Experimental Study of Ginkgo-Chunghyul-dan on Anti-oxidant, Anti-platelet Aggregation, and Anti-hyperlipidemic Activity

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Objectives: Ginkgo-Chunghyul-dan (GCHD) is newly developed herbal medicine to prevent and treat stroke. In this study, we investigated whether the GCHD had antioxidant activity and anti-platelet aggregation effect in vitro and hypolipidemic activities in vivo.

Methods: Anti-oxidant activity of GCHD was measured using the Blois method, anti-platelet effect of GCHD was assessed by the Born method, and hypolipidemic activities of GCHD were evaluated in corn oil- or Triton WR-1339-induced and cholesterol-fed rats.

Results: GCHD showed anti-oxidant activity in the study inhibiting the formation of 1-diphenyl-2-picrylhydrazyl radicals and xanthine oxidase activity. GCHD had anti-platelet aggregation activity. GCHD significantly lowered total cholesterol (TC), triglyceride (TG), and low density lipoprotein cholesterol (LDL-C) in high cholesterol diet and Triton WR-1339 induced model TG in corn oil-induced model. GCHD had no acute toxicity at a single dosage.

Conclusion: These results suggest that GCHD has the potential to treat hyperlipidemia and stroke.

Key Words: Ginkgo–Chunghyul–dan anti–oxidant activity; anti–platelet effect; hypolipidemic activities.

Introduction

The mortality of stroke and cardiovascular disease is increasing in Korea. Stroke and cardiovascular disease are the second and the third leading causes of mortality above 50 years old after cancer.¹ Hyperlipidemia, hypertension, diabetes mellitus, and smoking are common risk factors for stroke and cardiovascular disease.²,³ Lowering cholesterol levels can reduce the mortality of cardiovascular disease to 20-30% and decrease the incidence of stroke up to 16-30%.⁴,⁵ The incidence of hyperlipidemia is 11% in Korea.⁶

As our life expectancy grows and our diet and lifestyle change to higher calorie foods and sedentary lifestyles, it is likely to increase over time. Thus, it is quite necessary to treat hyperlipidemia.

HMG-CoA (3-hydroxy-3-methyl glutaryl-coenzyme) reductase inhibitor (statin) has a therapeutic effect on lowering cholesterol levels, but many adverse effects have been reported like gastric disturbance, liver function change, myopathy and rhabdomyolysis. Furthermore, cessation of administrating statins causes elevation of cholesterol levels.⁷

Chunghyul-dan (CHD) is made of extract of Scutellariae Radix, Coptidis Rhizoma, Phellodendri
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Cortex, Gardeniae Fructus, and Rhei Rhizoma. Previous studies revealed that it inhibited cholesterol biosynthesis, HMG-CoA reductase, pancreatic lipase, nitrite oxide synthesis, and scavenged free radicals.\(^{10-12}\) In clinical studies, CHD had [minor] adverse effects like renal or hepatic toxicity, lowered serum cholesterol levels, and elevated blood pressure.\(^{13-15}\)

GCHD is a newly developed prescription adding Ginkgo Folium to CHD. It has been known that Ginkgo Folium has effects for treating hyperlipidemia, improving blood circulation, and protecting brain cells.\(^{16-19}\)

In this study, we performed experimental study in vitro and in vivo with rats to evaluate the anti-oxidant activity, anti-platelet aggregation activity, and anti-hyperlipidemic effect of GCHD in comparison with CHD. We also assessed the acute toxicity of GCHD at a single dosage with mice.

**Materials & Methods**

1. Materials

   1) Chemicals and instruments

   Xanthine, xanthine oxidase, bovine serum albumin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), 2,2'-azobis-(2-amidinopropane) dihydro chloride (AAPH), and Triton WR-1339 were purchased from Sigma Chemical (U.S.A.). Cholesterol was purchased from Yakuri Pure Chemical (Japan). Cholic acid was purchased from Fluka Biochemica (Switzerland). Total cholesterol, triglyceride, and HDL-cholesterol assay kits were from Asan Pharm. (Korea). LDL-cholesterol assay kits were from BioMerieux Chemical (France). We used lovastatin (20mg/tab) from ChoongWae Pharma (Korea) and fenofibrate (100mg/tab) from Kuhnil Pharm. (Korea). We used high performance liquid chromatography (HLPC) (Waters Co., U.S.A.), spectrophotometer (Shimadzu Co., Model UV-160A, Japan),prime automatic clinical chemistry analyzer (BPC Biosed, Italy), aggregometer (Chrono-log Corporation, U.S.A.), rotary vacuum evaporator (EYELA Co., Model NE-1, Japan), and freeze dryer (EYELA Co., Model FD-1, Japan).

   2) Preparation of GCHD

   Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Gardeniae Fructus, Rhei Rhizoma, and Ginkgo Folium were purchased from Kyunghee Oriental Medical Hospital (Seoul, Korea) and identified by Dr. Nam-Jae Kim, East-West Medical Research Institute, Kyunghee Medical Center (Seoul, Korea). Voucher specimens were deposited at East-West Medical Research Institute, Kyunghee Medical Center (Seoul, Korea).

   CHD is composed of Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Gardeniae Fructus, and Rhei Rhizoma (4:4:4:1). CHD (510 g) was extracted twice with water or 80% ethanol (170 g) in boiling water for 2 hours. These extracts were then filtered and evaporated by using a rotary vacuum evaporator. Finally CHD (137.7 g, dry weight corresponding to 27%) was made after being lyophilized with a freezing dryer. To standardize the quality of CHD, berberine in Coptidis Rhizoma and Phellodendri Cortex, baicalin in Scutellariae Radix, and geniposide in Gardeniae Fructus were quantitatively assayed according to the previous methods.\(^{20}\) The contents were 0.51% for berberine, 6.93% for baicalin, and 2.28% for geniposide.

   Ginkgo Folium (1 kg) was extracted twice with 95% ethanol (5 L) in boiling water for 2 hours. These extracts were then filtered and evaporated by using the rotary vacuum evaporator, and then ethanol extract (205 g) (GE, dry weight corresponding to 20.5%) was made after being lyophilized. The ethanol extract was dissolved in distilled water, and that was extracted by 3 parts of saturated water n-BuOH, and then each part of n-BuOH layer was combined, and this n-BuOH layer was filtered and evaporated by using the rotary vacuum evaporator, and then n-BuOH fraction (50 g) (GEB, dry weight corresponding to 5%) was made by lyophilizing that n-BuOH. Subsequently, this n-BuOH fraction was dissolved in the distilled water, and that was fractionated by 3 parts of fraction with n-hexane, and then water layer without n-hexane was evaporated. Finally Ginkgo Folium fraction (42 g) (GEBW,
dry weight corresponding to 4.3%) was made after being lyophilized (Fig.1). Water extract (320 g) (GW, dry weight corresponding to 32%) was used as a sample extracted from *Ginkgo Folium* which was added to the distilled water and filtered and evaporated by using the rotary vacuum evaporator. It was then lyophilized. GCHD is composed of CHD and GEBW (10:1).

3) Animals

Male and female ICR mice (about 30 g weight) and male Sprague-Dawley rats (about 250 g weight) were bought from Samtaco (Korea) and fed a commercial diet (Samyang, Korea). These animals were kept for at least 7 days before the experiments.

2. Methods

1) Assay of anti-oxidant activity

Free radical scavenging activity was measured by DPPH using Blois’ method. A sample (4 ml) was added to the 1×10^{-4}M DPPH radical in methanol (1 ml) and the mixture was shaken and held for 30 minutes at room temperature. Absorbance was measured at 520 nm with a spectrophotometer.

The scavenging potential for superoxide anion was measured using the xanthine oxidase method. A sample solution was added to the mixture (0.02 ml), consisting of 3 mM xanthine, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.15% bovine serum albumin (BSA) and 0.24 mM NBT in 0.05 M sodium carbonate buffer (0.48 ml). The mixture (0.02 ml) was diluted with distilled water and held for 10 minutes at room temperature. Xanthine oxidase (6 mU) and CuCl_{2} (0.02 ml) were added, followed by incubation in a shaking water bath at 25°C for 20 minutes, the absorbance was monitored spectrophotometrically at 560 nm.

The inhibition of hemolysis caused by AAPH induced lipid oxidation in erythrocyte membranes was measured using Miki’s method. Blood was obtained from aortic vein of rat and collected into a heparinized tube. Erythrocytes were separated from the plasma and the buffy coat and then washed three times with 10 volumes of phosphate-buffered saline (PBS). During every wash, the erythrocytes were centrifuged at 1000 revolutions per minute (rpm) for 10 minutes to obtain a packed cell preparation. After the last wash, the packed erythrocytes were...
suspended in 10 volumes of PBS. Erythrocyte oxidative hemolysis was induced by AAPH, a peroxyl radical indicator. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and proteins, resulting in hemolysis. The erythrocyte suspension (0.2 ml) after pre-incubation for 5 minutes at 37°C was mixed with 0.2 ml PBS containing varying amounts of samples. 0.2 ml of 100 mM AAPH in PBS was then added to the mixture. The reaction mixture was incubated for 3 hours at 37°C with gentle shaking. After incubation, the reaction mixture was diluted with 20 volumes of PBS and centrifuged at 1000 rpm for 10 minutes. The antioxidant activity was measured at 540 nm using a spectrophotometer.

\[
\text{Hemolysis rate (\%)} = \left( \frac{A_{540nm} \text{ of 0.15 M sodium chloride supernatant}}{B_{540nm} \text{ of distilled water supernatant}} \right) \times 100
\]

The percent inhibition was calculated as follows:

\[
\% \text{ Inhibition} = \left( \frac{H_{\text{AAPH}} - H_{\text{sample}}}{H_{\text{AAPH}}} \right) \times 100
\]

Where: \(H_{\text{sample}}\) is the absorbance of the sample extracts and \(H_{\text{AAPH}}\) is the absorbance of the control group.

In the present study, 2 steps were taken. First, Ginkgo Folium fractions (GF) were compared. Then, CHD and GCHD (the GF which proved to be the most effective to anti-oxidant activity was added to CHD) were compared.

2) Assay of anti-platelet aggregation activity

The platelet aggregation induced by adenosine diphosphatase (ADP) and collagen was assessed by Born’s method. Specimens of blood samples were collected using 2.2% sodium citrate at the ratio 1:9 with the blood in syringe. Samples of blood and anticoagulants were gently inverted up and down, avoiding shaking. Platelet rich plasma (PRP) was prepared by centrifuging at 1000 rpm for 10 minutes. Platelet poor plasma (PPP) was prepared by centrifuging at 3000 rpm for 30 minutes. Platelet aggregation studies in PRP were performed at 37°C with a constant rate of stirring at 1200 rpm in the aggregometer using PPP as reference, and samples and control (distilled water) 10 \(\mu\)l were added to PRP. During the experiments the optical density was continuously recorded. After a stable baseline was observed for 3 minutes, 10 \(\mu\)l of an aggregation-inducing agent was added to 390 \(\mu\)l PRP. The following concentrations of aggregation inducing agents were used: 10 \(\mu\)g/ml collagen and 20 \(\mu\)M ADP.

In the current study, 2 steps were taken. First, each GF was compared. Then, CHD and GCHD (the GF which proved to be the most effective to anti-platelet aggregation activity was added to CHD) were compared.

3) Assay of anti-hyperlipidemic activity

To evaluate the anti-hyperlipidemic effect, three kinds of hyperlipidemic animal models were prepared. First, hyperlipidemic rat model by corn oil was prepared according to the method of Duhaault et al. Six rats were used per group. Corn oil (3g/kg) was orally administered 2 hours after each sample (CHD 400 mg/kg, GCHD 200 and 400 mg/kg, and fenofibrate 800 mg/kg) was orally administered. Each sample was orally administered once per experiment. At 2 hours after the administration of corn oil, blood sample of rats was drawn by cardiac puncture under ether anesthesia.

Second, hyperlipidemic rat model by Triton WR-1339 was prepared according to the method of Kusama et al. Triton WR-1339 was injected at the end of the regular 16-hour fasting period as a 10% solution in saline at a dose of 200 mg/kg into the tail veins of rats under light ether anaesthesia. Six rats were used per group. These rats were anesthetized with ether 18 hours after Triton WR-1339 injection and 1-1.5 ml blood was withdrawn by cardiac puncture. Tested samples (CHD 400 mg/kg, GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg) were administered orally once a day for 3 days. The final administrations of these samples were performed 1 hour before Triton WR-1339 injection.
Third, hyperlipidemic rat model by high cholesterol diets was prepared according to the method of Niiho et al.\textsuperscript{28} Rats were divided into 5 groups (normal, control group, GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg administered group). Each group contained 6 rats. Normal group received a solid normal diet (rodent chow: 32% protein, 5% fat, 2% fiber, and 60% nitrogen extract) alone. The control group was fed on normal diet supplemented with 1% cholesterol, 0.25% cholic acid, and 2.5% olive oil (CCO) for 21 days. The GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg administered groups were fed on normal diet supplemented with CCO and orally administered GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg. After 24 hour fasting period from final administration of sample, blood samples of rats were drawn by cardiac puncture under ether anesthesia.

4) Determination of serum lipid levels
Serum was obtained from a portion of the blood sample by centrifugation at 3000 rpm for 30 minutes. All serum samples were stored at room temperature before analysis.
Serum total cholesterol (TC) and triglyceride (TG) were measured by the enzyme method designed by Allain et al. and Vanhadel and Zilversmit.\textsuperscript{29,30} Serum density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were measured by the method designed by Mainard and Madec and Lopes et al.\textsuperscript{31,32}

5) Acute toxicity
Toxicity study was carried out using female and male ICR mice. Mice were divided into 6 groups (5 GCHD administered groups and control group) with 5 males and 5 females in each group. GCHD was orally administered by gavage at a dosage of 312.5, 625, 1250, 2500, and 5000 mg/kg. The general behavior of the mice was observed continuously for 6 hours immediately after administration and then intermittently observed for a period of 14 days. The mice were further observed once a day for up to 14 days after treatment. General condition, any signs of toxicity, and death were also recorded. All mice weighed 4 times; before treatment and 3, 10, and 14 days after treatment. At the end of the experiment, mice were anesthetized under ether and sacrificed by cervical dislocation. The outward appearance and abnormality of the organs were carefully examined with the unaided eye. The weight of the heart, liver, spleen, and kidney were measured and recorded.\textsuperscript{33}

6) Statistical analysis
All data were expressed as mean ± standard error and statistical significance was determined using Student’s t-test. P-values less than 0.05 were considered as significant.

### Results

1. Anti-oxidant activity of GF
The anti-oxidant activity of the sample was expr-

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH$^1$</th>
<th>IC$_{50}$ (mg/mL)</th>
<th>Xanthine oxidase$^3$</th>
<th>Hemolysis$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW</td>
<td>0.076</td>
<td>0.026</td>
<td>5.13</td>
<td></td>
</tr>
<tr>
<td>GE</td>
<td>0.087</td>
<td>0.024</td>
<td>5.42</td>
<td></td>
</tr>
<tr>
<td>GEB</td>
<td>0.050</td>
<td>0.011</td>
<td>2.22</td>
<td></td>
</tr>
<tr>
<td>GEBW</td>
<td>0.038</td>
<td>0.005</td>
<td>1.54</td>
<td></td>
</tr>
</tbody>
</table>

GW: water extract GE: EtOH extract GEB: n-BuOH fraction GEBW: HOH fraction
$^1$: Absorbance of DPPH radical at 520 nm
$^3$: Absorbance of NBT at 560 nm
$^4$: Absorbance of RBC at 540 nm
essed as IC\textsubscript{50}. The IC\textsubscript{50} value was defined as the concentration of extracts that scavenged the DPPH radicals, xanthine oxidase activity, and red blood cell (RBC) membrane hemolysis by 50%.

As shown in Table 1, IC\textsubscript{50} of GEBW was 0.038, 0.005, and 1.54 mg/mL for the DPPH scavenging activity, the inhibition of xanthine oxidase activity, and that of RBC membrane hemolysis respectively. GEBW proved to be the most effective to anti-oxidant activity among GF.

2. Anti-oxidant activity of CHD and GCHD
As shown in Table 2, IC\textsubscript{50} of CHD and GCHD were 0.048 and 0.021 mg/mL, respectively, for the inhibition of xanthine oxidase activity. GCHD proved to be more effective at inhibiting xanthine oxidase than CHD.

3. Anti-platelet aggregation of GF
The anti-platelet aggregation activity of the extract was expressed as IC\textsubscript{50}. The IC\textsubscript{50} value was defined as the concentration of extracts that inhibits platelet aggregation induced by ADP and collagen by 50%.

As shown in Table 3, IC\textsubscript{50} of GEBW was 2.2 and 6.2 mg/mL for the inhibition of ADP and collagen induced platelet aggregation, respectively. GEBW proved to be the most effective at inhibiting the platelet aggregation induced by ADP and second most at inhibition of platelet aggregation induced by collagen among GF.

4. Anti-platelet aggregation of CHD and GCHD
As shown in Table 4, IC\textsubscript{50} of CHD and GCHD were 25.5 and 22.4 mg/mL, respectively, for the inhibition of ADP induced platelet aggregation and

### Table 2. Anti-oxidant activity of CHD and GCHD

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH\textsuperscript{1}</th>
<th>Xanthine oxidase\textsuperscript{2}</th>
<th>Hemolysis\textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD</td>
<td>0.037</td>
<td>0.048</td>
<td>1.31</td>
</tr>
<tr>
<td>GCHD</td>
<td>0.032</td>
<td>0.021</td>
<td>1.48</td>
</tr>
</tbody>
</table>

CHD: Chunglyul-dan  GCHD: Ginkgo-Chunglyul-dan
\textsuperscript{1}: Absorbance of DPPH radical at 520 nm
\textsuperscript{2}: Absorbance of NBT at 560 nm
\textsuperscript{3}: Absorbance of RBC at 540 nm

### Table 3. Anti-platelet aggregation activity of GF

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC\textsubscript{50} (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
</tr>
<tr>
<td>GW</td>
<td>7.2</td>
</tr>
<tr>
<td>GE</td>
<td>4.3</td>
</tr>
<tr>
<td>GEB</td>
<td>4.1</td>
</tr>
<tr>
<td>GEBW</td>
<td>2.2</td>
</tr>
</tbody>
</table>

GW: water extract  GE: EtOH extract  GEB: n-BuOH fraction  GEBW: HOH fraction

### Table 4. Anti-platelet aggregation activity of CHD and GCHD

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC\textsubscript{50} (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
</tr>
<tr>
<td>CHD</td>
<td>25.5</td>
</tr>
<tr>
<td>GCHD</td>
<td>22.4</td>
</tr>
</tbody>
</table>

CHD: Chunglyul-dan  GCHD: Ginkgo-Chunglyul-dan
44.7 and 34.9 mg/mL, respectively, for that of collagen-induced platelet aggregation. GCHD proved to be more effective at the inhibition of platelet aggregation than CHD.

5. Anti-hyperlipidemic effects of CHD and GCHD in corn oil-induced hyperlipidemic rats

As shown in Table 5, there was no significant effect on serum level of TC 2 hours after treatment with corn oil (3g/kg). However, TG level increased by 230 mg/dL compared with normal. Conversely, when rats were administered CHD 400mg/kg and GCHD 400mg/kg, serum level of TG decreased significantly by 102.8 and 123.3 mg/dL, respectively, compared with the group with corn oil alone. There was no significant difference between the CHD 400 mg/kg and the GCHD 400 mg/kg groups.

6. Anti-hyperlipidemic effects of CHD and GCHD in Triton WR-1339-induced hyperlipidemic rats

As shown in Table 6, when rats were treated with Triton WR-1339 (200 mg/kg) for 18 hours, the amount of TG and TC increased by 741.3 and 172.9 mg/dL, respectively, compared with the normal group. In contrast, when rats were administered CHD 400 mg/kg and GCHD 400 mg/kg, serum levels of the CHD and the GCHD group markedly reduced by 249 and 267.1 mg/dL, respectively, for TG and 42.2 and 56.2 mg/dL respectively for TC, compared with the group treated with Triton WR-1339 alone. There was no significant difference between the CHD and the GCHD groups, however GCHD had a tendency to lower serum TG and TC levels more than CHD.

7. Anti-hyperlipidemic effects of GCHD in cholesterol diet induced rats

As shown in Table 7, when rats were treated with a high cholesterol diet for 3 weeks, the amount of TC, TG, and LDL-C increased by 198.4, 24, and 111.3 mg/dL, respectively, compared with the normal group. In contrast, when rats were administered with GCHD 200, 400 mg/kg, and lovastatin 50 mg /kg, serum levels of lipoproteins in the GCHD 200

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg, p.o.)</th>
<th>TG (mg/dL)</th>
<th>TC (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>114.3±8.92</td>
<td>77.8±4.43</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>344.3±46.2</td>
<td>83.0±6.93</td>
</tr>
<tr>
<td>CHD</td>
<td>400</td>
<td>241.5±17.7</td>
<td>75.2±6.71</td>
</tr>
<tr>
<td>GCHD</td>
<td>400</td>
<td>221.0±14.7</td>
<td>81.7±5.31</td>
</tr>
</tbody>
</table>

CHD: Chunghyul-dan  GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats.
*: Significantly different from the normal value (*: p<0.001)
†: Significantly different from the control value (†: p<0.05)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg, p.o.)</th>
<th>TG (mg/dL)</th>
<th>TC (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>163.0±12.3</td>
<td>80.3±10.1</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>904.3±79.4</td>
<td>253.2±13.4</td>
</tr>
<tr>
<td>CHD</td>
<td>400</td>
<td>655.3±71.1</td>
<td>211.0±10.1</td>
</tr>
<tr>
<td>GCHD</td>
<td>400</td>
<td>637.2±54.0</td>
<td>197.0±19.3</td>
</tr>
</tbody>
</table>

CHD: Chunghyul-dan  GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats.
*: Significantly different from the normal value (*: p<0.001)
†: Significantly different from the control value (†: p<0.05)
mg/kg group reduced by 30.7, 20, and 27 mg/dL for TC, TG, and LDL-C, respectively, compared with the group treated with a high cholesterol diet. The GCHD 400 mg/kg and lovastatin 50 mg/kg groups significantly reduced by 35.7 and 34 mg/dL, respectively, for TC, 27.3 and 18 mg/dL, respectively, for TG, and 38.3 and 36.2 mg/dL, respectively, for LDL-C, compared with the group treated with a high cholesterol diet. HDL-C level decreased by 16.5 mg/dL compared with the normal group. However, after treatment with GCHD 200, 400 mg/kg and lovastatin 50 mg/kg, the serum concentrations of HDL-C increased by 2.5, 6.0, and 1.2 mg/dL, respectively, compared with the group with a high cholesterol diet alone.

8. Anti-hyperlipidemic effects of GCHD in corn oil-induced rats

As shown in Table 8, there was no significant increase in serum level of TC 2 hours after treatment with corn oil (3 g/kg). However, TG level increased by 210 mg/dL, compared with normal. When rats were administered GCHD 200 or 400 mg/kg, or fenofibrate 800 mg/kg, TC level significantly decreased by 23.4 mg/dL only in the fenofibrate 800 mg/kg group, compared with the group with corn oil alone. Serum levels of TG decreased significantly by 79.4, 88.9, and 192.9 mg/dL, respectively, compared with the group with corn oil alone. Fenofibrate 800 mg/kg was most effective at lowering serum TG levels.


As shown in Table 9, when rats were treated with Triton WR–1339 (200 mg/kg) for 18 hours, the amount of TC, TG, and LDL-C increased by 227.9, 767.3, and 81.6 mg/dL, respectively, compared with
the normal group. In contrast, when rats were administered with GCHD 200, 400 mg/kg, and lovastatin 50 mg/kg, serum levels of lipoproteins in the GCHD 200 mg/kg group reduced by 31.2, 78.3, and 12.3 mg/dL for TC, TG, and LDL-C, respectively, compared with the group treated with Triton WR-1339 alone. The GCHD 400 mg/kg and the lovastatin 50 mg/kg group significantly reduced by 72.4 and 67.4 mg/kg, respectively, for TC, 181.3 and 120 mg/dL, respectively, for TG, and 21.3 and 14 mg/dL, respectively, for LDL-C, compared with the group treated with Triton WR-1339 alone. The GCHD 400 mg/kg and the lovastatin 50 mg/kg, the serum concentrations of HDL-C increased by 1.7, 4.4 and 6.7 mg/dL, respectively, compared with the group treated with Triton WR-1339 alone.

10. Acute toxicity of GCHD

Oral administration of GCHD in a dose of 312.5 to 5000 mg/kg did not produce behavioral changes

Table 9. Anti-hyperlipidemic effects of GCHD in Triton WR-1339-induced hyperlipidemic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg, p.o.)</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>71.8±10.4</td>
<td>57.0±5.87</td>
<td>49.0±2.99</td>
<td>19.7±1.25</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>299.7±16.8</td>
<td>824.3±30.9</td>
<td>39.8±0.72</td>
<td>101.3±6.22</td>
</tr>
<tr>
<td>GCHD</td>
<td>200</td>
<td>268.5±16.8</td>
<td>746.0±64.7</td>
<td>41.5±3.56</td>
<td>89.0±3.54</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>227.3±22.2</td>
<td>643.0±44.3</td>
<td>44.2±1.56</td>
<td>80.0±6.72</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>50</td>
<td>232.3±18.4</td>
<td>704.3±33.9</td>
<td>46.5±2.91</td>
<td>87.3±1.99</td>
</tr>
</tbody>
</table>

GCHD: Ginkgo-Chunghyul-dan

Values are mean ± standard error of 6 rats

*: Significantly different from the normal value (\( p<0.001 \))

\( \dagger \): Significantly different from the control value (\( p<0.05 \))

\( \ddagger \): Significantly different from the control value (\( p<0.01 \))

Table 10. Survival time and body weight changes in mice orally administered with single dosage of GCHD (5 male and 5 female mice per group)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dose (mg/kg)</th>
<th>Number of deaths</th>
<th>Days after administration</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>31.8±0.82</td>
</tr>
<tr>
<td></td>
<td>312.5</td>
<td>0</td>
<td>0</td>
<td>30.4±0.57</td>
</tr>
<tr>
<td></td>
<td>625</td>
<td>0</td>
<td>0</td>
<td>30.6±0.27</td>
</tr>
<tr>
<td></td>
<td>1250</td>
<td>0</td>
<td>0</td>
<td>30.6±0.84</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>0</td>
<td>0</td>
<td>31.2±0.42</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>0</td>
<td>0</td>
<td>31.4±0.57</td>
</tr>
<tr>
<td>Female</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>25.2±0.74</td>
</tr>
<tr>
<td></td>
<td>312.5</td>
<td>0</td>
<td>0</td>
<td>26.2±0.42</td>
</tr>
<tr>
<td></td>
<td>625</td>
<td>0</td>
<td>0</td>
<td>25.2±0.89</td>
</tr>
<tr>
<td></td>
<td>1250</td>
<td>0</td>
<td>0</td>
<td>25.0±0.61</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>0</td>
<td>0</td>
<td>23.6±0.82</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>0</td>
<td>0</td>
<td>25.6±1.04</td>
</tr>
</tbody>
</table>

Values are mean ± standard error
nor did death of mice occur (Table 10). Autopsy study with naked eye showed no visible change. There was no significant difference in body weight and organ weight after the oral administration of GCHD 312.5 to 5000 mg/kg (Table 10, Fig. 2, 3). These effects were observed during the experimental period. These results showed that the medium lethal dose (LD50) was higher than 5000 mg/kg for male and female mice.

**Discussion**

The purpose of this study was to develop new herbal medicine having a therapeutic effect on prevention of stroke through the mechanisms of scavenging free radicals, inhibiting platelet aggregation, and lowering cholesterol levels. Thus, we investigated anti-oxidant activity, anti-platelet aggregation activity, and anti-hyperlipidemic activity of GCHD in comparison with CHD.

First, we performed comparatively studies with GW, GE, GEB, and GEBW to find which *Ginkgo Folium* fraction was the most effective to anti-oxidant and anti-platelet aggregation activity. GEBW proved to be the most effective, so we made GCHD by adding GEBW to CHD.

Second, we compared GCHD with CHD for effects on inhibiting cholesterol biosynthesis, HMG-
CoA reductase, pancreatic lipase, nitrite oxide synthesis, and scavenging free radicals to evaluate antioxidant activities.\textsuperscript{10-12} GCHD showed a more potent xanthine oxidase inhibiting activity than CHD. This result suggests the possibility that GCHD could be used for the treatment and prevention for stroke because oxygen-derived free radicals seem to be involved in the pathophysiology of cerebral ischemia as well as in reperfusion\textsuperscript{34}, and agents with a capacity to scavenge their action proved to be effective to the recovery of stroke.\textsuperscript{35,36}

We also conducted platelet aggregation study with GCHD and CHD. Platelet activation plays a multifactorial role in the production of cerebral ischemia. Platelet activation contributes to the formation of atherosclerosis of the carotid artery.\textsuperscript{37} During the acute stage of ischemic cerebrovascular disease, a significant increase in platelet aggregation occurs.\textsuperscript{38,39} Ischemic damage may be propagated following an acute ischemic event as platelet aggregates may contribute to ongoing ischemic damage.\textsuperscript{40} Determination of platelet aggregation by aggregometers involves adding an aggregating agent to plasma-containing platelets. The aggregating agents used in this study included ADP and collagen. ADP acts on platelets via ADP receptor and collagen acts via the combined glycoprotein Ia/IIa receptor.\textsuperscript{41} When high concentrations of ADP and collagen are present, platelet aggregability is increased. In this study, GCHD had a more anti-platelet aggregation effect than CHD. This result suggests the possibility that GCHD could be used for stroke prevention because many anti-platelet agents have been used for treatment and prevention of stroke.\textsuperscript{42-44}

We also investigated which one was more effective in lowering serum cholesterol levels between GCHD and CHD. Though there was no significant difference in lowering serum cholesterol levels between CHD and GCHD in corn oil and Triton WR-1339-induced hyperlipidemic models, GCHD had a tendency to reduce the serum cholesterol levels more than CHD. Thus, GCHD could be used as an agent for treating hyperlipidemia and preventing stroke because CHD is used for lowering serum cholesterol levels and prevention of stroke. We further investigated whether GCHD had anti-hyperlipidemic potential at the level of serum lipids in high cholesterol diet-, corn-oil-, and Triton-WR 1339-induced rats, too. Triton WR-1339 is one of the many well-known non-ionic detergents that induce increases in plasma cholesterol and TG by increasing hepatic cholesterol biosynthesis. The mechanisms underlying this phenomenon have been suggested to be the consequence of the trapping of cholesterol and TG in the blood compartment and the subsequent reduction of the influx of plasma in the liver.\textsuperscript{45,46} Corn oil contains abundant oleic acid and linoleic acid, which are known to influence plasma TG levels in mammals.\textsuperscript{47}

In the present study, the results showed that GCHD 400 mg/kg significantly reduced TG, TC, and LDL-C levels in high cholesterol diet-, corn oil-, and Triton WR-1339-induced hyperlipidemic rats, indicating that GCHD was a potent anti-hyperlipidemic agent.

With respect to the hypolipidemic activity of GCHD, there are two possible explanations. One is a synergistic effect of the six components. \textit{Scutellariae Radix} has anti-oxidant activity by DPPH radical scavenging activity\textsuperscript{48,49} and anti-hyperlipidemic activity by inhibiting HMG-CoA reductase and pancreatic lipase.\textsuperscript{10} \textit{Coptidis Rhizoma} has anti-hyperlipidemic activity by reducing lipid peroxidation\textsuperscript{50} and inhibiting HMG-CoA reductase and pancreatic lipase.\textsuperscript{10} \textit{Phellodendri Cortex} has antithrombic activity.\textsuperscript{51} \textit{Gardeniae Fructus} has anti-hyperlipidemic activity by inhibiting pancreatic lipase.\textsuperscript{52} \textit{Rhei Rhizoma} has anti-oxidant activity by direct scavenging of nitric oxide\textsuperscript{53} and anti-hyperlipidemic activity by inhibiting HMG-CoA reductase and pancreatic lipase.\textsuperscript{10} \textit{Ginkgo Folium} has anti-oxidant activity by scavenging free radicals, anti-platelet aggregation activity, and anti-hyperlipidemic effect by reducing oxidized LDL-C and circulating free cholesterol level.\textsuperscript{54-57} The other explanation is that the hypolipidemic activity of GCHD is involved
with the inhibition of cholesterol synthesis and interference with lipoprotein distribution through anti-oxidant activity of GCHD.

In the current study, LDL-C level was dose-dependently decreased in the GCHD administered groups in high cholesterol diet hyperlipidemic rats, and GCHD 400 mg/kg was better to lower LDL-C level than lovastatin 50 mg/kg. This result suggested the possibility that GCHD 400 mg/kg could be used for treating hyperlipidemia and preventing stroke. This possibility can be explained by the fact that LDL-C level is a target therapy point for hyperlipidemia, elevated LDL-C is associated with increasing the incidence of stroke,58-61 lowering LDL-C level can prevent the attack and recurrence of stroke62) and statin appears to be associated with significant reduction in stroke risk.4,6,63)

There was no acute toxicity at a single dosage in the present study; GCHD proved to be a safe drug.

In conclusion, GCHD had anti-oxidant, anti-platelet aggregation and anti-hyperlipidemic activities. GCHD was a safe and effective herbal medicine used for treating hyperlipidemia and preventing stroke. But, in this study, there was some limitation, as this was in vitro and in vivo experimental study, so further research will be needed to be undertaken to elucidate the biochemical mechanism of anti-oxidant, anti-platelet aggregation, and hypolipidemic activity of GCHD and clinical study will be needed. Further study will also be needed to determine GF dose.

### Conclusions

1. GCHD proved to have anti-oxidant activity in the study inhibiting the formation of DPPH radicals, xanthine oxidase activity, and RBC membrane hemolysis.
2. GCHD proved to have anti-platelet aggregation activity in ADP and collagen-induced platelet aggregation study.
3. GCHD 400 mg/kg significantly lowered TC, TG, and LDL-C levels in high cholesterol diet-induced model.
4. GCHD 400 mg/kg lowered TG level in corn oil-induced model.
5. GCHD 400 mg/kg significantly lowered TC, TG, and LDL-C levels in Triton WR-1339-induced model.
6. GCHD had no acute toxicity at a single dosage.

### References

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