Chronic Administration of Cardanol (Ginkgol) Extracted from *Ginkgo biloba* Leaves and Cashew Nutshell Liquid Improves Working Memory-Related Learning in Rats

Seisho Tobinaga,[a,1)] Michio Hashimoto,*[,b] Iku Utsunomiya,*[,a] Kyoji Taguchi,[a] Morihiko Nakamura,[c] and Tokugoro Tsunematsu[d]

[a] *Showa Pharmaceutical University; Higashi-tamagawagakuen, Machida, Tokyo 194–8543, Japan:* [b] *Faculty of Medicine, Shimane University;* [c] *Collaboration Center, Shimane University; Enya-cho, Izumo, Shimane 693–8501, Japan: and* [d] *Shimane Institute of Health Science; Enyacho, Izumo, Shimane 693–0021, Japan.*
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Cardanol (ginkgol) extracted from *Ginkgo biloba* leaves and cashew nutshell liquid enhances the growth of NSC-34 immortalized motor neuron-like cells and, when chronically administered to young rats, improves working memory-related learning ability as assessed by eight-arm radial maze tasks. These findings suggest that cardanol is one of the components in *Ginkgo biloba* leaves that improves cognitive learning ability.

Key words *Ginkgo biloba*; *Anacardium occidentale*; cardanol; motor neuron-like cell; working memory-related learning ability

Ginkgo biloba L., although grown mainly in China and Japan, is currently well-known in various European countries. The plant is believed to be one of the oldest living species trees and the only living member of the family Ginkgoaceae. It is called a living fossil because its fossils, as much as two hundred million years old, are found throughout the world. In the 19th century it was shown to be a dioecious plant that propagates by a unique and ancient mechanism.[2)] In Europe the extract of its leaves, referred to as EGb 761, is commonly used to alleviate symptoms of dementia.

Various chemical components have been isolated from the plant, including flavonoids,[3)] terpenoids (ginkgolides, bilobalides)[4,5)] and organic acids (anacardic acid, cardanol).[6,7)] Several biomedical studies on its activity have been carried out,[8—11)] however, its pharmacologic effects appear to be due more to the synergistic action of multiple components than to a single compound.[12)] Studies on its therapeutic effect have also yielded conflicting results.[12—15)] Therefore, in the current study, we sought a particular component in the extract that alone has activity related to the treatment of dementia.

Diterpenoid ginkgolides have recently received a great deal of attention because of their unique structures and specific ability to antagonize the effect of a platelet activating factor (PAF). We examined the effect of cardanol and related organic acids (Fig. 1) in the current study because of their ability to cross the blood–brain barrier.[11)] On the other hand, the organic acids, anacardic acid and cardanol (gingkol acid and ginkgol; assigned the name derived more from cashew nuts *Anacardium occidental* L., *Anacardiaceae*, than from *Ginkgo biloba* L. in this study) and related compounds have not been discussed from the viewpoint of active ingredients because of their unfavorable side effects, such as allergenic properties.

We first examined the neurotrophic effect of cardanol (isolated from *Ginkgo* leaves and the crude organic acids extracted from cashew nutshell) on NSC-34 murine immortalized motor neuron-like cells established by fusing mouse neuroblastoma N18TG2 with mouse neuron-enriched embryonic spinal cord cells.[16)]

According to favorable results obtained from *in vitro* studies, large amounts of cardanol and cardanol acetate were prepared from commercially available cashew nutshell liquid for *in vivo* studies. The effect of cardanol acetate was investigated by administering it to young rats and then assessing their learning ability through radial-maze tasks. Two types of memory, reference memory and working memory are estimated by the maze task experiments, without any harmful stress to the rats.[17)] Reference memory involves using information that remains constant over time; working memory involves holding information that is pertinent only within a short period of time.[18)]

MATERIALS AND METHODS

Compounds Anacardic acid and cardanol were obtained as described previously[6)] from the leaves of *Ginkgo biloba* L.

Crude organic acids of cashew nutshell were obtained by direct extraction with ether, then with aqueous 5% NaOH, acidification with diluted HCl, and re-extraction with ether.

Cardanol was prepared from cashew nutshell liquid (Bola Raghavendra Kamath and Sons, Kukundur, Karkala, India) in which anacardic acid decarboxylates to cardanol by heating.

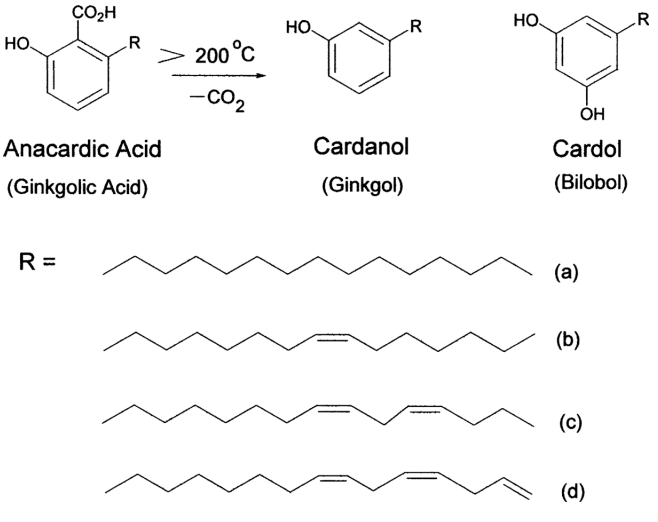

Fig. 1. Organic Acids Obtained from *Anacardiaceae*

* To whom correspondence should be addressed. e-mail: michiol@med.shimane-u.ac.jp; utsunomi@ac.shoyaku.ac.jp

The liquid (30 g) was subjected to SiO_2 column chromatography and eluted with $CHCl_3$ to yield the liquid of cardanol (22 g).

Cardanol acetate was prepared by treating 3 g of cardanol with 9 mL pyridine and 15 mL acetic anhydride at room temperature for 1 d; the solution was then poured into water, extracted with ether, washed with aqueous $NaHCO_3$, and then dried with Na_2SO_4 to afford 3 g of acetate which was purified by SiO_2 column chromatography and eluted with 1 : 1 $CHCl_3$–hexane. Commercially available cardanol contains a mixture of approximately 0.09% cardanol (Fig. 1a) (3-pentadecylphenol), 24.7% cardanol monoene (Fig. 1b) (3-[8[*Z*]-pentadecenyl]phenol), 15.6% cardanol diene (Fig. 1c) (3-[8(*Z*),11(*Z*)-pentadeca-dienyl]phenol) and 35.3% cardanol triene (Fig. 1d) (3[8(*Z*),11(*Z*),14-pentadeca-trienyl]phenol). (Test plan for cashew nutshell liquid, CAS No. 8007-24-7, submitted to the U.S. Environmental Protection Agency (EPA) by Cardolite Corporation, Inc.) The structure of cardanol was confirmed by ^1H- and ^{13}C-nuclear magnetic resonance spectra. The Cardanol acetate used in this study was of like composition.

Growth Assay NSC-34 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. NSC-34 cells were plated on flat-bottomed 96-well microtiter plates at a density of 1×10^4 cells per 100 μL medium and incubated with various concentrations of the compounds for 72 h. Cell viability was measured by Cell Counting Kit-8 (Dojin Chemical Co., Tokyo, Japan), which modifies 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and absorbance was measured at 490 nm. The results are expressed as means±S.D. The significance of differences was analyzed by one-way analysis of variance combined with Dunnett's multiple comparison test for *post hoc* analysis of intergroup comparisons. Differences were considered significant at $p<0.05$.

Animals All animal protocols were carried out in accordance with the guidelines for animal experimentation of Shimane University, compiled from the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science. Thirty-two male Wistar rats (five weeks old; Jcl; Clea Japan, Osaka, Japan) were randomly divided into two groups. One group (cardanol group) was orally administered 100 mg/kg/d of cardanol acetate emulsified in 5.5% gum Arabic solution for 2 months. The second (control) group was administered a similar volume of the vehicle only. The rats were maintained in an air-conditioned animal room with a 12-h light/12-h dark cycle under controlled temperature (23±2°C) and humidity (50±10% relative humidity) and given free access to normal laboratory diet, (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Radial Maze-Learning Ability Two months after starting the administration of cardanol acetate, the rats were tested for learning ability in an eight-arm radial maze (Toyo Sangyo Co., Toyama, Japan) as described previously.[19,20] The rats were trained to acquire a reward (food-pellet) at the end of four arms of an eight-arm radial maze and tested for two parameters of memory function: reference memory error (RME), entry into unbaited arms; and working memory error (WME), repeated entry into arms that had already been visited in the same trial. Each rat was given two trials per day, 6 d per week for a total of 2.5 weeks (total 30 trials). The body weight of the animals in the two groups did not differ (control group, 369±5 g; cardanol acetate group, 364±5 g) during the tests.

Behavioral data was analysed by a randomized two-factor (group and block) randomized block factorial analysis of variance (ANOVA). ANOVA was followed by Fisher's protected least significant differences test for *post-hoc* comparisons. GB-STAT™ 6.5.4 (Dynamic Microsystems, Inc., Silver Spring, MD, U.S.A.) was used for the statistical analyses. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

We first examined the effects of cardanol and cardanol acetate on the growth of NSC-34 cells *in vitro* and found that a 3-d treatment of the cells with 1 μg/mL of cardanol increased cell growth by approximately 40% (Fig. 2). Moreover, cardanol acetate was more effective than free cardanol in promoting cell growth.

We next examined the effect of the chronic administration of 100 mg/kg/d cardanol acetate for 2 months on learning ability through radial maze tasks. Randomized two-factor (block and group), two-way analysis of variance, revealed significant main effects of blocks of trials ($p<0.0001$) on the number of WMEs (Fig. 3A), but no significant main effect of groups ($p=0.0946$), with a significant block×group interaction ($p=0.0337$). Similarly, a significant main effect of trials ($p<0.0001$) was observed on the number of RMEs (Fig. 3B), but without a significant main effect of both groups and block×group interaction. These results suggest that long-term administration of cardanol acetate improves working, but not reference, memory-related learning ability in young rats.

Our present examination of the effects of cardanol acetate *in vitro* and *in vivo* revealed, for the first time, evidence supporting the effectiveness of a specific compound from Ginkgo leaves, cardanol (ginkgol), in improving working memory-related learning ability, indicating that it has potential in the treatment of dementia. As cardanol and related organic acids have the ability to cross the blood-brain barrier,[11] cardanol acetate may directly act to the neuronal cell functions in brain

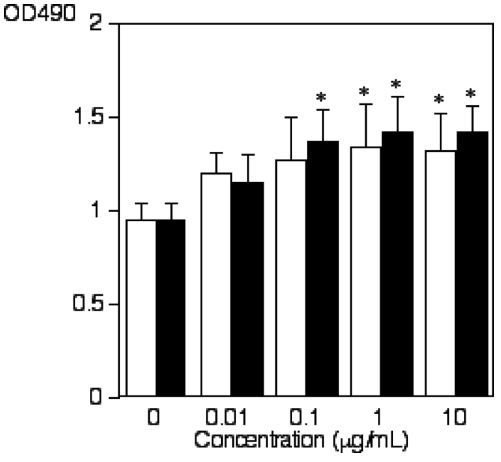

Fig. 2. Effects of Cardanol (Open Column) and Cardanol Acetate (Closed Column) in the NSC-34 Cell Growth Assay

The results are expressed as means±S.D. ($n=4$). *$p<0.05$ *vs.* 0 μg/mL group. OD_{490}, optical density at 490 nm.

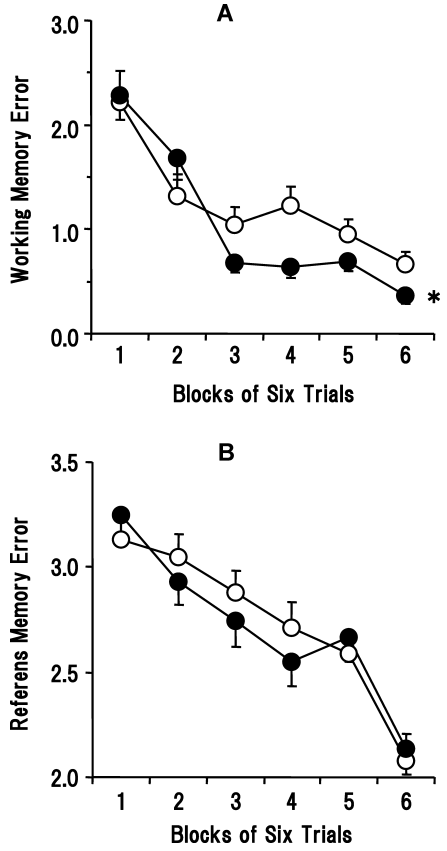

Fig. 3. Effects of Chronic Administration of Cardanol Acetate on Working (A) and Reference (B) Memory-Related Learning Ability of Young Rats Tested in the Radial Maze

○ control rats (n=15); ● cardanol acetate (100 mg/kg/d)-administrated rats (n=17). Each value represents the number of working and reference memory errors; mean±S.E. in each block of six trials. *A significant difference of the main effect of block×group interaction at $p<0.05$ *vs.* 0 μg/mL group.

and modify the memory-related learning ability. Therefore, we assume that cardanol acetate has the potential to improve neuronal function by promoting neuronal cell growth, although the precise mechanism of its pharmacological activity is not clear. Our study suggests that diverse evaluation of the extract through clinical trials will disclose the content of particular ingredients in organic acids.

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