Effects of Ninjin-yoei-to (Rensheng-Yangrong-Tang),
a Kampo medicine, on brain monoamine and nerve
growth factor contents in mice with
olfactory bulb lesions

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Abstract

We used mice with olfactory bulb lesions induced by 5 % intranasal zinc sulfate (ZnSO\textsubscript{4}) perfusion, and investigated the effects of Ninjin-yoei-to (人参養栄湯, Rensheng-Yangrong-Tang), a Kampo medicine administered orally, on the learning, memory, and content of monoamine in the brain. Learning and memory tests were carried out with the step-through type passive avoidance apparatus. We observed a decrease of memory acquisition and retention in mice with olfactory bulb lesions. Ninjin-yoei-to was administered orally at 800 mg/kg/day \textit{ad libitum} for 2 weeks. A beneficial effect of Ninjin-yoei-to on learning and memory was observed. At the same time, the reductions of DA, DOPAC, HVA, 5-HT and 5-HIAA in the olfactory bulb, hippocampus and substantia nigra were found in mice with olfactory bulb lesions but Ninjin-yoei-to inhibited these reductions, it was also observed that the content of nerve growth factor in the olfactory bulb was increased in the Ninjin-yoei-to group. This suggested that Ninjin-yoei-to relieved the ZnSO\textsubscript{4}-induced injury of the nerve cells. The reason was guessed to be that the repair was due to an increase in nerve growth factor, which was expressed as an improvement in learning and memory.

Key words learning and memory, monoamine, nerve growth factor, Ninjin-yoei-to (人参養栄湯, Rensheng-Yangrong-Tang), olfactory bulb lesion, steroidal hormones.

Introduction

With the aging of the population, the number of patients with senile dementia is increasing rapidly, and becoming a big social problem. An increase of scent threshold and reduction of scent distinction are observed in elderly people. \textsuperscript{1) It is known that mitral cells, the center of scent, show a marked decrease with age. \textsuperscript{2) It has been reported that olfactory bulb (OB) neurons are dysfunctional and scent is obstructed in Alzheimer-pattern dementia patients. \textsuperscript{3) It is suggested that the progress of Alzheimer’s disease is related to OB. \textsuperscript{4) In animal experiments, a reduction of memory retention in rats with olfactory bulbectomy has been reported. \textsuperscript{5) This may be useful as a model of dementia in research. \textsuperscript{6) Kampo prescriptions have also been used for the treatment of dementia patients. Both in basic research and clinical study, the actions of Toki-shakuyaku-san (当帰芍薬散, Danggui-Shaoyao-San), Choto-san (荻藤散, Gouteng-San) and Kami-untan-to (加味温胆湯, Jiawei-Wendan-Tang) have been reported. \textsuperscript{7) Ninjin-yoei-to (NYT) has been applied to elderly people in

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traditional Oriental medicine. In such patients, respiratory and digestive disease may occur together with fever, accompanied by symptoms of neurosis in addition to decline of strength and vitality. In basic research, it has been suggested that NYT may have various effects on the nervous, immune and endocrine systems.\textsuperscript{8}

In the present study, we used mice after olfactory bulb lesions (OBL) and investigated the effects of NYT administrated orally on learning and memory and neurotransmitters in the mouse brain.

**Materials and Methods**

*Experimental animals*: C57BL/6 male mice were obtained from Nippon SLC (Hamamatsu, Japan) at 6 weeks of age, and were provided with commercial pellets (CE2 Clea Japan) through the whole experiment at a temperature of 24±1°C.

*Olfactory bulb lesions*: OBL was performed according to the modified method of Alberts et al.\textsuperscript{10} In brief, they were induced by 20 μl/mouse of 5 % zinc sulfate intranasal perfusion. Normal control mice were intranasally perfused with physiological saline.

*Preparation of Ninjin-yoei-to*: NYT was supplied by Tsumura Co., Ltd., Japan as dried powder, and was composed of *Ginseng radix* (人参, Rensheng) 3 g, *Angelicae radix* (当帰, Danggui) 4 g, *Rehmannia radix* (地黄, Dihuang) 4 g, *Atractylodis lanceae rhizoma* (白朮, Baizhu) 4 g, *Hoelen* (茯苓, Fuling) 4 g, *Cimicifugae cortex* (桂枝, Guipi) 2.5 g, *Polygalae radix* (远志, Yuanzhin) 2 g, *Paoniae tuber* (芍藥, Shaoyao) 2 g, *Aurantii fructus immaturus* (陳皮, Chenpi) 2 g, *Astragali radix* (黄芪, Huangqi) 1.5 g, *Glycyrrhizae radix* (甘草, Gancao) 1 g, *Schisandrae fructus* (五味子, Wuweizi) 1 g. The powder was suspended in distilled water, and was adjusted to 800 mg/kg/day as 10 times the dose for human adults. The extract was given from the day of OBL as drinking water. The herbal medicines were administered consecutively for two weeks. Normal mice and those with olfactory bulb lesions were provided with water in the same way.

*Passive avoidance test*\textsuperscript{11}: Learning and memory tests were carried out with the step-through type passive avoidance apparatus (Muromachi, Japan).

The acquisition tests were performed with an AC 3 mA electric shock via the metal grid floor for 3 sec. Tests were performed before noon. If latency longer than 300 sec were recorded, the experiment was stopped and a value of 300 sec was recorded. Learning and memory tests were differentiated into acquisition test and retention tests. In acquisition test, olfactory bulb lesions were induced by intranasal zinc sulfate perfusion before passive avoidance test. In the retention test, zinc sulfate perfusion was carried out after the latency of all mice had become more than 300 seconds.

*Sectioning of brains*: The brains of each group were removed 2 weeks after the beginning of administration of NYT. Specific brain regions were sectioned for the cerebral cortex (CC), the hippocampus (Hip), the hypothalamus (Hyp), the substantia nigra (SN) the olfactory bulb (OB) on dry ice using the method of Glowinski & Iversen\textsuperscript{13} for assay of monoamine contents.

*Measurement of monoamines*: Monoamine contents were measured by high performance liquid chromatography using the electron chemical detector (HPLC-ECD) method. In brief, individual brain regions (30-50 mg) were placed in test tubes in an ice-water bath, and homogenized in 1.0 ml of 0.5 M perchloric acid, containing 1×10^{-3} M sodium bisulphite, 4×10^{-3} M EDTA and 3, 4-dihydroxy benzylamine (DHBA as an internal standard; 3×10^{-7} M). The homogenate was centrifuged at 4°C for 5 min at 3000 rpm. The supernatant was transferred to a screw-cap vial containing 0.5 ml pH 8.6 Tris buffer (1.25 M) and adjusted to pH 8.6 with 1 M-sodium carbonate, and 20 mg of acid-washed alumina. After addition of the supernatant, the vial was immediately shaken for 10 min. After the alumina had settled the supernatant was removed by aspiration. The alumina was washed twice with distilled ice-cold water. Then 200 ml of 0.13 n-HClO\textsubscript{4} was added to the alumina and centrifuged for 5 min. The supernatant was filtered through a membrane filter (0.45 mm, Millipore, Co. Ltd.). An aliquot of the eluate was used for HPLC.

The HPLC system consisted of a Shimadzu LC-10AD pump (Shimadzu Co., Ltd.) and reversed phase column (Eicom MA- 5 ODS; 150 mm×4.6 mm, ID, Eicom Co., Ltd.). The mobile phase was a mixture of
0.1 M citrate buffer (pH 3.5) containing, sodium octyl sulphonate (Kanto Kagaku Co.) and methanol with a flow rate of 1.0 ml/min. The contents of the eluting solvent were as follows: 0.1 M CH3COONa 393 ml, 0.1 M citric acid 437 ml, methanol 150~170 ml, 100 mg/ml sodium octyl sulphonate solution 2.3 ml, 5 mg/ml EDTA-2Na solution 1.0 ml, pH 3.5. The applied potential of ECD was 750 mV.13)

Measurement of nerve growth factor (NGF) in OB: Each OB tissue was placed in a test tube in an ice-water bath, and an aliquot of phosphate buffer saline (PBS) was added. The tissue was homogenated ultrasonically at 4°C, and the supernatant of each section was taken. The contents of NGF in the supernatants were measured by the sandwich ELISA method of Yabe et al.7 using affinity-purified anti-NGF polyclonal antibody. Protein concentrations in OB were measured according to the method of Bradford14 with bovine serum albumin as a standard. The data of NGF concentrations were used after adjustment according to protein concentrations.

Measurement of steroid hormones: Two weeks after the administration of NYT extracts, blood was collected from the trunk, allowed to clot for 1 hour and centrifuged at 1000×g for 15 min at 4°C. Serum was stored at -20°C for assay. The contents of estradiol, testosterone and progesterone in the serum were measured by enzyme immunoassay kit (Cayman Chemical company, MI, USA), and the steroid contents were calculated.

Statistical analysis: Data were analyzed by Student’s t-test or Mann-Whitney U-test to determine significance.

Results

Effects of NYT administration on learning and memory
All normal control mice acquired learning after two shocks, but in the OBL group not all mice acquisition was achieved at two shocks, and 3 mice out of 7 could not acquire it completely after three shocks. In the NYT group, all mice acquired it completely, NYT counteracted the reduction of learning and memory due to OBL (data was not showed). On the other hand, in the retention test, ZnSO4 was applied after the latency of all mice became more than 300 seconds.

The latency after 48 hours was 227±91 sec in the normal control, and 65±46 sec in the OBL controls. A reduction was thus observed due to OBL. The latency in the NYT group was 150±113 sec, showing a tendency to suppress this reduction by NYT (Fig. 1).

Effects of NYT on the body weight
The body weight of OBL control was acutely reduced the day after intranasal zinc sulfate perfusion. The decrease of body weight continued for one week, but later recovered to the level of normal mice. A tendency to suppress the reduction due to OBL was observed both in NYT treatment after intranasal zinc sulfate perfusion (Fig. 2 a) and in NYT treatment before such perfusion (Fig. 2 b). The recovery of body weight was also rapid in the NYT group.

Effects of NYT on monoamine content of the brain
No significant difference of the content of catecholamine was observed between the normal and OBL groups in CC. Decreases of the contents of DA, DOPAC, HVA and 5-HT, but not of NE were observed in Hip in the OBL group. NYT treatment significantly restored the contents of dopamine and its metabolites (DOPAC and HVA). A decrease of 5-HT in Hyp was observed due to OBL. However, NYT did not show any effects compared to the OBL group.
catecholamines measured in SN were decreased in the OBL group compared with the normal group, but NYT counteracted these decreases. In OB decreases of dopamine, especially DOPAC and HVA were observed even under detectivity in the OBL group. NYT
countered these decreases (Table I).

Effects of NYT on steroid hormones in serum

No significant changes of estrodiol and testosterone were observed in the OBL or NYT groups. The content of progesterone in the serum was significantly

<table>
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<tr>
<th>Table 1</th>
<th>Effects of Ninjin-yoei-to on monoamine contents (p mol/mg tissue) in brain regions of mice with olfactory bulb lesions (mean±S.E., n=7-8).</th>
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<td></td>
<td>NE</td>
</tr>
<tr>
<td>CC</td>
<td>Control</td>
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<td></td>
<td>OBL</td>
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<td></td>
<td>OBL+NYT</td>
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<tr>
<td>Hip</td>
<td>Control</td>
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<td>OBL</td>
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<td>OBL+NYT</td>
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<td>OBL+NYT</td>
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</table>

Olfactory bulb lesions were induced by intranasal zinc sulfate perfusion (5% ZnSO4, 20 µl/mouse). The normal control mice were intranasal physiological saline perfusion. The mice received tap water (Control and OBL) or Ninjin-yoei-to (NYT, 800 mg/kg/day) for 2 weeks. CC: Cerebral Cortex, Hip: Hippocampus, Hyp: Hypothalamus, SN: Substantia Nigra, OB: Olfactory Bulb, NE: Norepinephrine, DA: Dopamine, DOPAC: 3,4-Dihydroxy Phenyl-Acetic Acid, HVA: Homovanillic Acid, 5HT: Serotonin, 5-HIAA: 5-Hydroxyindole-3-Acetic Acid. * or **: Significantly different from the control at p < 0.05 or 0.01. * or **: Significantly different from the OBL at p < 0.05 or 0.01. N.D.: not detectable.
Table II  Effects of Ninjin-yoei-to on steroidal serum hormones in mice with olfactory bulb
(Mean±S.E., n=7-8.)

<table>
<thead>
<tr>
<th></th>
<th>Testosterone (pg/ml)</th>
<th>Estradiol (pg/ml)</th>
<th>Progesterone (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.03±0.91</td>
<td>30.30±4.10</td>
<td>9.40±0.88</td>
</tr>
<tr>
<td>OBL</td>
<td>2.64±1.56</td>
<td>36.50±5.30</td>
<td>4.40±0.63**</td>
</tr>
<tr>
<td>OBL+NYT</td>
<td>2.22±1.28</td>
<td>38.80±5.50</td>
<td>5.40±0.61</td>
</tr>
</tbody>
</table>

Olfactory bulb lesions were induced by intranasal zinc sulfate perfusion (5% ZnSO₄, 20 μl/mouse). The control mice were intranasal physiological saline perfusion. The mice received tap water (Control and OBL) or Ninjin-yoei-to (NYT, 800 mg/kg/day) for 2 weeks. **: Significantly different from the control group at p<0.01.

decreased in the OBL group compared with normal control. It was higher in the NYT group than in the OBL group, but the difference was not significant (Table II). The same result was observed in a repeated experiment (data is not shown).

Effects of NYT on NGF in the olfactory bulb

The content of NGF in OB was 6.8±1.5 pg/mg protein in normal control, but increased significantly to 13.4±2.7 pg/mg protein in the OBL group. It was 16.0±1.7 pg/mg protein in the NYT group, a tendency to increase compared with the OBL group (p<0.1) (Fig. 3).

![Chart](chart.png)

Fig. 3  Effect of Ninjin-yoei-to on nerve growth factor (NGF) in mice with olfactory bulb lesions (Mean±S.D., n=7-8). Olfactory bulb lesions were induced by intranasal zinc sulfate perfusion (5% ZnSO₄, 20 μl/mouse). The mice were irrigated with physiological saline (normal control) or zinc sulfate solution (OBL and OBL+NYT). The normal control and the OBL groups received tap water, and the OBL+NYT group received Ninjin-yoei-to (NYT, 800 mg/kg/day) for 2 weeks. **: Significantly different from the control at p<0.01.

Discussion

In animal experiments, reduction of memory retention in after olfactory bullectomy rats has been reported. This is useful as a model of dementia in research. By spraying the ZnSO₄ into the nasal cavity, secretion of glutamate is promoted, so that the sense of smell is impaired by obstruction of the regeneration of OB epithelial cells. In this case, it is thought that ZnSO₄ has little effect on any functions except the sense of smell. Therefore, in the present study, we used mice with olfactory bulb lesions induced by ZnSO₄ but not those which had undergone olfactory bullectomy to investigate the effect on neurotransmitters and learning and memory, and further to research the effects of NYT. Reductions of body weight, learning and retention were observed in OBL mice. NYT administered orally counteracted these reductions.

To study the beneficial effect of NYT on learning and memory, we measured the content of monoamine in OBL mice brains. Monoamines and the turnover may play a role in improvement in memory. The obstacle of passive avoidance learning of rat induced by olfactory bullectomy is improved by the medicines which induce the excitement of 5-HT nerve. When exhaustion of 5-HT is caused by anti-5-HT regent, the obstacle of passive avoidance learning is recognized. It is thought that one reason for learning and memory reduction may be the decrease of DA, NE and 5-HT and their receptors. In the present experiment, the decrease of monoamine in OBL mice was most severe in the OB, Hip and SN. The Hip plays a central role in learning and memory. It has been reported that the inhibition learning and memory occurs when the Hip is extracted or destroyed. In the same way there is intellectual inhibition when the SN is destroyed. Reduction of DA, DOPAC, HVA in the OB and Hip, in addition to 5-HT, 5-HIAA in SN was observed. It was suggested that the reduction of learning and memory may be due to these changes. Moreover, it was shown that the damaging effects of ZnSO₄ were not limited to the OB, but were also seen in the Hip and SN, although these are remote areas. NYT can counteract the reduction of DA, DOPAC, HIAA 5-HT and NE in OB and SN.
On the other hand, it is known that NGF plays an important role in maintaining the function of nerve cells. NGF can promote differentiation of the undifferentiated neuron, and can encourage the survival in the differentiated neuron. It also promotes a regeneration of the nerve fibers. Moreover, as a nerve nutrition factor, it can repair the neuron and improve its function when the brain is injured.\(^{33}\) NGF is produced in the OB, and works as a nutrition factor to the olfactory epithelium, and it can promote protein synthesis and growth in olfactory epithelial cells.\(^{24}\) It has also been reported that the content of NGF in the Hip was reduced in rats of advanced age,\(^ {25}\) and that NGF could counteract the reduction of learning and memory in such rats.\(^ {26}\) In this experiment, we measured the content of NGF in the OB, and observed that NGF was increased in the OBL group compared with the normal group. It was suggested that the purpose of the increase was to repair the neurons injured by ZnSO\(_4\). NYT increased the content of NGF in OB, and it was presumed that it can repair the neurons by increasing NGF. NGF is a high molecule protein, so that if it is given peripherally, the passage through the brain blood barrier is difficult, and no physiological action can be expected. In the present experiment, NYT, a Kampo prescription was administered orally to increase NGF in the central nervous system.

Steroid hormones are relative to learning and memory ability,\(^ {27}\) and neurotransmitters in the brain.\(^ {28}\) We therefore measured the contents of hormones in serum. We found that the content of progesterone in serum was decreased but there were no changes in testosterone or estradiol in the OBL group compared with normal control. Effects of NYT were not observed. It was presumed that the improvement of NYT on learning and memory is relative to NGF but not hormones.

It has been reported that Kami-untan-to, another Kampo medicine, can have a beneficial effect on the reduction of learning and memory, reinforce the activity of ChAT in brain and promote the production of NGF and BDNF.\(^ {29,30}\) Kami-untan-to is composed of 13 kinds of crude medicines, of which Ginseng radix, Hoelen, Rehmannia radix, Polygalae radix, Aurantii fructus immaturus and Glycyrrhizae radix are common to NYT. Ginseng radix increases the survival rate of nerve cells\(^ {31}\) and the content of monoamines in the brain,\(^ {32}\) promotes the production of NGF in the brain\(^ {33}\) and reinforces learning memory.\(^ {34}\) Polygalae radix can also augment the activity of ChAT in brain and promoted the production of NGF and BDNF.\(^ {30}\)

It may be thought that the effects on the central nervous system may be due to Ginseng radix and Polygalae radix. It is necessary to research the active components and the difference of these two prescriptions.

**Acknowledgement**

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**和文抄録**

5％ZnSO\(_4\)（20µl/mouse）を鼻首に点鼻することで嗅覚障害を惹起したマウスを用い、嗅覚障害による記憶学習能の低下及び脳内モノアミン類の変動に対する漢方方剤・人参養栄湯の経口投与の影響を検討した。記憶学習能は回避型ステップスルー装置により検討した。その結果、ZnSO\(_4\)を点鼻した群では記憶獲得および記憶保持能の低下が観察された。人参養栄湯は800mg/kg Bwtの用量になるよう2週間経口投与した。その結果、記憶学習能の改善が認められ、またその時の脳内モノアミン含量を測定したところ、ZnSO\(_4\)点鼻では、嗅球、海馬、黒質などでDA、DOPAC、HVAや5-HT、5-HIAAの低下が認められた。また、嗅球の神経成長因子（NGF）含量は人参養栄湯の投与により増加する傾向が観察された。人参養栄湯の経口投与によりZnSO\(_4\)点鼻により惹起される神経細胞の障害を緩和する作用を示すことが認められた。またその背景にはNGFを増加することで、神経細胞の修復に作用することが推定された。その結果、低下した記憶学習能を改善するものと考えられた。

**References**


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