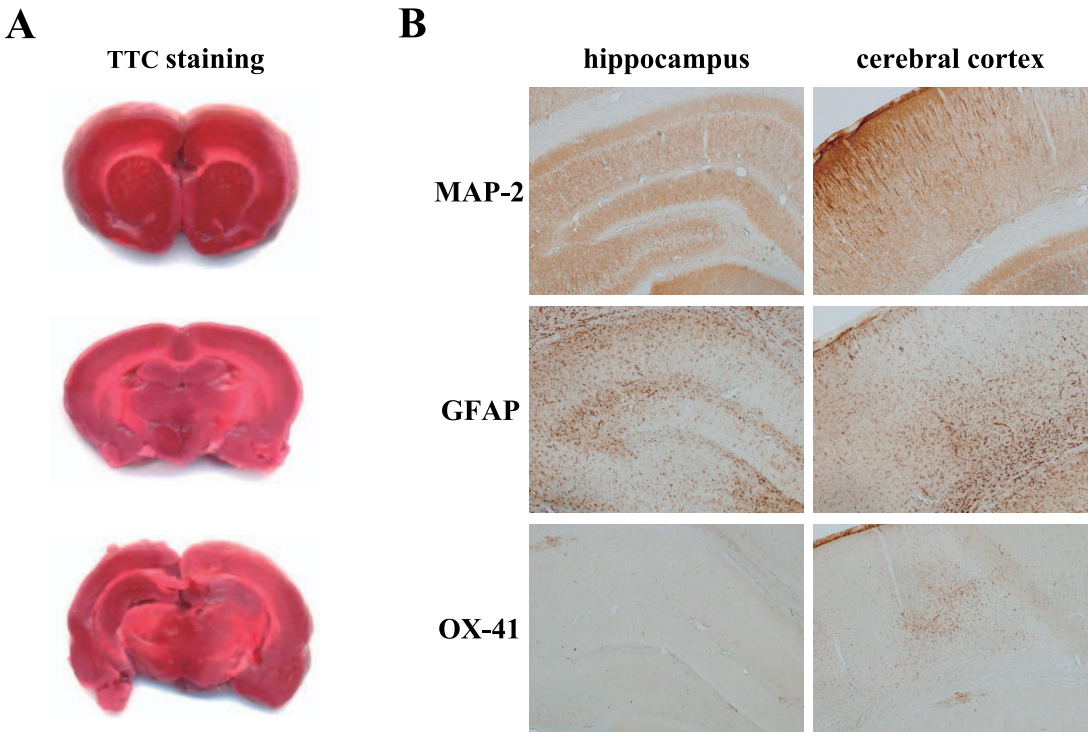

Figure 3. Histological changes after constant blood flow in the brain
(A) 2, 3, 5-triphenyltetrazolium chloride (TTC) staining 24 h after constant blood flow revealed no mitochondrial dysfunction in CTX and HIP, indicating that no apparent cell death.
(B) A neuronal marker (MAP-2), an astrocytic marker (glial fibrillary acidic protein, GFAP) and a microglial marker (OX-41) were immunostained in HIP and CTX 24 h after constant blood flow. No apparent cell damage in MAP-2 positive neurons and GFAP-positive astrocytes are shown in HIP and CTX.

staining but a slight change in argyrophilic III staining.

Our finding that average blood pressure in the brain was 19. 5 mmHg without blood flow by the pump is comparable to previous reports in large animal models [16-18], indicating that our model would be suitable for investigating cellular damage. Furthermore, our model may be advantageous for investigating the effect of cerebral blood flow, because a more stable blood flow would be obtained by the cannulation method [19].

In our constant blood flow model (arterial blood from the tail artery was returned to the right CA with left CA occlusion), there are several points that should be noted in consideration of the use. In this model, chest opening, cardiac arrest, hypothermia, or circulatory arrest was not taken into account. Also, the blood supply to the hippocampus in rats is anatomically different from that in humans. The posterior cerebral artery comes from the proximal intracranial portion of the internal CA [20], and thus, ischemia in the hippocampus seems to be related to the internal CA rather than the vertebral arteries.

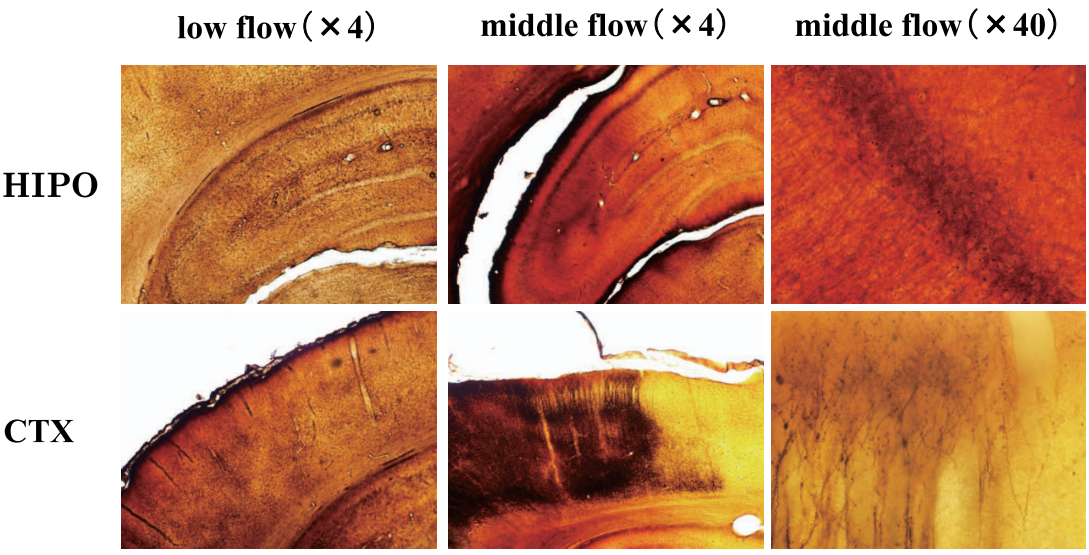

Figure 4. Argyrophil III staining after constant blood flow in the bain
Very early neuronal damage was detected as argyrophilic dark neurons (DNs). Typical DNs with corkscrew-like dendrites were found in HIP and CTX even at moderate-flow mode (10 mL/kg/min). Atypical DNs showing a stress pattern were detected in HIP.

Argyrophil-III staining reflects very early neuronal damage after various brain insults, such as ischemia, and shows typical morphological features (e.g., shrunken somata and corkscrew-like dendrites). It identifies damage mainly through disarray of electric charges in the neurofilaments and microtubules [9-11]. In this study, TTC staining, MAP-2 staining, GFAP staining, and OX-41 staining did not indicate that there was any apparent brain dam-

Table 2. Cellular level damage by various blood flows in a SCP rat model

	Hippocampus	Cerebral Cortex	Brainstem
No perfusion	-	-	-
Low-flow (2 mL/kg/min)	++	++	-
Middle-flow (10 mL/kg/min)	+	+	-
High-flow (25 mL/kg/min)	++	++	+

age. However, very early neuronal damage in the hippocampus and the cortex (sensory motor area) was detected after 24-h in argyrophil-III staining.

In our previous studies, two types of DNs were detected in the brain [12, 14]. One was a typical pattern found in severe damage; the other was an atypical pattern that appeared to reflect stress. Although we detected a minor change in the hippocampus after moderate-flow perfusion, it was the atypical staining pattern (faint staining in the cell body with weakly-stained brush fibers), indicating that it was probably associated with a stress response. However, a typical staining pattern of DNs (strong staining in the cell body with corkscrew-like dendrites) was shown in CTX with moderate-flow perfusion. It is probable that weak edema is produced even by the optimal constant blood flow rate, causing the appearance of typical DNs in CTX. The minor cell damage detected as DNs in the cortex might be related to the side effects of heart operation with CPB such as postoperative delirium and higher brain dysfunction. Additional treatment with neuroprotective agents such as erythropoietin and radical scavengers [21-23], which are drugs used clinically for stroke, may also be effective in heart surgery, especially in the aged. Further studies will be needed to prove the neuroprotective effect at the cellular level in our rat model in future.

In high-blood flow mode (25 mL/kg/min), DNs were detected even in the brainstem. It is likely that high-flow mode causes the increase of intracranial pressure (ICP). Higher ICP might be related to cerebral edema, provoked by excessive blood flow, causing more cellular damage in the brainstem. ICP is known as an important prognostic indicator of later neurologic function [24]. It is also probable that high blood flow mode causes in stress reactivity in the brain stem. Thus, excessive cerebral blood flow by high-flow condition, even under constant flow, may be associated with cellular damage in the brain.

In summary, we investigated the effect of constant blood flow on brain damage at cellular level and focused on cellular damage in the hippocampus and the cortex. Constant moderate-blood flow at 10−15 mL/kg/min seems to be better to prevent brain edema by avoiding excessive blood flow, and minimizing neuronal damage in the brain.

REFERENCES

1. Ebert AD, Walter TA, Huth C, Herrmann M. Early neurobehavioral disorders after cardiac surgery: a comparative analysis of coronary artery bypass graft surgery and valve replacement. J Cardiothorac Vasc Anesth. 2001 Feb; 15 (1): 15-19.
2. Lassen NA. Cerebral blood flow and oxygen consumption in man. Physiol Rev. 1959; 39: 183-238.
3. Baumbach GL, Heistad DD. Cerebral circulation in chronic arterial hypertension. Hypertension. 1988; 12: 89-95.
4. Sungurtekin H, Boston US, Orszulak TA, Cook DJ. Effect of cerebral embolization on regional autoregu-

lation during cardiopulmonary bypass in dogs. Ann Thorac Surg. 2000; 69: 1130-4.
5. Mutch WA, Sutton IR, Teskey JM, Cheang MS, Thompson IR. Cerebral pressure-flow relationship during cardiopulmonary bypass in the dog at normothermia and moderate hypothermia. J Cereb Blood Flow Metab. 1994; 14: 510-8.
6. Howard R, Trend P, Russell RW. Clinical features of ischemia in cerebral arterial border zones after periods of reduced cerebral blood flow. Arch Neurol. 1987; 44: 934-40.
7. O'Dwyer C, Prough DS, Johnes WE. Determinants of cerebral perfusion during cardiopulmonary bypass. J Cardiothorac Vasc Anesth. 1996; 10: 54-64.
8. Halsted JC, Spielvogel D, Meier DM, Weisz D, Bodian C, Zhang N, Griepp RB. Optimal PH strategy for selective cerebral perfusion. Eur J Cardiothracic Surg. 2005; 28: 266-273.
9. Gallyas F. Physico-chemical mechanism of the argyrophil III reaction. Histochemistry. 1982; 74 (3): 409-421.
10. Gallyas F, Guldner FH, Zoltay G, Wolff JR. Golgi-like demonstration of "dark" neurons with an argyrophil III method for experimental neuropathology. Acta Neuropathol 1990; 79 (6): 620-628.
11. Gallyas F, Zoltay G, Dames W. Formation of "dark" (argyrophilic) neurons of various origin proceeds with a common mechanism of biophysical nature (a novel hypothesis). Acta Neuropathol. 1992; 83 (5): 504-9.
12. Ishida A, Ueda Y, Ishida K, Misumi S, Masuda T, Fujita M, Hida H. Minor neuronal damage and recovered cellular proliferation in the hippocampus after continuous unilateral forelimb restraint in normal rats. J Neurosci Res 2011; 89: 457-465.
13. Ishida K, Ungusparkorn C, Hida H, Aihara N, Hida H, Ida K, Nishino H. Argyrophilic dark neurons distribute with a different pattern in the brain after over hours treadmill running and swimming in the rat. Neurosci Lett. 1999; 277 (3): 149-52.
14. Ishida K, Shimizu H, Hida H, Urakawa S, Ida K, Nishino H. Argyrophilic dark neurons represent various states of neuronal damage in brain insults: some come to die and others survive. Neuroscience 2004; 125 (3): 633-644.
15. Urakawa S, Hida H, Masuda T, Misumi S, Kim TS, Nishino H. Environmental enrichment brings a beneficial effect on beam walking and enhances the migration of doublecortin-positive cells following striatal lesions in rats. Neuroscience. 2007; 144: 920-933.
16. Halstead, J. C. Meier, M. Wurm, M. Zhang, N. Spielvogel, D. Weisz, D, Bodian C, Griepp RB. Optimizing selective cerebral perfusion: deleterious effects of high perfusion pressures. J Thorac Cardiovasc Surg. 2008; 135: 184-91.
17. Sakurada, T. Kazui, T. Tanaka, H. Komatsu, S. Comparative experimental study of cerebral protection during aortic arch reconstruction. Ann Thorac Surg. 1996; 61 (5): 1348-54.
18. Tanaka H, Kazui T, Sato H, Inoue N, Yamada O, Komatsu S. Experimental study on the optimum flow rate and pressure for selective cerebral perfusion. Ann Thorac Surg. 1995; 59 (3): 651-7.
19. Gourlay T, Modine T, The influence of cannulation technique on blood flow to the brain in rats undergoing cardiopulmonary bypass: A cautionary "Tail". Artificial Organs 2010; 34 (6): 498-522.
20. Rice J E, Vannuci C, Brierley J B. The influence of immatuarity on hypoxic-ishemic brain damage in rat. Annals Neurology. 1981; 92 (2): 131-141.
21. Masuda T, Hida H, Kanda Y, Aihara N, Ohta K, Yamada K, Nishino H. Oral administration of metal

chelator ameliorates motor dysfunction after a small hemorrhage near the internal capsule in rat. J. Neurosci Res 2007; 85: 213-22.

22. Mizuno K, Hida H, Masuda T, Nishino H. Pretreatment with low doses of erythropoietin ameliorates brain damage in periventricular leukomalacia by targeting late oligodendrocytes progenitor: a rat model. Neonatology 2008; 94: 255-66.
23. Spinnewyn B, Cornet S, Auguet M, Chabrier PE. Synergistic protective effects of antioxidant and nitric oxide synthase inhibitor in transient focal ischemia. J Cereb Blood Flow Metab 1999; 19 (2): 139-43.
24. Hagl C, Khaladj N, Wesz DJ, Zhang N, Guo LJ, Bodian CA, Spielvogel D, Griepp RB. Impact of high intracranial pressure on neurophysiological recovery and behavior in a chronic porcine model of hypothermic circulatory arrest. Eur J Cardiothorac Surg. 2002; 22 (4): 510-6.