Kangenkaryu Prevents the Decrease of Cholinergic Markers Following the Nucleus Basalis Magnocellularis Lesion

Masanori Takahashi, Kiminobu Sugaya* and Kazuhiko Kubota

Research Institute for Biosciences, Science University of Tokyo, 2669 Yamazaki, Noda, Chiba 278, Japan

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ABSTRACT—The nucleus basalis magnocellularis (nbm)-lesioned rat is considered to be a model of the cholinergic dysfunction observed in the cerebral cortices of Alzheimer's disease patients. The cholinergic markers, acetylcholine release and choline acetyltransferase activity, were decreased in the cerebral cortex of the nbm-lesioned rat. Kangenkaryu (KAN), a Chinese traditional medicine, is a typical prescription for the treatment of symptoms related to blood circulation deficiency. Orally administered KAN following the nbm lesion significantly preserved the cholinergic markers. The present results indicate that KAN may preserve the activity of cholinergic neurons in the cerebral cortex after the nbm lesion.

Keywords: Chinese traditional medicine, Alzheimer's disease, Nucleus basalis magnocellularis lesion

Alzheimer's disease (AD) is characterized by degenerative changes in the brain (1) and the impairment of learning and memory function (2). It is widely accepted that learning and memory are deeply associated with the functional activity of the cholinergic system in the central nervous system (CNS) (2–4). The basal forebrain (BF) provides the major source of cholinergic input to the neocortex (2, 5, 6) and hippocampus (2), and the cholinergic neurons in the nucleus basalis of Mynert (nbM) are markedly degenerated in AD (1). Because the nucleus basalis magnocellularis (nbm) in the rat is analogous to the nbM in humans, the nbm-lesioned rat has been regarded as an animal model for cholinergic dysfunction in the cerebral cortex of patients with AD (7). The nbm-lesioned rat shows decreases in cholinergic markers, such as acetylcholine (ACh) release (8) and choline acetyltransferase (ChAT) activity (9), in the cerebral cortex and shows learning and memory impairment (10). Kangenkaryu (KAN) is a prescription of Chinese traditional medicine, being composed of substances extracted with aquas solution from Cyperi Rhizoma (cyperus rhizome), Cnidii Rhizoma (cnidium rhizome), Paeoniae Radix (peony root), Carthami Flos (safflower), Saussureae Radix (saussurea root), and Salviae Miltiorrhizae Radix (salvia root). KAN is a modification of Kanjin II (KJN) and it has wider application than KJN. KJN is also a Chinese traditional medicine and has been used to treat thrombosis, myocardial infarction and cerebral infarction in China. The mechanism of action for KJN is considered to be improvement of the micro-circulation through lowering the blood viscosity. In the present study, we examined whether KAN was effective in improving the deficit of the cholinergic markers in the rat cerebral cortex following the nbm lesion.

Male Sprague-Dawley rats weighing 200–260 g at the start of the experiment were used. They were housed in the cages maintained at 22 ± 2°C with a 12/12 hr light-dark cycle. The rats had free access to laboratory chow (CE2, Nihon Clea Co.). For the lesion operation, animals were anesthetized by sodium pentobarbital (50 mg/kg, i.p.) and mounted on a stereotaxic apparatus (David Kopf). Unilateral nbm lesion was performed via injection of ibotenic acid (Sigma, 10,ug/ul) at the nbm located AP = −2.3 mm from the bregma, ML = ± 3.7 mm, 7.5 mm below the dura. KAN was dissolved in purified water at 5% (w/v). Following the operation, the KAN solution was orally administered to the rats through a catheter at a dose of 2.5 ml/rat, twice a day for 2 days, because the animals require several days to recover from the effects of the operation. Then the rats were fed for 12 days under free access to the KAN

*To whom correspondence should be addressed.
solution. Two weeks after the lesion, rats were sacrificed by decapitation. The brains were quickly taken out and harvested into the ice-cold Krebs Ringer bicarbonate buffer. The Krebs Ringer bicarbonate buffer had the following composition: 118 mM NaCl, 4.70 mM KCl, 1.50 mM MgSO4, 1.15 mM KH2PO4, 1.25 mM CaCl2, 25.0 mM NaHCO3 and 11.1 mM glucose, and was pre-bubbled with 95% O2 and 5% CO2. The brains were sliced with a McILWAIN tissue chipper (Brinkman) into 400-μm-thick slices. Each of the ten sections ranging from +2.0 mm bregma to −2.0 mm bregma was dissected. The cerebral cortices were named C.C.-1, C.C.-2, C.C.-3, C.C.-4, and C.C.-5 from the anterior to the posterior, respectively. [3H]-ACh release and ChAT activity were measured in every other one of the serial cortical slices. Three 2-mm micro punches were taken from the cerebral cortex of each hemisphere. A total of sixty punches were taken, and thirty of them were stored at −80°C until the measurement of ChAT activity, while the remaining thirty punches were immediately used for the ACh release experiment. These micro-punches were pre-incubated with the Krebs Ringer bicarbonate buffer containing 0.1 μM [3H]-choline chloride (80 Ci/mmol, Amersham) for 30 min. Three micro-punches per one chamber were transferred into the superfusion system (Brandel) and continuously superfused with the oxygenated Krebs buffer containing 10 μM hemicolinium-3. The flow rate was 0.6 ml/min, and the fractions were collected every 2.5 min. After the forty min washing period, the micro punches were stimulated by Krebs buffer containing 40 mM K+ for 5 min. At the end of the experiment, the micro punches were solubilized by Soluen-350 (Packard) at 80°C for 30 min. The radioactivities in the each collected fraction and solubilized tissue were measured with a liquid scintillation counter. For the measurement of ChAT activity, the micro punches were homogenized by an ultrasonicator in 50 μl of 50 mM Tris-HCl buffer (pH 7.4). The mixture of 20 μl micro-punch homogenate, 20 μl 10 mM EDTA-2Na (pH 7.4) and 8 μl 2.5% Triton X-100 was incubated for 15 min at room temperature. The protein concentration of each aliquot of the homogenate was measured by a Bio-Rad protein assay kit. Eight microliters of the above mixture and 20 μl 50 mM sodium phosphate buffer (pH 7.4) which contains 0.2 mM [3H]-Acetyl CoA (NEN, 1 μCi/mmoll), 20 mM EDTA-2Na, 300 mM NaCl, 8 mM choline chloride and 0.1 mM physostigmine salicylate, were added into a scintillation mini vial. Following a 15 min incubation at 37°C, the vials were put into an ice bath, and the reaction was stopped by adding 1 ml of ice-cold 10 mM sodium phosphate buffer (pH7.4). Then 400 μl of acetonitrile containing 2 mg kalig nost was put into the vials. Synthesized [3H]-ACh was extracted with 2 ml of toluene scintillation cocktail (PPO 4 g/l and POPOP 0.1 g/l). The radioactivity was measured with a liquid scintillation counter. For statistical analyses, Student’s t-test was used.

The total [3H]-choline uptake and [3H]-ACh basal release of each micro-punch were not significantly changed following the nbm lesion (data not shown). These results indicate that most of the cholinergic neurons in the cerebral cortex still survive even after the nbm lesion. However, potassium evoked [3H]-ACh release and ChAT activity in the rat cerebral cortices were significantly decreased at two weeks after the nbm lesion (Figs. 1 and 2). The means of ChAT activity were 0.764–0.840 (nmol/mg protein/min) in the control group and 0.473–0.551 (nmol/mg protein/min) in the lesioned group. This neuronal dysfunction may be partly explained by the reduction of the blood flow in the cerebral cortex that was caused by the loss of the projection of cholinergic neurons from the nbm. The electrical stimulation of nbm increased blood flow in the cerebral cortex (11, 12), and the nbm lesion causes a reduction in the cortical blood flow (13). In addition, the existence of the close association between cholinergic projection fibers and the cortical blood vessels has been reported (14). These findings suggest that the projection of cholinergic neurons from the nbm may be involved with the regulation of the cortical blood flow. The reduction of the brain blood flow may cause a decrease in the oxygen supply. As a result, the deficiency of oxygen may cause a shrinkage of the neuronal function in the cerebral cortex since the neuron needs sufficient oxygen to keep its activity. KAN preserved the decrease of the K+ evoked [3H]-ACh release (Fig. 1) and the ChAT activity (Fig. 2) in a wide area of the cortex of the nbm-lesioned rat. These results suggest that the KAN treatment preserved the cholinergic system in the cerebral cortex after the nbm lesion. It is generally known that the components of KAN have the following effects that are concerned with the blood flow regulation. Cnidii Rhizoma, Paeoniae Radix and Salviae Miltiorrhizae Radix have a vasodilatation effect. Carthami Flos has an inhibitory effect on noradrenaline evoked vasoconstriction. Cyperi Rhizoma strongly prevents platelet aggregation. Cnidii Rhizoma strongly prevents platelet aggregation.
cholinergic markers of the cerebral cortex shown in this study may be explained by not only the neutropic action but also the blood viscosity decreasing action to increase the cortical blood flow. Since KAN prevents the decrease of the cholinergic markers in the cerebral cortex after the nbm lesion, KAN is considered to be a possible anti-dementia drug.

Fig. 1. The effect of KAN on the decrease of K⁺ evoked [3H]-ACh release from the micro-punches of the rat cerebral cortices (C.C.-1–C.C.-5) 2 weeks after the nbm lesion. [3H]-ACh release from the intact hemisphere was taken as 100%. Each bar represents the percent of [3H]-ACh release from the micro-punches of the lesioned hemisphere. *P < 0.05, Significant difference between the untreated group and the KAN administered group.

Fig. 2. The effect of KAN on the decrease of ChAT activity in the micro-punches from the cerebral cortices (C.C.-1–C.C.-5) 2 weeks after the nbm lesion. Each bar represents the ChAT activity in the micro-punches of the lesioned hemisphere. The ChAT activity in the intact hemisphere was taken as 100%. *P < 0.05, Significant difference between the untreated group and the KAN administered group.
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