Prophylactic Effects of Ajoene on Cerebral Injury in Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)

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As part of a basic study on the prevention of cerebral injury, ajoene (0.5 mg/d) and oil-macerated garlic extract (OMGE, containing 0.5 mg ajoene/d) were administrated to stroke-prone spontaneously hypertensive rats (SHRSP) among 8 weeks from 9 weeks of age. In the control group, 3 of 10 rats died (30%), whereas all SHRSP treated by ajoene or OMGE survived. Our results suggested that ajoene and OMGE-treatment reduced the mortality and cerebral injury in SHRSP. The levels of thiobarbituric acid reactive substance (TBARS) and the enzymatic activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) in the serum of stroke stage of SHRSP were measured. The results obtained were as follows; the TBARS level of the ajoene and OMGE-treated groups were lower than those of control groups. On the other hand, the GSH-Px and SOD activities of the ajoene and OMGE-treated groups were higher. Our results suggested that ajoene and OMGE were capable of having prophylactic effects on cerebral injury in SHRSP.

Key words ajoene; oil-macerated garlic extract; thiobarbituric acid reactive substance; glutathione peroxidase; superoxide dismutase, stroke-prone spontaneously hypertensive rat

Garlic (Allium sativum L.) has been used world-wide as a medicinal plant since ancient times. Some epidemiologic reports showed that high intake of Allium vegetables including garlic reduce the risk of thrombus formation by blood platelet condensation effect. And recent studies have validated many of the medicinal properties attributed to garlic and its potential to lower the risk of disease. Garlic has been shown to have antithrombotic activity. It is well known that garlic has been used as folk medicine. The medicinal effects of garlic are summarized in the reviews. Oil-maceration is one method for processing garlic, and this type of garlic product is common as a health food. Ajoene [([Z,E]-4,5,9-trithiadodeca-1,6,11-triene-9-oxide)], one of the derivatives of allicin, has been found as a major compound in oil-macerated garlic extract (OMGE), and has antithrombotic effect.

Stroke is one of the major diseases that were induced by thrombus. Therefore, prevention of thrombus was the first step of stroke reperfusion. Stroke-prone spontaneously hypertensive rats (SHRSP) were used an excellent animal model about the studies of hypertension and stroke effects. It was originally isolated from a colony of spontaneously hypertensive rats (SHR) and characterized by severe spontaneous hypertension and the development of cerebrovascular disease.

Ajoene has been reported the one of the strongest compound having antithrombotic effect. However, no one has demonstrated the preventive effect of ajoene against stroke. Therefore, in this study, we attempted to examine the preventive effect of cerebral injury of ajoene and OMGE using SHRSP.

MATERIALS AND METHODS

Animals Male SHRSP (8 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals, weight 170—220 g, were housed individually in plastic cage in controlled-temperature environment (25 ± 1 °C) with a 12 h light/dark cycle. Water was provided ad libitum for 1 week of adaptation. In this study, ajoene and OMGE were administrated to SHRSP among 8 weeks from 9 weeks of age. After the 9 weeks growing period, the rats were anesthetized with diethyl ether, and their blood was collected in a heparinized syringe from the abdominal aorta, and their brains were immediately removed. The serum was separated by centrifugation at 3000×g at 4 °C for 10 min. The animal experiments were done according to institutional guidelines of Committee on Animal Research at Nagoya Seiraku.

Drugs Ajoene, separated from OMGE, had a purity of 99.2%, was used in the series of experiments. Preparation and the check of purity were done by the method described previously.

An OMGE used for administration was prepared according to the method of Yoshida et al. In this study, ajoene (0.5 mg/d) was kept on a laboratory chow diet consisting of 52.7% carbohydrate, 23.6% protein, 4.4% fat, 4.9% fiber, and 6.6% minerals and vitamins (CE-2, CLEA Japan, Inc., Tokyo) powdered diet containing 0.5 mg/d. The diet containing ajoene was prepared as follows. The solution suspended ajoene in 0.5% calboxymethyl cellulose (CMC, Wako Pure Chemical Industries, Osaka, Japan) was well mixed with CE-2 (ajoene-treated group).

The diets of control animals were only 0.5% CMC mixture with CE-2 (control A group). OMGE was kept containing 64 mg/d including 0.5 mg of ajoene (OMGE-treated group). The diet of control animals were only middle chain fatty acids triglyceride (MCT, Panacete 810, Japan Oil and Fat Co. Ltd., Tokyo, Japan) mixture with CE-2 (control B group).

Thiobarbituric acid (TBA) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Diagnosis of Stroke Diagnosis of stroke was made by the susceptibilities of the animals to stimuli such as sound and soft touch. Animals with stroke were also recognized by softens and bleeds of brain. Here, we emphatically examined the brain hemorrhage. We judged it as the brain hemorrhage, we judged by investigating strictly the existence of internal
hemorrhage on the brain surface by stroke.

**TBARS Measurement** The lipid peroxides in blood serum were measured as the TBARS and was performed according to the method of Ohkawa et al.\(^{11}\)

**Glutathione Peroxidase (GSH-Px) Activity** The GSH-Px activity was measured according to the method of Donald et al.\(^{12}\) with some improvement. An assay procedure is described in which the serum GSH-Px was measured by spectrophotometer (Shimadzu Co. Ltd., UV-160 type). One unit of GSH-Px was expressed as the amount of enzyme for decreasing 1 \(\mu\)mol of NADPH per min.

**Superoxide Dismutase (SOD) Activity** The SOD activity in the serum was measured according to the method reported by Fridovich et al.\(^{13}\) using a SOD test kit purchased from Wako Pure Chemical Industries. The activity that the reduction reaction of nitro blue tetrazolium was calculated from the rate inhibited by SOD.

**Catalase (CAT) Activity** The CAT activity was measured according to the method of Luck\(^{14}\) with some improvement. On decomposition of \(H_2O_2\) with catalase the absorption decreases with time and from this decrease the enzyme activity can be calculated.

**Protein Assay** Protein assay was done similar to that described previously.\(^{15}\) The protein standards were made by dissolving bovine serum albumin (Wako Pure Chemical Industries) in distilled water. Optical density was measured at 595 nm in the spectrophotometer. The values were plotted against protein standard concentrations and regression analysis performed to determine protein concentrations for the samples. Using the protein values, TBARS level, GSH-Px and CAT activities were then calculated according to the levels of protein in the serum samples.

**Statistics** Each data value is presented as the mean± standard deviation (S.D.), and stroke incidence compared control and administered groups by a Chi-square test. \(p<0.05\) was considered to be statistically significant. The treatment effects, in the case of the TBARS level, GSH-Px, SOD and CAT activity were analyzed by two-way ANOVA, and the differences between means were tested by Duncan’s multiple-range test\(^{16}\) when the \(F\)-value was significant. Other data were analyzed by one-way ANOVA. Student’s \(t\) test and Aspin–Welch’s test were used to evaluate significant differences between means with either the same or different variance, respectively.\(^{17}\) Differences of \(p<0.01\) are considered to be significant.

**RESULTS AND DISCUSSION**

**Growth and Food Intake in SHRSP** The average body weight gain and food intake in SHRSP for 9 weeks are shown in Table 1. No differences were found in the average body weight gain and food intake between those control groups and two (ajoene and OMGE-treated) experimental groups. However, stroke incidence was significant clearly in the ajoene and OMGE-treated groups than in the control A and B groups. As signs of stroke, spasm and paralysis of one lateral left half of the body etc. were seen (data not shown). It was notably that symptoms became increased with aging. Some rats of both control A and B groups died, but ajoene and OMGE-treated groups all survived (Table 1).

**Effects of Ajoene and OMGE-Treated on SHRSP** Comparison of the brain surface of a control and ajoene group are shown in Fig. 1. About Fig. 1A (control), as the arrow showed, the brain internal hemorrhage that is one of the symptoms of stroke is seen everywhere. By Fig. 1B (ajoene), the bleeding from a brain is not seen. Therefore, we guessed that ajoene-treatment reduced the mortality and cerebral injury in SHRSP.

**TBARS Level, GSH-Px, SOD and CAT Activities** To elucidate the preventive mechanism of cerebral injury, we investigated the level of TBARS revealing lipid peroxide and also analyzed the activities of GSH-Px, SOD and CAT in the serum of SHRSP. The results of the TBARS level, GSH-Px, SOD and CAT activities are shown in Table 2. The ajoene-treated group showed 31.4% lower TBARS levels \((p<0.01)\) than that of control A group. Whereas the GSH-Px and SOD activities were 12.8% \((p<0.01)\) and 5.2% \((p<0.05)\) higher than those of control A group, respectively. Similarly,

<table>
<thead>
<tr>
<th>Groups</th>
<th>(n)</th>
<th>Body weight gain (g/d)</th>
<th>Final body weight (g)</th>
<th>Food intake (g/d)</th>
<th>Stroke incidence(^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>10</td>
<td>1.92±0.08</td>
<td>310.0±12.0</td>
<td>19.1±2.5</td>
<td>3</td>
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<tr>
<td>Ajoene-treated</td>
<td>10</td>
<td>1.86±0.06</td>
<td>304.0±9.8</td>
<td>17.4±2.5</td>
<td>0**</td>
</tr>
<tr>
<td>Control B</td>
<td>10</td>
<td>1.16±0.07</td>
<td>297.6±6.2</td>
<td>18.5±1.4</td>
<td>3</td>
</tr>
<tr>
<td>OMGE–treated</td>
<td>10</td>
<td>1.20±0.10</td>
<td>292.3±9.4</td>
<td>18.0±1.6</td>
<td>0**</td>
</tr>
</tbody>
</table>

\(^{a}\)Values are means±S.D. \(n\): number of rats. \(^{b}\)The number of death by stroke. Significance: **\(p<0.05\) vs. control.

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**Fig. 1.** Comparison of the Brain Surface of SHRSP

Some arrows show the internal hemorrhage part of the brain of SHRSP. A: control SHRSP, B: ajoene-treated SHRSP.
OMGE-treated group showed 31.7% lower TBARS levels ($p<0.01$) than that of the control B group. Whereas the GSH-Px and SOD activities were 7.2% ($p<0.05$) and 7.7% ($p<0.01$) higher than those of control B group, respectively. From these results, ajoene and OMGE suppressed the rise of TBARS level, and kept higher GSH-Px and SOD activities than the control. However, CAT activity, significant difference was not seen at both ajoene and OMGE-treated groups.

The relationship between the TBARS levels and GSH-Px activity was then investigated (Fig. 2). The tendency of this relationship for GSH-Px activity differs by ajoene and OMGE-treated groups. OMGE-treated group has correlation to TBARS levels (Fig. 2 below). Ajoene and OMGE-treated group have a tendency in TBARS levels show the low value (Fig. 2). Tomita et al.\(^{18}\) also have reported a significant correlation between the TBARS levels and GSH-Px activity. Thus, our result is in agreement with their report. By treated with ajoene, TBARS level is kept low than the value of control. The relationship about both the TBARS levels and SOD activity is shown in Fig. 3. Higher correlation was obtained than that of GSH-Px activity between TBARS levels. However, the CAT activity did not have correlation to TBARS levels in both ajoene and OMGE-treated groups (data not shown).

From these results, ajoene and OMGE treatment decreases TBARS levels, and is maintaining or increasing the activity of GSH-Px and SOD. Ajoene or OMGE treatment showed the correlation of TBARS levels and GSH-Px or SOD activities. Moreover, in the TBARS level and GSH-Px activity, although it is weak correlativity, the significant difference was observed to control by ajoene treatment. We surmised that the major function of ajoene was maintaining GSH-Px and SOD activities. Thus, our result has suggested that there is an effect whose ajoene or OMGE containing ajoene inhibits generation of lipid peroxide or which activates the GSH-Px and SOD from which the product lipid peroxide is removed. The mechanism of action remained to be further investigated.

Finally, although we examined using OMGE (64 mg/d) in which ajoene (0.5 mg/d) and other compounds (containing about 4 mg of dialkyl sulfides and vinyldithiins/d) are contained, a synergistic effect of ajoene and their compounds were not obtained. Therefore, it is guessed that the effective compound of OMGE to the stroke in SHRSP is ajoene.

REFERENCES


<table>
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<tr>
<th>Groups</th>
<th>n</th>
<th>TBARS (nmol/ml serum)</th>
<th>GSH-Px (U/ml serum)</th>
<th>SOD (% inhibition)</th>
<th>CAT (U/ml serum)</th>
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<tr>
<td>Control A</td>
<td>10</td>
<td>$2.10\pm0.4^{a}$</td>
<td>36.3 $\pm3.1$</td>
<td>50.7 $\pm2.3$</td>
<td>49.7 $\pm7.0$</td>
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<td>Ajoene-treated</td>
<td>10</td>
<td>$1.44\pm0.2^{*}$</td>
<td>41.7 $\pm1.5^{*}$</td>
<td>53.5 $\pm2.2^{**}$</td>
<td>43.5 $\pm16.6$</td>
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<tr>
<td>Control B</td>
<td>10</td>
<td>$2.16\pm0.2$</td>
<td>36.0 $\pm3.2$</td>
<td>58.3 $\pm2.1$</td>
<td>61.9 $\pm9.3$</td>
</tr>
<tr>
<td>OMGE-treated</td>
<td>10</td>
<td>$1.47\pm0.4^{*}$</td>
<td>38.8 $\pm1.5^{**}$</td>
<td>63.2 $\pm1.5^{*}$</td>
<td>59.1 $\pm3.5$</td>
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\(a\) Values are means $\pm$ S.D. \(n\) number of rats. Significance: \(^* p<0.01\) vs. control, \(^{**} p<0.05\) vs. control.