Effect of constraint-induced movement therapy on the neurogenesis and functional recovery after hypoxic-ischemic injury in mice

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Finally, I dedicate this paper and my love to my wife and my son, Kyung Min.

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<ABSTRACT>

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In the hemiparetic patients with brain injury such as stroke and cerebral palsy (CP), the motor deficit of impaired limb is the result not of the damage per se, but of a learned suppression of movement. The constraint-induced movement therapy(CIMT) has been demonstrated to be effective for counteracting this learned non-use phenomenon in various hemiplegic conditions. Although many studies reported the clinical benefits for improving the function of impaired limb, there are few and inconclusive reports about the influence of CIMT for the injured brain tissue. This study investigated whether the CIMT would improve motor recovery in mice after early hypoxic-ischemic brain injury and whether the CIMT would induce the endogenous neurogenesis in the brain.

Postnatal day 7 CD1(ICR) mice were subjected to unilateral common carotid artery ligation to induce permanent ischemic brain injury. After surgery, the pups were exposed to 8% oxygen for 90 minutes which made hypoxic brain damage. At 3 weeks after injury, the mice with hypoxic-ischemic brain injury were assigned to the following 3 groups; control group(n=15) which was housed in the standard cage, enriched-environment group(EE, n=17) which was housed in the cage with enriched environment and constraint-induced

movement therapy group(CIMT, n=15) which was housed in the cage with enriched environment after restraint of unimpaired forelimb.

After 2 weeks of intervention, the horizontal ladder rung walking test which was sensitive for quantifying skilled locomotor movements showed that the percentage of foot-falls in the impaired forelimbs was significantly less in the CIMT group than in control and EE groups at 2 weeks and 4 weeks after end of intervention. Contrarily, the Rotarod test revealing the general motor function and balance and the grip strength test measuring the muscle strength of impaired forelimb showed no difference between all groups in all time points of measurement.

In the immunohistochemical analysis, a significant number of BrdU+ cells were observed in the subventricular zone and the striatum of all 3 experimental groups. In contrast, a significant increase in the density of BrdU+ βIII-tubulin^{+} cells in the subventricular zone of EE and CIMT groups compared to control group was observed.

In conclusion, the CIMT appears to be beneficial for facilitating the coordination of impaired forelimb, although there was no additional gain in muscle power and general motor function. The functional improvement was only obtained in CIMT group, although there was a significant increase of subventricular neurogenesis in both CIMT and EE groups. The mechanism that brings a functional improvement to only CIMT group remains to be clarified.

Key words : hypoxic-ischemic brain injury, constraint-induced movement therapy, enriched environment, induced neurogenesis, functional recovery

I. INTRODUCTION

Hemiparesis affects the majority of patients with nervous system injury, such as stroke and cerebral palsy. The general principle for the impairment of hemi-side is that a certain portion of the motor deficit resulting from damage to the nervous system is the result not of the damage per se, but of a learned suppression of movement.[1, 2] This result came from repeated failure with the impaired limb combined with successful use of the normal limb.[3]

Restraint of the unimpaired limb to force the use of an impaired limb during normal daily activities and rehabilitation therapy counteracts this "learned non-use" phenomenon.[3] This technique is termed constraint-induced movement therapy (CIMT) and it has been demonstrated to be effective for various hemiplegic conditions in multiple studies.

CIMT has been reported to be an effective treatment for improving the use of extremities affected by such neurologic injuries as stroke and traumatic brain injury in adults.[4-6] Since the year of 2003, it has been reported that CIMT also produced major and sustained improvement in motoric function in the young children with hemiplegic cerebral palsy.[7, 8] CIMT may work not only through behavioral changes, but also cortical reorganization.[9] However, the CIMT-induced tissue level changes of injured brain have not been fully demonstrated.

Actually, the environmental modifications such as enriched environment (EE) and application of exercises have been reported to have beneficial effects on the endogenous neurogenesis in the brain of rodents.[10-12] But, the excessive physical activities were reported to have no benefit on the functional improvement and the neurogenesis of brain.[13] Therefore, it is still questioned whether CIMT, which is more intensive physical activity than EE, would promote the neurogenesis in the injured brain although there have been many evidences showing clinical benefits of CIMT in human researches. Currently, there are few and inconclusive reports about the anatomical changes of brain

tissue after CIMT in the adult brain injury model.[3, 14] There has been no report about the effect of CIMT on the pediatric brain injury model and on the neurogenesis of injured brain.

The objective of this study was to demonstrate whether the environmental modification such as CIMT and EE would bring the functional benefits and also induce the endogenous neurogenesis in mice after early hypoxic-ischemic brain injury.

II. MATERIALS AND METHODS

1. Hypoxic-ischemic Model

Experimental protocols were approved by the local ethics committee. CD1(ICR) mice of both sexes were used for the experiments. These mice were maintained on a regular 12 hour light/12 hour dark cycle and accessed to food pellets and tap water *ad libitum*. A previously described hypoxic ischemia injury procedure was modified to suit immature mice.[15] At postnatal day 7, mice weighing 4 to 5 g were anesthetized with ketamine in the peritoneum and injured. In rodents, the 7-day-old pup has been variously likened to a 32- to 34-week-old human fetus.[16] The right common carotid artery was dissected out, and occluded by suture. The incision was sutured, and total surgery time usually did not exceed 5 minutes. After surgery, the pups were placed in an incubator within a chamber (Jeungdo B & P Co. Ltd, Seoul, Korea), through which a humidified atmosphere of 8% O_2 and 92% N_2 was flushed during the 90-minute exposure to hypoxia.(fig 1, a) The concentration of O_2 was monitored with Oxygen Monitor (Cat No. 5800; Hudson RCI, Temecula, CA, USA).(fig 1, b) The chamber temperature was kept constant at 37℃. Seven days after injury, the scalp was opened and the severity of brain injury was checked. The mice whose extents of injury were more than half of hemisphere were only included. (47 of total 91 mice)

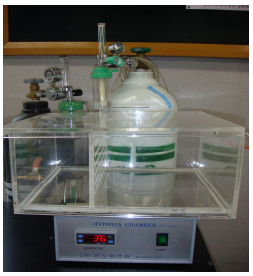
(a)  (b) 

Fig. 1. Hypoxic chamber and oxygen monitor. After surgery, the pups were placed in a hypoxic chamber (a) with a humidified atmosphere of 8% O_2 and 92% N_2. The concentration of O_2 was monitored with Oxygen Monitor (b).

2. Rehabilitation Therapies

Three weeks after injury, animals were assigned in a fully randomized and blinded fashion to the following 3 groups: control group(n=15), enriched-environment group(EE, n=17), and constraint-induced movement therapy (CIMT, n=15)

Control group mice housed in 25 x 20 x 11 cm-sized standard cages with bedding and *ad libitum* access to food and water.(fig 2, a) They received no additional therapy. EE group housed in a large cage (84 x 74 x 29cm) specially prepared with various stuffs enhancing novelty and complexity, such as plastic tunnels, a running wheel and wooden blocks, for 14 days.(fig 2, b)[17, 18] This condition is known that facilitate enhanced sensory, cognitive and motor stimulation.[19] CIMT group received CIMT therapy in enriched environmental condition for 14 days. CIMT involved restraining the forelimb ipsilateral to the lesion using the Elastic tape with Liner (3M Corp., Seoul, Korea) that wrapped around the forelimb and upper torso of the mice.(fig 2, c) The bandage was continuously applied for 7 days. After one day of the rest, the bandage was applied again for 7 days, mimicking human therapy.

(a) (b) (c)

Fig. 2. Housing conditions of experimental groups. (a) Standard cages with bedding and *ad libitum* access to food and water. (b) Enriched environment in a large cage (84 x 74 x 29cm) specially prepared with various stuffs enhancing novelty and complexity, such as plastic tunnels, a running wheel and wooden blocks. (c) Restraints of constraint-induced movement therapy group were made by wrapping with the elastic bandage.

3. Behavioral Testing

All behavioral tests were performed before (baseline) and 0, 2 and 4 weeks after 14 days of intervention.

A. Rotarod test

Motor coordination and balance were measured using rotarod analysis.[20] For this test, mice were placed on an Rotarod treadmill (Cat No. 47600, UGO Basile, VA, Italy), and the time the animals remained on the Rotarod was measured.(fig 3, a)[21, 22] The mice were placed on the rod and then ran while it was rolling. Speed was set at 56 and 64 rpm. Additionally, speed was increased from 4 to 80 rpm within 5 minutes. The trial ended if the animal fell off the rungs or gripped the device and spun around for 2 consecutive revolutions without attempting to walk on the rungs. An arbitrary time limit of 300 seconds was set for the mice on the Rotarod cylinder in the testing procedures. The mean duration of 3 trials was analyzed.

B. Horizontal ladder rung walking test

Horizontal ladder rung walking is a sensitive test for quantifying skilled locomotor movements, and it is known that the degree and duration of motor dysfunction following brain injury can be measured by counting the number of foot-faults made by the affected limb over 50 steps.[21, 23]

The previously described apparatus for rats[23] was modified to suit mice. Metal rungs (2 mm diameter) could be inserted to create a floor with a minimum distance of 7 mm between rungs. The side walls were 70 cm long and 15 cm high measured from the height of the rungs. The ladder was elevated 11 cm above the ground. The width of the alley was 3 cm, which was about 1cm wider than an animal to prevent the animal from turning around.(fig 3, b) A regular pattern of the rungs could allow the animals to learn the pattern over several trials and to anticipate the location of the rungs.

So we made the spacing of the rungs change irregularly (maximal inter-rung distance did not exceed 14 cm) and used it as a template for test. Analysis was made by inspection of the video recordings. An error represents any kind of foot slip or total miss between rungs. The number of errors and the number of steps was counted. From these data, the percentage of errors through all the steps was calculated and averaged by 5 trials, which was defined as fall rate.

C. Grip strength test

The apparatus comprises a push-pull strain gauge, SDI Grip Strength System (San Diego Instruments Inc., San Diego, CA, USA). A 2 mm diameter triangular piece of metal wire was used as the grip bar.(fig 3, c) A mouse was held near the base of its tail and approached toward the bar until it gripped the bar with one forepaw. The mouse was then gently pulled away from the bar at a steady rate until the bar was released. Peak force was automatically registered in grams-force by the apparatus. The mean peak force of 3 trials was analyzed.

(a) (b) (c)

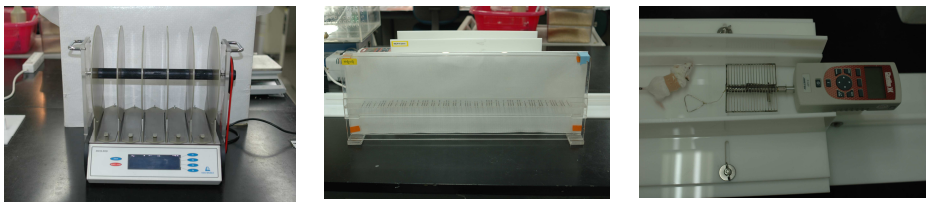


Fig. 3. Functional tests. (a) Rotarod test measured the motor coordination and balance. Rolling speed was set at 56 and 64 rpm. Additionally, speed was increased from 4 to 80 rpm within 5 minutes. (b) Horizontal ladder rung walking quantified the skilled locomotor movements. The percentage of errors through all the steps was calculated and averaged by 5 trials. (c) Peak grip force was automatically registered by the computerized apparatus. The mean peak force of 3 trials was analyzed.

4. Neurogenesis Detection and Histological Quantification In Vivo

During the 14 days of intervention, mice were injected daily with the mitotic marker BrdU (100mg/kg, i.p.). Mice were sacrificed 14 days after the last BrdU injection and perfusion fixed with 4% paraformaldehyde. Their brains were cryosectioned coronally at 16-㎛ thickness (every 256 ㎛) on dry ice, then stained for BrdU and neuronal marker βIII-tubulin, using rat anti-BrdU (1:100; Oxford Biotechnology, Oxfordshire, UK) and mAb TuJ1 (mouse IgG, 1:400; Covance, NJ, USA). Secondary antibodies were goat anti-rat Alexa 488 (1:400, Molecular Probes, Eugene, OR, USA) and goat anti-mouse Alexa 594 (1:400, Molecular Probes, Eugene, OR, USA).

First we analyzed the total number of $BrdU^+$ cells, which represents all new cells including neurons, astrocytes, oligodendrocytes, microglia, and macrophages. In sections double-stained for BrdU and βIII-tubulin, single $BrdU^+$ cells were selected for confocal imaging. Briefly, the images of every cell double-immunostained with BrdU and βIII-tubulin were observed orthogonally in both the vertical and horizontal plane using LSM 510 confocal microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA). When a cell as double-labeled, with central BrdU immunoreactivity surrounded by neuronal staining from all observation angles in every serial optical section and in each merged and rotated composite, the cells were scored as newly generated neurons.

Striatal and subventricular $BrdU^+$ cells counts were done on three 16-㎛ coronal sections at 256-㎛ interval per animal. First, we analyzed all the $BrdU^+$ cells in each area. The striatal region sampled began with the first appearance of striatal fascicles. In each striatum and subventricular zone, total $BrdU^+$ nuclei and cells colabeled with βIII-tubulin were counted; these results were converted into $BrdU^+$ cells/mm^3 after determining the striatal surface area using MetaMorph Imaging System (Molecular Device, Sunnyvale, CA, USA), with which the net volume of each striatum was estimated.

5. Data and Statistical Analysis

Comparisons of functional measurements and the number of $BrdU^+$ βIII-$tubulin^+$ cells/mm3 in each group were performed using a 1-way ANOVA or Kruskal-Wallis test for each time point.

The statistical significance of differences in functional parameters and neurogenesis was determined using post hoc Tukey's test. Statistical calculations and analyses were done with SPSS 12.0 for windows. A p-value < 0.05 was considered significant.

III. RESULTS

1. Rotarod test

Comparison of the latency to fall off using 1-way ANOVA showed no significant difference between all groups before intervention. The latency to fall off at 2 rolling speeds and accelerated mode revealed no difference between groups in all time points of measurement. (Table 1)

Table 1. Comparison of the latency to fall off in Rotarod test between control, EE and CIMT groups.

		Pre-intervention (seconds)	0 weeks (seconds)	2 weeks (seconds)	4 weeks (seconds)
56rpm	Control	147.7±119.5	166±116.5	116.8±112.9	120.1±129.8
	EE	89.9±110.9	135.1±121.2	65.0±85.7	142.3±148.3
	CIMT	92.2±104.8	96.2±101.2	119.7±111.5	144.4±115.8
64rpm	Control	108.9±106.7	76.3±82.7	55.8±41.4	103.6±93.2
	EE	61.5±78.4	76.9±85.0	45.4±92.1	97.2±104.9
	CIMT	83.5±107.4	80.8±104.1	86.9±121.7	104.7±117.8
Accelerated	Control	158.5±59.2	167.5±52.9	112.2±44.0	154.6±34.4
	EE	151.8±82.6	165.5±65.1	126.8±71.4	139.8±81.0
	CIMT	126.7±63.2	143.3±67.5	138.4±85.7	165.5±78.8

Values are mean±S.D.

Abbreviations: EE, enriched environment; CIMT, constraint-induced movement therapy

2. Horizontal ladder rung walking test

The fall rates of unaffected-side forelimbs were not different between control, EE and CIMT groups in the measurements of pre-intervention (5.1±5.1% vs. 4.4±4.2% vs. 4.4±5.0%), 0 week after intervention (5.5±5.5% vs. 4.4±5.5% vs. 3.7±4.0%), 2 weeks after intervention (4.2±4.9% vs. 5.0±5.6% vs. 5.2±4.2%) and 4 weeks after intervention (3.3±4.7% vs. 3.7±4.7% vs. 2.3±3.3%). The percentage of falls in the hemi-side forelimbs was significantly less in CIMT group than in control and EE groups. (Pre-intervention; 8.8±7.8% vs. 10.6±10.1% vs. 8.1±7.2%, 0 week after intervention; 9.8±7.2% vs. 12.7±14.6% vs. 4.8±4.7%, 2 weeks after intervention; 14.3±7.3% vs. 12.4±7.7% vs. 5.4±3.6%, 4 weeks after intervention; 12.3±7.1% vs. 17.3±13.1% vs. 4.0±3.1%) It appeared to diverge at 2 weeks after the end of intervention. ($P < 0.05$)(fig 4)

(a) (b)

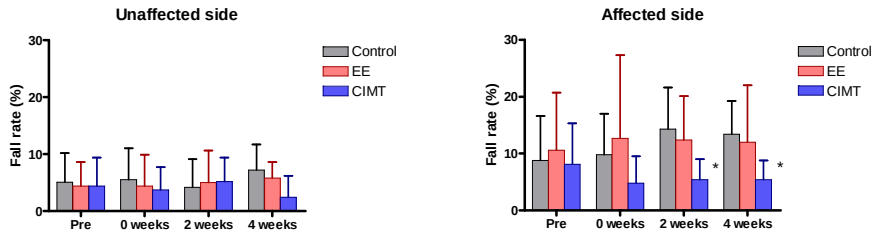


Fig. 4. The percentage of falls in forelimbs while crossing the ladder. (a) The fall rates of unaffected-side forelimbs at each time point. (b) The fall rates of affected-side forelimbs at each time point. The fall rate was defined by percentage of number of errors from the number of steps during walking through the horizontal ladder. Constraint-induced movement therapy (CIMT) group revealed the improvement of affected forelimb's locomotion from 2 weeks after intervention compared to both control and enriched environment (EE) groups. ($P < 0.05$)

3. Grip strength test

The grip strengths of unaffected-side forelimbs were not different between control, EE and CIMT groups in the measurements of pre-intervention (24.0±11.4grams vs. 31.8±15.6grams vs. 24.7±13.7grams), 0 week after intervention (35.7±9.0grams vs. 45.0±8.5grams vs. 32.2±9.0grams), 2 weeks after intervention (28.0±11.5grams vs. 46.5±15.1grams vs. 40.3±14.0grams) and 4 weeks after intervention (55.3±13.0grams vs. 59.0±9.7grams vs. 43.9±7.0grams). The grip strength of affected-side forelimbs also revealed no difference between control, EE and CIMT groups in the measurements of pre-intervention (24.0±20.4grams vs. 26.5±14.6grams vs. 20.1±14.3grams), 0 week after intervention (35.1±20.6grams vs. 40.5±18.1grams vs. 32.2±9.6grams), 2 weeks after intervention (35.2±25.6grams vs. 44.9±21.8grams vs. 50.8±11.6grams) and 4 weeks after intervention (50.6±8.9grams vs. 45.4±15.2grams vs. 57.3±10.3grams). (fig 5)

(a) (b)

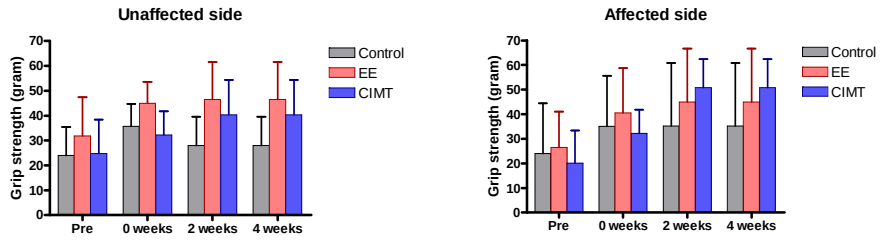


Fig. 5. The grip strengths of unaffected- and affected-side forelimbs. (a) The grip strengths of unaffected-side forelimbs at each time point. (b) The grip strengths of affected-side forelimbs at each time point. The grip strength revealed no difference between groups in all time points of measurement.

4. Number of proliferated endogenous neural stem cells

The total number of $BrdU^+$ cells, which represents all new cells including neurons, astrocytes, oligodendrocytes, microglia, and macrophages, and $BrdU^+$ βIII-tubulin$^+$ cells, which represents neurons, were counted in the subventricular zone and striatum. In the subventricular zone, although a significant number of $BrdU^+$ cells were observed in all 3 experimental groups, there was no difference between groups. In contrast, a significant increase in the density of $BrdU^+$ βIII-tubulin$^+$ cells in the EE and CIMT groups compared to control group was observed. ($P < 0.05$)(Table 2)(fig 6, 7)

In the striatum, the number of $BrdU^+$ cells and $BrdU^+$ βIII-tubulin$^+$ cells showed no significant difference in all 3 experimental groups. (Table 2)(fig 6)

Table 2. Comparison of cell proliferation and neurogenesis between control, EE and CIMT groups in the SVZ and striatum at 2 weeks after intervention.

		$BrdU^+$ cells/mm^3	$BrdU^+$ βIII-tubulin$^+$ cells/mm^3
Control group	SVZ	6466.07 ± 4097.62	735.60 ± 1158.98
	Striatum	1208.88 ± 1140.34	92.50 ± 195.02
EE group	SVZ	15628.75 ± 16707.84	6488.42 ± 4202.15*
	Striatum	1241.27 ± 53.81	476.37 ± 509.26
CIMT group	SVZ	12735.43 ± 7175.53	4798.73 ± 3989.91*
	Striatum	2458.73 ± 3714.82	565.89 ± 702.53

Values are mean ± SD. Comparisons were made by one-way ANOVA(Analysis of Variance) with Tukey *post hoc* test.

Abbreviations: EE, enriched environment; CIMT, constraint-induced movement therapy; SVZ, subventricular zone

*$P < .05$; control vs. EE groups and CIMT group

(a) (b)

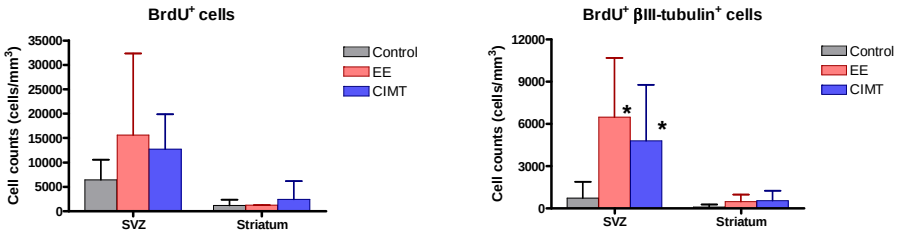
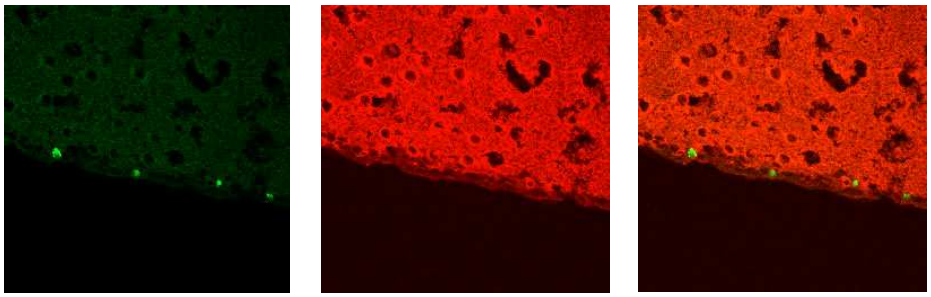


Fig. 6. Comparison of cell proliferation and neurogenesis in the subventricular zone (SVZ) and striatum at 2 weeks after intervention. (a) Although a significant number of $BrdU^+$ cells were observed in all 3 experimental groups, there was no difference between groups in both SVZ and striatum. (b) A significant increase in the density of $BrdU^+$ βIII-$tubulin^+$ cells in the enriched environment (EE) and constraint-induced movement therapy (CIMT) groups compared to control group was observed in the SVZ, not in the striatum. ($P < 0.05$)

(a)



BrdU (green) βIII-tubulin (red) Merged

(b) (c)

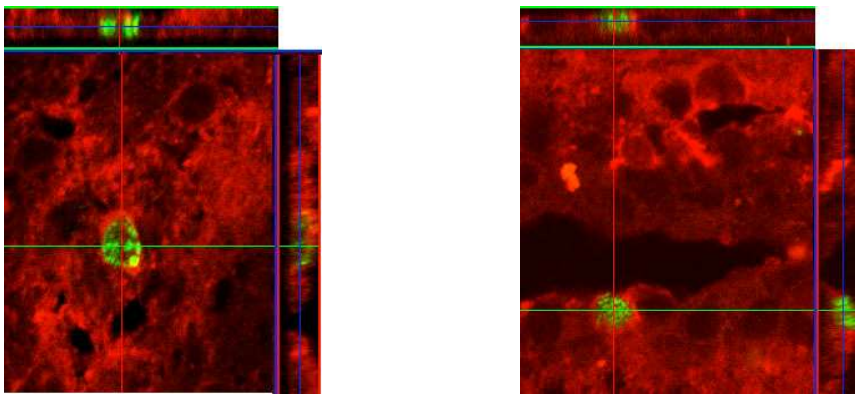


Fig. 7. Cell proliferation and neurogenesis in the subventricular zone and the striatum. At 2 weeks after intervention, many cells in the subventricular zone of affected hemisphere were double-stained with $BrdU^+$ (green) and $\beta III\text{-tubulin}^+$ (red) in the CIMT group (a). $BrdU^+$ and $\beta III\text{-tubulin}^+$ double-stained cells were confirmed using confocal microscope in both the striatum (b) and subventricular zone (c).

IV. DISCUSSION

CIMT is meant that the unaffected limb is restrained while intensive motor training for affected limb. This therapy has been reported to be an effective treatment for improving the use of extremities in the patients with hemiplegia due to various brain injury.[4-6] It is also known to produce major and sustained functional improvement in the young children with hemiplegic cerebral palsy.[7, 8] However, the possibility of organic changes in brain after CIMT was recently investigated and have not been fully demonstrated.

DeBow et al.[3] investigated the efficacy of CIMT for the adult rats with intracerebral hemorrhage(ICH) in the aspect of functional and histological recovery. In their study, the combination of rehabilitation exercises and CIMT 7 days after injury substantially and persistently improved functional recovery of affected limb. In addition, they found that this group had a statistically smaller volume of tissue loss than untreated ICH rats.

The cellular level changes of injured brain after CIMT with rehabilitation exercises have not been investigated yet. Actually, for the normal brain, the changes of environmental factors and therapeutic exercise have been reported to increase the neurogenesis especially in the hippocampus of adult rodents.[10-12] However, if the physical activity is too intense, it cannot induce beneficial effect on the brain tissue. In normal adult mice, the EE and voluntary wheel running were reported to increase neurogenesis in the dentate gyrus, while the more intensive physical activity such as forced exercise using water mazing test had no beneficial effect on neurogenesis contrarily.[13] Accordingly, although some studies reported the clinical and histological benefits of CIMT, it seems like that CIMT which is more intensive physical activity than EE can have both beneficial and adverse effects on the brain tissue according to the factors such as intensity and timing of application.

Additionally, for the injured brain, there have been controversies for the effect of environmental factors on neurogenesis. In the 2 studies using adult

rodents with middle cerebral artery infarction, one reported that EE increased the neural stem/progenitor cell pool and neurogenesis in the subventricular zone.[24] On the contrary, the other reported that an EE increased neurogenesis in the hippocampus, not in the subventricular zone, and decreased cell genesis and migration of neuroblasts and newborn astrocytes in the striatum instead.[25] However, both of them could not explain the mechanisms behind their findings. Moreover, both studies were performed using the rodents of adult brain injury model and there have been no report for the effect of environmental factors on neurogenesis in the early brain injury model.

For this reason, it has been investigated whether the CIMT would improve motor recovery in mice after early hypoxic-ischemic brain injury and whether the CIMT would induce the endogenous neurogenesis in the brain. The CIMT was applied 3 weeks after injury because the CIMT immediate after injury was reported to aggravate the volume of injury by localized events including hyperthermia in the research using the rats with devascularization lesions in brain.[14]

Classical method of constraint therapy is meant that the unaffected limb is restrained while intensive motor training. This motor training can partially be mimicked in animal studies by housing the animals in an EE. Accordingly, the mice were housed in the cage of EE after restraining the unaffected limb.

In this study, mice in the CIMT group performed significantly better than control and EE groups in the horizontal ladder rung walking test, but not in the Rotarod test and the grip strength test by comparing group means at each time point. Rotarod test is known to reveal the general function of motor coordination and balance.[20] Grip strength test was used to quantify the strength of the muscle.[26] In contrast, the horizontal ladder rung walking test is known to be sufficiently challenging to reveal subtle impairments in forelimb use and unmask impairments that require forebrain control.[23] Furthermore, the animal's task is relatively simple, as they spontaneously walk across the

ladder to reach a refuge, and therefore no special training is required. A reliable discrimination between the different experimental groups can be achieved by just counting the number of footfall errors made. Accordingly, the advantage of CIMT is thought to facilitate the coordination of affected limb, but not to strengthen the muscle power and not to promote the general motor function. Actually, in the studies investigating the effect of CIMT for humans with CP, CIMT improved the amount of use and the quality of movement in affected upper extremities, not strength and general motor function.[8, 27]

For investigating the effect of CIMT on neurogenesis of injured brain, the 2 target regions, subventricular zone and striatum, were focused. Since Kempermann et al.[28] reported that mice exposed to an EE have more granule cell neurons in dentate gyrus, many researchers have studied the EE-induced neurogenesis in this 2 regions, subventricular zone and striatum.[10-13, 24] In this study, although $BrdU^+$ cells were frequently observed in the subventricular zone and the striatum of all 3 experimental groups, there was no difference between groups. $BrdU^+$ cells represents all newly proliferated cells including neurons, astrocytes, oligodendrocytes, microglia, and macrophages. To verify the neuronal cells from these $BrdU^+$ cells, the incorporation of BrdU into βIII-tubulin-expressing cells was analyzed using confocal microscopy and 3-dimensional analysis. In contrast, a significant increase in the density of $BrdU^+$ βIII-$tubulin^+$ cells in the subventricular zone of EE and CIMT groups compared with control group was observed, but there was no difference between EE and CIMT groups. In the human who walk upright, the learned non-use phenomenon can be significant in the hemi-paretic upper limb. Contrarily, in the rodents which walk on all fours, the walking activity itself forces the use of affected forelimb and this may result in the similar induction of neurogenesis in both EE and CIMT groups.

Although the subventricular neurogenesis of both EE and CIMT groups increased more than the control group, the EE group was insufficient to

produce the statistically significant improvement in the results of all 3 functional tests. However, EE group also showed the tendency of decreased foot fall rate in the horizontal ladder rung walking test and increased strength in the grip strength test compared to the control group. For that reason, the increased subventricular neurogenesis after CIMT might contribute to the functional improvement of affected forelimb although this alone did not easily account for all of the improved recovery.

CIMT is a rehabilitation regime that has been shown to improve the motor function of upper limb in patients with hemiplegic CP. However, neural mechanism of this improvement was poorly understood. Many researchers have tried to reveal the mechanism of improvement after CIMT. In the study of adult stroke patients[29], functional gain produced by CIMT in chronic stroke patients was suggested to be associated with a shift in laterality of motor cortical activation toward the undamaged hemisphere. Recently, increased cortical activation in the ipsilateral motor field was reported with increased contralateral movement evoked field after CIMT in the patients with spastic hemiplegic CP.[9]

Accordingly, the question remains how CIMT-induced neurogenesis in the subventricular zone corresponds to improved motor behavior of paralyzed limb after hypoxic-ischemic injury. Additionally, while EE also promoted the subventricular neurogenesis, EE group showed no significant functional improvement compared to the control group. As a result, although our study showed increased neurogenesis in CIMT group, the additional mechanism of functional improvement after CIMT should be considered.

This is the first report of neurogenesis after CIMT in a mouse model with early hypoxic-ischemic brain injury. However, this study is limited by the fact that enhancement of post-injury neurogenesis is not quantitatively analyzed to be correlative to an improvement in motor recovery of paralyzed limb. While an increase of neurogenesis in subventricular zone would be expected to result

in fewer behavioral deficits, it is unlikely to account solely for the functional improvements in the CIMT group. Further experiments are needed to specifically block the neurogenesis to dissociate neurogenetic effects from other plastic factors after hypoxic-ischemic brain injury and the additional mechanism of functional improvements should be investigated.

V. CONCLUSION

Postnatal day 7 CD1 (ICR) mice were subjected to unilateral common carotid artery ligation to induce permanent ischemic brain injury. After surgery, the pups were exposed to 8% oxygen for 90 minutes which made hypoxic brain damage. At 3 weeks after injury, the mice with hypoxic-ischemic brain injury were assigned to the following 3 groups; control group (n=15) which was housed in the standard cage, enriched-environment group (EE, n=17) which was housed in the cage with enriched environment and constraint-induced movement therapy group (CIMT, n=15) which was housed in the cage with enriched environment after restraint of unimpaired forelimb.

After 2 weeks of intervention, Rotarod test, horizontal ladder rung walking test and grip strength test for measuring functional changes and immunohistochemical analysis for observing the cell proliferation and neurogenesis were performed. The results were as follows;

1. In the horizontal ladder rung walking test which was sensitive for quantifying skilled locomotor movements showed that the percentage of foot-falls in the impaired forelimbs was significantly less in the CIMT group than in control and EE groups at 2 weeks and 4 weeks after end of intervention.
2. Contrarily, the Rotarod test revealing the general motor function and balance and the grip strength test measuring the muscle strength of impaired forelimb showed no significant difference between all groups in all time points of measurement.
3. In the immunohistochemical analysis, a significant number of BrdU+ cells were observed in the subventricular zone and the striatum of all 3 experimental groups. In contrast, a significant increase in the density of BrdU+ βIII-tubulin^{+} cells in the subventricular zone of both EE and CIMT groups compared to control group was observed.

REFERENCES

1. Taub E, Miller NE, Novack TA, Cook EW, Fleming WC, Nepomuceno CS et al. Technique to improve chronic motor deficit after stroke. Archives of physical medicine and rehabilitation 1993;74(4):347-54.

2. Taub E, Uswatte G, Mark VW, Morris DM. The learned nonuse phenomenon: implications for rehabilitation. Europa medicophysica 2006;42(3):241-56.

3. DeBow SB, Davies ML, Clarke HL, Colbourne F. Constraint-induced movement therapy and rehabilitation exercises lessen motor deficits and volume of brain injury after striatal hemorrhagic stroke in rats. Stroke; a journal of cerebral circulation 2003;34(4):1021-6.

4. Kunkel A, Kopp B, Muller G, Villringer K, Villringer A, Taub E et al. Constraint-induced movement therapy for motor recovery in chronic stroke patients. Archives of physical medicine and rehabilitation 1999;80(6):624-8.

5. Miltner WH, Bauder H, Sommer M, Dettmers C, Taub E. Effects of constraint-induced movement therapy on patients with chronic motor deficits after stroke: a replication. Stroke; a journal of cerebral circulation 1999;30(3):586-92.

6. Taub E, Uswatte G, Pidikiti R. Constraint-Induced Movement Therapy: a new family of techniques with broad application to physical rehabilitation--a clinical review. Journal of rehabilitation research and development 1999;36(3):237-51.

7. Eliasson AC, Bonnier B, Krumlinde-Sundholm L. 'Clinical experience of constraint induced movement therapy in adolescents with hemiplegic cerebral palsy--a day camp model'. Developmental medicine and child neurology 2003;45(5):357-9.

8. Taub E, Ramey SL, DeLuca S, Echols K. Efficacy of constraint-induced movement therapy for children with cerebral palsy with asymmetric motor impairment. Pediatrics 2004;113(2):305-12.

9. Sutcliffe TL, Gaetz WC, Logan WJ, Cheyne DO, Fehlings DL. Cortical reorganization after modified constraint-induced movement therapy in pediatric hemiplegic cerebral palsy. Journal of child neurology 2007;22(11):1281-7.
10. Kempermann G, Brandon EP, Gage FH. Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. Curr Biol 1998;8(16):939-42.
11. Kempermann G, Kuhn HG, Gage FH. Experience-induced neurogenesis in the senescent dentate gyrus. J Neurosci 1998;18(9):3206-12.
12. Rosenzweig MR, Bennett EL, Hebert M, Morimoto H. Social grouping cannot account for cerebral effects of enriched environments. Brain Res 1978;153(3):563-76.
13. van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 1999;2(3):266-70.
14. DeBow SB, McKenna JE, Kolb B, Colbourne F. Immediate constraint-induced movement therapy causes local hyperthermia that exacerbates cerebral cortical injury in rats. Canadian journal of physiology and pharmacology 2004;82(4):231-7.
15. Aden U, Dahlberg V, Fredholm BB, Lai LJ, Chen Z, Bjelke B. MRI evaluation and functional assessment of brain injury after hypoxic ischemia in neonatal mice. Stroke 2002;33(5):1405-10.
16. Yager JY. Animal models of hypoxic-ischemic brain damage in the newborn. Seminars in pediatric neurology 2004;11(1):31-46.
17. Dahlqvist P, Ronnback A, Bergstrom SA, Soderstrom I, Olsson T. Environmental enrichment reverses learning impairment in the Morris water maze after focal cerebral ischemia in rats. Eur J Neurosci 2004;19(8):2288-98.
18. Grabowski M, Sorensen JC, Mattsson B, Zimmer J, Johansson BB. Influence of an enriched environment and cortical grafting on functional

outcome in brain infarcts of adult rats. Exp Neurol 1995;133(1):96-102.

19. Nithianantharajah J, Hannan AJ. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nature reviews 2006;7(9):697-709.

20. Andreassen OA, Dedeoglu A, Ferrante RJ, Jenkins BG, Ferrante KL, Thomas M et al. Creatine increase survival and delays motor symptoms in a transgenic animal model of Huntington's disease. Neurobiology of disease 2001;8(3):479-91.

21. Ikeda R, Kurokawa MS, Chiba S, Yoshikawa H, Hashimoto T, Tadokoro M et al. Transplantation of motoneurons derived from MASH1-transfected mouse ES cells reconstitutes neural networks and improves motor function in hemiplegic mice. Exp Neurol 2004;189(2):280-92.

22. Schabitz WR, Berger C, Kollmar R, Seitz M, Tanay E, Kiessling M et al. Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke 2004;35(4):992-7.

23. Metz GA, Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 2002;115(2):169-79.

24. Komitova M, Mattsson B, Johansson BB, Eriksson PS. Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. Stroke 2005;36(6):1278-82.

25. Nygren J, Wieloch T, Pesic J, Brundin P, Deierborg T. Enriched environment attenuates cell genesis in subventricular zone after focal ischemia in mice and decreases migration of newborn cells to the striatum. Stroke 2006;37(11):2824-9.

26. Bertelli JA, Mira JC. The grasping test: a simple behavioral method for

objective quantitative assessment of peripheral nerve regeneration in the rat. Journal of neuroscience methods 1995;58(1-2):151-5.
27. Taub E, Griffin A, Nick J, Gammons K, Uswatte G, Law CR. Pediatric CI therapy for stroke-induced hemiparesis in young children. Developmental neurorehabilitation 2007;10(1):3-18.
28. Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. Nature 1997;386(6624):493-5.
29. Schaechter JD, Kraft E, Hilliard TS, Dijkhuizen RM, Benner T, Finklestein SP et al. Motor recovery and cortical reorganization after constraint-induced movement therapy in stroke patients: a preliminary study. Neurorehabilitation and neural repair 2002;16(4):326-38.

< ABSTRACT(IN KOREAN)>

저산소성-허혈성 뇌손상을 받은 생쥐에서 건측제한 치료가 신경분화 및 기능적 회복에 미치는 효과

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나 동 욱

뇌졸중이나 뇌성마비와 같은 뇌 손상으로 인하여 발생한 편마비 환자에서 이환측 상지의 운동기능 소실은 뇌 손상 자체뿐 아니라 학습된 비사용 (learned non-use)에 기인한다고 알려져 있다. 건측제한 운동치료(constraint-induced movement therapy)는 다양한 편마비 환자에서 이러한 학습된 비사용을 극복하게 하는 데에 효과적인 치료로 알려져 있다. 많은 기존의 임상연구들에서 건측제한 운동치료가 이환측 상지의 기능을 증진시킨다고 보고하고 있지만, 손상된 뇌 조직에 미치는 영향에 대한 연구는 제한적으로만 보고되고 있으며 뇌성마비 모델에 해당하는 조기 뇌 손상 동물에서의 연구는 보고된 바가 없다. 본 연구에서는 이러한 건측제한 운동치료가 조기 저산소성-허혈성 뇌손상을 입은 생쥐의 운동기능 회복 및 뇌조직의 신경재생에 어떤 영향을 주는지 연구하고자 하였다.

생후 7일째의 CD1(ICR) 생쥐에서 편측 총경동맥을 결찰하여 영구적인 허혈성 뇌손상을 유발한 후 8%의 산소농도를 유지한

저산소방에서 90분 동안 유지하여 저산소성 뇌손상을 유발하였다. 손상 유발 3주 후에 저산소성-허혈성 뇌손상을 입은 생쥐들을 각각 대조군 (n=15), 강화환경군 (n=17), 건측제한 운동치료군 (n=15)으로 나누었다. 대조군은 일반적인 표준사육환경에서 사육하였고, 강화환경군은 보다 넓고 다양한 운동, 감각 및 인지 등의 자극을 줄 수 있는 사물들이 제공되는 우리에서 사육하였으며, 건측제한 운동치료군은 건측 앞발을 탄력붕대로 몸통에 고정시켜 사용할 수 없도록 제한하면서 강화환경 우리에서 사육하였다. 사육기간은 모두 2주로 하였다. 2주간의 사육기간이 끝난 후 시행한 기능적 평가 중, 이환측 앞발의 기술적인 운동기능을 정량화 할 수 있는 평행사다리 건너기 검사(horizontal ladder rung walking test)에서 건측제한 운동치료 군이 대조군과 강화환경군에 비해 앞발의 실수 횟수가 의미 있게 감소하였다. 반대로 전반적인 운동기능 및 균형기능을 평가하는 Rotarod 검사와 이환측 앞발의 근력을 측정하는 잡기 근력 검사에서는 각 군간에 뚜렷한 차이를 보이지 않았다.

면역조직학적 검사에서는 모든 실험군에서 뇌실 하 구역 및 기저핵에서 $BrdU^+$ 세포들이 다수 관찰되었다. 반면 $BrdU^+$ $\beta III\text{-}tubulin^+$ 세포는 강화환경군과 건측제한 운동치료군의 뇌실 하 구역에서 대조군에 비하여 의미 있게 증가된 소견이 관찰되었다.

결론적으로 건측제한 운동치료는 이환측 앞발의 운동조절기능을 의미 있게 증진시키지만 근력 및 전반적인 운동기능에는 영향을 미치지 않음을 알 수 있었으며, 강화 환경과 건측제한 운동치료 모두 뇌실 하 구역의 세포증식 및 신경분화를 증진시킴을 알 수 있었다. 이러한 뇌실 하 구역의 신경분화 증진은 건측제한 운동치료군에서 기능적 회복에 기여할 것으로 기대되지만, 추후

신경분화와 기능적 회복간의 연관성에 대한 분석 및 이 외의 추가적인 회복기전에 대한 연구가 필요하다.

핵심되는 말: 저산소성-허혈성 뇌 손상, 건측제한 운동치료, 강화환경, 신경분화, 기능적 회복