Effects of Motor Skill Learning and Treadmill Exercise on Motor Performance and Synaptic Plasticity in Harmaline Induced Cerebellar Injury Model of Rat

This study is intended to examine the motor skill learning and treadmill exercise on motor performance and synaptic plasticity in the cerebellar injured rats by harmaline. Experiment groups were divided into four groups and assigned 15 rats to each group. Group I was a normal control group(induced by saline); Group II was a experimental control group(cerebellar injured by harmaline); Group III was a group of motor skill learning after cerebellar injured by harmaline; Group IV was a group of treadmill exercise after cerebellar injured by harmaline. In motor performance test, the outcome of group II was significantly lower than the group II, IV especially group III(p<0.01). In histological finding, the experimental groups were destroy of dendrites and nucleus of cerebellar neurons. Group III, IV were decreased in degeneration of cerebellar neurons especially group II. In immunohistochemistic response of synaptophysin in cerebellar cortex, experimental groups were decreased than group I. Group III’s expression of synaptophysin was more increased than group II, IV. In electron microscopy finding, the experimental groups were degenerated of Purkinje cell. These result suggest that improved motor performance by motor skill learning after harmaline induced is associated with dynamically altered expression of synaptophysin in cerebellar cortex and that is related with synaptic plasticity.

Key words: Cerebellar; Harmaline; Motor Performance; Motor Skill Learning; Synaptic Plasticity

INTRODUCTION

Excessive sustained activation of glutamate receptors can kill neurons, particularly under conditions of reduced energy, availability and increased oxidative stress(1). This phenomenon, which is called excitotoxicity, can be dramatically demonstrated by exposing cultured neurons to high concentrations of glutamate and by exposing animals to excitotoxins such as kainic acid and domoic acid which, in contrast to glutamate, induced non-desensitizing ion currents and are not activity removed from the extracellular space(2). NMDA(N-methyl-D-aspartate), ibotenate, kainate, harmaline are substances that cause the neurotoxicity. Especially, harmaline is typical excitatory substance that associated with cerebellar damage(3).

The indole alkaloid harmaline has been to cause tremor and ataxia, and produced cerebellar neurotoxicity(4). The harmaline induced tremor model is regarded as the best model of essential tremor is that the olivocerebellar system plays a key role in pathophysiological mechanisms underlying both harmaline induced tremor in animals and essential tremor in humans(5). Important pharmacological action of harmaline is the selective induction of cerebellar Purkinje cell death. Harmaline primarily excites inferior olivary nucleus(ION), thereby providing excessive release of glutamate from nerve terminals of the olivocerebellar system of Purkinje cells(6).

The cerebellar receive inputs from spinal cord, medullar oblongata, pons, midbrain, hypothalamus, cerebrum cortex and generate outputs according to their internal rules of information processing(7). This
computation-centered view is consistent with a variety of proposed functions of the cerebellar, including sensory–motor integration, motor coordination, motor learning and timing. Moreover, feed-forward processing could be the common link between motor and non–motor function of the cerebellar(8). One of the most characteristic signs of cerebellar damage is walking ataxia(9). Physical therapists should mediate these movement disorders. Therefore, patients with cerebellar damage mediated method is focus on qualitative rather than quantitative movement for functional recovery.

In order to perform certain actions that should be made about the behavior of motor learning. After consisting motor learning, in order to perform an action is necessary to motor control(10). Based on this theory, aggressive treatment is done in clinical practice for patients with cerebellar damage. In addition, a variety of intervention methods and techniques are becoming. Modifications of neural properties, synchrony and synaptic efficacy are all related to the development and maintenance of motor skill(11). Motor skill learning is an experience that induces synaptogenesis in higher–order brain region involved in motor learning, such as the motor cortex and cerebellar(12). Proliferation of synapses is not merely the result of increased locomotor activity, as animals that engage in unskilled motor movement such as on a running wheel or treadmill exercise do not show any changes in the number of synaptogenesis in these brain areas(13).

The role of synaptic plasticity is particular depres- sion and facilitation, in sculpting network activity(14). Synaptophysin is a 38–kDa calcium–binding glycoprotein found in the membranes of neurotrans- mitter containing presynaptic vesicle, and increases in synaptophysin immuno–reactivity have most often been interpreted as reflecting an increase in presynaptic terminals(15).

The purpose of this study were to test the effect of motor skill learning on motor performance and histological change after neuronal toxicity cerebellar injury in the rat. And clinically applicable method of intervention is to evaluate the clinical effectiveness, light/dark cycle. Food and water were freely available. All experimental protocols were carried out according to the guidelines of the Dongshin University Animal Care and Use Committee. Care was taken to minimize numbers of animals used.

Harmaline(Sigma–Aldrich, H1392, USA), dissolved in physiological saline immediately before use, was administered intraperitoneally(30mg/kg)(6). After injection of harmaline, animals’ behavior, particularly the appearance of tremors, was observed.

**Table 1.** Classification of experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n=15)</td>
<td>Normal control(saline induced)</td>
</tr>
<tr>
<td>II (n=15)</td>
<td>Experimental control(harmaline induced)</td>
</tr>
<tr>
<td>II (n=15)</td>
<td>Harmaline induced with motor skill learning</td>
</tr>
<tr>
<td>IV (n=15)</td>
<td>Harmaline induced with treadmill exercise</td>
</tr>
</tbody>
</table>

**Motor Skill Learning**

The motor skill learning group was run daily on an acrobatic course designed to encourage problem solving and coordination and balance. The course was based on that used by Kleim et al, and was composed of 5 task(16). Rats traverse an elevated obstacle course consisting of rod, rope, barrier, parallel rods, grid platform. One session lasting approximately 30 min was conducted per day. The experimenter gently held the rat by the tail to prevent them from falling.

**Table 2.** Obstacles used for acrobatic training

<table>
<thead>
<tr>
<th>Task</th>
<th>Shape</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task–1</td>
<td>Rod</td>
<td>2.7cm diameter</td>
</tr>
<tr>
<td>Task–2</td>
<td>Rope</td>
<td>2cm diameter</td>
</tr>
<tr>
<td>Task–3</td>
<td>Barrier</td>
<td>13 to 21cm height</td>
</tr>
<tr>
<td>Task–4</td>
<td>Parallel rods</td>
<td>1.1cm each diameter</td>
</tr>
<tr>
<td>Task–5</td>
<td>Grid platform</td>
<td>2–3cm rectangle</td>
</tr>
</tbody>
</table>

**Treadmill Exercise**

Treadmill exercise of brain–damaged animals consisted of gradual adaptation to the running schedule: the first day they ran for several minutes and, by 5 day, they were able to complete 1 hr running(17).
Motor Performance Test

Rota-rod test
To evaluate the motor coordination, the rota-rod test was used, which measure balance, coordination, and motor control(18). Each rat was placed on a rod (diameter 7.5cm, length 10cm, height 35cm) covered with rubber to evaluate rota-rod performance. Rats were left for 5 min on the rod for habituation. The speed was slowly increased from 5 rpm to 20 rpm for 5 min and the dependent measure was the length of time on the rod. Three trials were conducted for each individual. Rats that stayed on the rota-rod for 300 S were considered complete responders, and their latencies were recorded as 300S. The trial was terminated when rat fell from the apparatus or after a maximum of 5 min.

Water maze-test
The water maze protocol was based on the work by Maaroufi et al,(19). The maze was made of opaque Plexiglas, measuring 90cm in diameter, with walls 30 cm in height and water level maintained at 15cm. The escape platform(diameter 8cm) was covered with a grid in order to provide firm gripping. Small black and white plastic beads covered the water surface in order to hide the escape platform from the animal’s view. The escape platform was hidden 1cm beneath water level in the middle, The rats were placed in the centre of the pool and the time spent in the training quadrant was measured in a single trial of 60S.

Histological Assessment
For light microscopy, 4 weeks after the behavioral studies, the rats were deeply anesthetized with halothane/nitrous oxide and killed by transcardiac perfusion, following a brief flush of saline, with formalin–acetic acid solution(10%:2%) in distilled water at a pressure of 100–120mmHg for 12 min, The cerebellar was removed and fixed by 4% paraformaldehyde in 0.1% phosphate buffer for a week, Then the block of cerebellar was immersed in 30% sucrose–buffer solution overnight. Mid-sagittal sections at the vermal level were cut. The sections were subsequently stained with Cresyl violet, and examined for histological changes.

Immunohistochemical Assessment
Immunohistochemistry for synaptophysin was carried out using the avidin–biotin–peroxidase method. Briefly, the free-floating sections were treated with 0.15% Triton–X and incubated in 1% hydrogen peroxide to block endogenous peroxidase. After pre-incubation in 5% blocking serum, the sections were incubated for 24h at 4℃ with a rabbit polyclonal anti-synaptophysin primary antibody(Sigma–Aldrich, S5768, USA) diluted 1:200, Sections were then incubated for 3 h with an appropriate secondary biotinylated antibody, followed by the avidin–biotin–peroxidase detection method(ABC Elite, Vector Laboratories). The positive signal was developed in a solution containing 3,3’–Diamino benzidine(DAB) in the presence of hydrogen peroxide(0.002%). In our preliminary study, immunoreactivity specificity was tested by incubating rat brain sections with no primary antibody, in which no immunostaining was observed.

Electron Microscopy Assessment
For analysis by electron microscopy, the animals were perfused with 2.5% glutaraldehyde in 0.1M cacodylate buffer(pH 7.2), and the brains were later dissected as described above. Parasagittal slices 1mm thick were cut through the cerebellar, postfixed in 1% OsO4 and embedded in Epon–araldite, Semithin(1um) sections were stained with toluidine blue, Thin sections were stained with lead citrate and uranyl acetate. Electron micrographs were taken at an original magnification of ×4000 or ×8000 using an electron microscope(Hitachi H–600, Japan).

Data Analysis
Results are expressed throughout as mean±standard error of mean, The behavioral data were analysed with a one–way ANOVA(SPSS 12.0 ver, for window), Post hoc analyses were performed using least significant difference(LSD). Differences were considered significant if p<0.05.

RESULTS
Results of Motor Performance Test

Results of rota-rod test
The rota-rod test was used to assess balance and motor coordination in each groups. Table 3 present each groups duration of stay on rota-rod. The duration in the experimental groups were shorter compared with group 1. After 4 weeks, the duration in the experimental group III, IV were significantly (p<0.001) longer compared with experimental group II.
Table 3. The changes of rota–rod test score in each groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Pre</th>
<th>1 day</th>
<th>2 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>293.00±4.93</td>
<td>293.0±5.28</td>
<td>293.67±5.81</td>
<td>294.33±5.94</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>294.67±4.81</td>
<td>13.80±2.29</td>
<td>26.80±3.23</td>
<td>42.67±3.06</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>291.00±6.60</td>
<td>14.60±3.14</td>
<td>55.06±4.33*</td>
<td>75.60±6.13*</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>292.67±5.63</td>
<td>14.80±2.78</td>
<td>40.80±4.52</td>
<td>56.27±8.01*</td>
<td></td>
</tr>
</tbody>
</table>

There were significant differences among the four groups (p<0.001)
*Statistically significant as compared with group II

Results of water maze test
The water maze test was used to assess motor ability and cognition in each groups. Table 4 present each groups spent time of reaching escape. The spent time in the experimental groups were longer compared with group I. After 4 weeks, the spent time in the experimental group III and IV were significantly (p<0.001) longer compared with experimental group II.

Table 4. The changes of water maze test score in each groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>25 day</th>
<th>26 day</th>
<th>27 day</th>
<th>28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>48.47±3.94</td>
<td>39.93±4.61</td>
<td>30.13±5.04</td>
<td>25.93±3.77</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>45.27±4.06</td>
<td>33.60±4.08*</td>
<td>26.47±5.01*</td>
<td>12.13±3.78*</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>46.87±5.34</td>
<td>37.20±4.48</td>
<td>29.40±3.98</td>
<td>21.20±2.93*</td>
<td></td>
</tr>
</tbody>
</table>

There were significant differences among the four groups (p<0.001)
*Statistically significant as compared with group II

Histological Finding
Light microscopy of the central lobule of the cerebellar cortex of all groups of rats showed relatively uniform structure. A typical three layers arrangement of neurons was noticed in the form of a superficial molecular layer which was lightly stained, a deeply placed granular layer, with dark staining granule cells, and single row of Purkinje neurons in between the molecular and granule cells. However, harmaline induced experimental groups the alignment of Purkinje cells was degenerated and Purkinje cell layer showed scattered, irregular and fined many vacuolated molecular layer(arrows). Especially, the experimental group II, IV severe degeneration in Purkinje layer. In contrast, experimental group III increased soma and dendrite than the experimental groups II, IV in Purkinje layer(Fig. 1).

Fig. 1. The histological findings of cerebellar cortex in each groups (Cresyl violet stain, ×400)
I: Normal control
II: Harmaline induced
III: Harmaline induced with motor skill learning
IV: Harmalined induced with treadmill exercise
Immunohistochemical Finding

Figure 2 illustrates each groups differences in synaptophysin levels. The group I exhibited higher synaptophysin levels than the experimental groups in cerebellar cortex. Especially, the experimental group II, IV exhibited lower synaptophysin levels in cerebellar cortex. In contrast, experimental group III exhibited higher synaptophysin levels than the experimental groups II, IV in cerebellar Purkinje layer and granular layer.

Fig. 2. The immunohistochemical findings of synaptophysin reaction in cerebellar cortex (immunohistochemical stain, ×200)
I: Normal control
II: Harmaline induced
III: Harmaline induced with motor skill learning
IV: Harmaline induced with treadmill exercise

Electron Microscopy Finding

Purkinje cell and around organelles observed by electron microscopy, group I observed uniform nuclear and cytoplasm and Purkinje cell and evenly 6–7 of Bergman neuroglia. However, experimental groups observed ultrastructurally to be swollen organelles and vacuolization(arrow), became very large and almost universal, although this did not lead to cell death. Also dendrites of Purkinje cells are shrunken and densely staining. Especially, the experimental group II, IV severe degeneration in Purkinje cell. In contrast, experimental group III observed mild degeneration the experimental groups II, IV in Purkinje cell(Fig. 3).

Fig. 3. The electron microscopic findings of Purkinje cells in each groups(Bar represent 500nm)
I: Normal control
II: Harmaline induced
III: Harmaline induced with motor skill learning
IV: Harmaline induced with treadmill exercise

DISCUSSION

The term "excitotoxicity" was coined by Olney to describe the "excitation to death" produced by the acidic amino acids glutamate, aspartate and their analogs(20). The field of excitotoxicity is one of the most active areas in neuroscience, particularly in relation to its clinical significance in neurological disorders(21). In this study, neurons apoptosis were considered to be the actually pathogenesis in the central nervous system disorders. The present study demonstrates the induction of nitric oxide synthase in Purkinje cells using a non–invasive trans–synaptic method. This method is based on the system administration of the harmaline, which selectively activates neurons of the inferior olive, O’Hearn and Molliver have demonstrated previously that protracted activation of inferior olivary neurons by harmaline or ibogaine result in a selective degeneration of subpopulation of Purkinje cells in the midline cerebellar cortex(22). Several lines of evidence indicate that the excitotoxicity amino acids glutamate and/or aspartate are the predominant neurotransmitters of climbing fibers and presumably these are released in response to harmaline administration(23). In this study, cerebellar injury model by harmaline were considered to indirectly method causing excitotoxicity. Through the evaluation of neuro–behavioral response were identified presence of cerebellar injury. We tried to select the rats that represents tremor
in the hole body. The results raised confidences as reducing the variation between subjects.

In this study, we observed that the effect of motor skill learning on motor performance and synaptic plasticity in excitotoxicity injury model of rat, in the rota--rod test which designed to measure the ability to balance and coordination, the duration in the experimental groups were shorter compared with group I and after 4 weeks, the duration in the group III, IV were significantly(p<.001) longer compared with group II. Our results were consistent with the results of Cotman and Berchtold study that complex motor training increased coordination and balance(24). In the water maze test which designed to measure the ability to motor learning and cognition, the spent time in the experimental groups were longer compared with group I and after 4 weeks, the spent time in the group III, IV were significantly longer compared with group II (p<.001). These results were consistent with previous studies that repetitive motor learning were improve cognition and motor learning(25). In motor performance test, the outcome of the motor skill learning group and treadmill exercise group was significantly better than experimental control group(p<.001). In particular, motor skill learning group showed a more rapid improve than treadmill exercise group. Motor skill learning, as opposed to motor activity, increases the number of synapses/Purkinje cell within the molecular layer of the cerebellar of animals trained to complete a complex motor learning task, in comparison to both active and inactive controls(26).

The structural transformations are thought to represent qualitative and/or quantitative changes in information processing that occur in association with changes in behavior. Frick and Fernandez were reported that environmental enrichment is to improve synaptophysin expression, as well as the spatial memory(27). We tried to observe the histologic change and synaptic plasticity of cerebellar. In Cresyl violet stain, experimental groups the alignment of Purkinje cells was showed scattered, irregular and fined many vacuolated molecular layer. Especially, the group II, IV severe degeneration in Purkinje layer. In contrast, group III increased soma and dendrites than the groups II, IV in Purkinje layer. These histological changes were consistent with motor performance results. The increase in synapse number appears to primarily involve increased parallel fiber to Purkinje cell synapse(28). These results were consistent with previous studies(29). Motor skill learning, and not mere motor activity, appears to cause a profound synaptic remodeling of the cerebellar cortex that includes changes in the structure of both Purkinje cells. In immunohistochemical reaction of synaptophysin, the group II, IV observed lower synaptophysin levels in cerebellar cortex. In contrast, group III observed higher synaptophysin levels than the groups II, IV in cerebellar Purkinje layer and granular layer. Changes in the strength of the connections between parallel fibers and the interneurons within the molecular layer have also been incorporated into various models of learning within the cerebellar cortex(30). In the cerebellar, parallel fiber stimulation induces plasticity in Bergmann glia extrasynaptic currents(31), and stimulation of Bergmann glia is relatively long--lasting consequences for excitatory postsynaptic currents in Purkinje cells(32). In electron microscopy, experimental groups observed ultrastructurally to be swollen organelles and vacuolization and dendrites of Purkinje cells are shrunken and densely staining. Especially, the group II, IV severe degeneration in Purkinje cell. These results can be considered that synaptic problem by induced excitotoxic and degeneration of Purkinje cells affect the nerve conduction in cerebellar. Group III observed mild degeneration the groups II, IV in Purkinje cell. These results can be considered that motor skill learning lead out the positive changes in cerebellar nerve cells by increase Purkinje cell synaptic activity. Our results were consistent with the results of Kleim et al, study that the stellate cells of animals trained on the acrobatic task had more extensive dendritic arborizations than those of activity control animals, indicating that motor learning is associated with an increase in the amount of postsynaptic space on these neurons(16).

CONCLUSION

This study is intended to examine the motor skill learning on motor performance and synaptic plasticity in the cerebellar injured rats by excitotoxicity. In each group, motor performance test, histologic observations, synaptophysin expression and electron microscopy observation were analyzed. The following result were obtained,

1. In motor performance test, the outcome of the motor skill learning group and treadmill exercise group was significantly better than experimental control group(especially motor skill learning group(p<.001)).
2. In histological finding, the excitotoxicity groups were destroy of dendrites and nucleus of cerebellar neurons (especially experimental control group). Motor skill learning group and treadmill exercise group were decreased in degeneration of cerebellar neurons (especially motor skill learning group).

3. In immunohistochemistic response of synaptophysin in cerebellar cortex, the outcome of excitotoxicity groups were decreased than the normal control group (especially experimental control group). Motor skill learning group and treadmill exercise group were more improved than experimental control group (especially motor skill learning group).

4. In electron microscopy finding, the excitotoxicity groups were degenerated of Purkinje cell and cell organelles (especially experimental control group).

These result suggest that improved motor performance by motor skill learning after excitotoxicity induced is associated with dynamically altered expression of synaptophysin in cerebellar cortex and that is related with synaptic plasticity.

REFERENCES