Effects of *TongBiEum*(TBE) on Hyperlipidemia Induced by a Sucrose-rich Diet

Yong-Seong Oh, Yoon-Sik Kim, In-Chan Seol

Department of Circulatory Internal Medicine, College of Oriental Medicine, Daejeon University, Daejeon, Korea

**Introduction**

As the economy advances and eating habits become more indulgent, the mortality rate due to vascular disease, heart disease, hypertension, hyperlipidemia and arteriosclerosis is increasing.1,2

Hyperlipidemia refers to an elevation of lipids in the bloodstream. These lipids include cholesterol, cholesterol esters, phospholipids and triglycerides, all of which are risk factors in developing heart disease. Other factors such as genetics, the environment, lifestyle, and the presence of other diseases such as diabetes and hypertension may also contribute to the development of heart disease. Hyperlipidemia is a major risk factor for coronary arterial disease, cerebrovascular disease, and peripheral vascular disease. Thus, the management of plasma cholesterol levels as a potential risk factor has been the focus within the field of such diseases indicted above research.2-5

Recently, the effectiveness of a new class of powerful hypolipidemic agents has been tested in several large clinical trials.6 Unfortunately, chemical therapeutics causes serious organ toxicity including adrenocortical
degeneration. In many cultures, herbal remedies are increasingly being employed in an attempt to achieve the same purpose with less toxicity\textsuperscript{7}.

From the inference that herbal remedies may be less toxic than therapeutic chemicals, a growing interest in oriental medicine has emerged with an increase in the number of non-pharmacological therapies within lipid management\textsuperscript{7,8}.

A medicine named TongBiEum (TBE) is used at Daejeon University Oriental Medical Hospital for patients during the recovery phase after a stroke, numbness or pain and hyperlipidemia. We have evaluated its therapeutic effects through patient-based clinical trials, but the scientific basis of its effect on hyperlipidemia has not yet been examined in the laboratory. Therefore, this study aimed to determine the effects of TBE on hyperlipidemia through gene expressions related with lipid metabolism, serum triglyceride as HDL-cholesterol levels, and perceived histological changes.

**Materials and Methods**

1. Materials

Medicinal herbs were purchased from Daejeon Oriental Medical Hospital. The composition of TongBiEum (TBE) formation is described in Table 1. After drying, 1 day’s dosage (168g) of the formulation for a human adult was mixed with 600 ml of distilled water and left for 1 hr at room temperature, and the whole mixture was then boiled twice for 1 hr each time. The TBE extract was then freeze-dried. The yield of TBE extract was 10.71% (w/w) in terms of the dried medicinal herbs. The TBE extract was suspended in distilled water and given orally to mice once daily for five weeks.

Mice in the control group were orally given distilled water. DNA Taq polymerase was obtained from Bioneer (Cheong-Won, Korea), M-MLV reverse transcriptase was obtained from Promega (Madison, U.S.A.). TRIzol reagent was obtained from Gibco (Maryland, U.S.A.). Lest reagent was purchased from Sigma Inc. (St. Louis, U.S.A.).

2. Experimental animals

5-week-old male Sprague-Dawley rats were purchased from a commercial animal breeder (Daehan BioLink, Korea). After one week of acclimation, 24 rats were used for this experiment. The rats were housed in an environmentally controlled room at $22 \pm 2^\circ C$.

<table>
<thead>
<tr>
<th>Scientific Name</th>
<th>Part used</th>
<th>Voucher specimen number</th>
<th>Dosage (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehmannia glutinosa (\text{Radix})</td>
<td>Radix</td>
<td>RG-2001-01-Ra</td>
<td>12</td>
</tr>
<tr>
<td>Paonia lactiflora (\text{Radix})</td>
<td>Radix</td>
<td>PL-2001-01-Ra</td>
<td>8</td>
</tr>
<tr>
<td>Cinnamomum cassia (\text{Ramulus})</td>
<td>Ramulus</td>
<td>CC-2001-01-Ra</td>
<td>8</td>
</tr>
<tr>
<td>Eucommia ulmoides (\text{Cortex})</td>
<td>Cortex</td>
<td>EU-2001-01-Co</td>
<td>4</td>
</tr>
<tr>
<td>Achyranthes bidentata (\text{Ramulus})</td>
<td>Radix</td>
<td>AB-2001-01-Ra</td>
<td>4</td>
</tr>
<tr>
<td>Angelica gigas (\text{Radix})</td>
<td>Radix</td>
<td>AG-2001-01-Ra</td>
<td>4</td>
</tr>
<tr>
<td>Lycium chinense (\text{Fructus})</td>
<td>Fructus</td>
<td>LC-2001-01-Fr</td>
<td>4</td>
</tr>
<tr>
<td>Poria cocos (\text{Ramulus})</td>
<td>Radix</td>
<td>PC-2001-01</td>
<td>4</td>
</tr>
<tr>
<td>Asarum sieboldii (\text{Ramulus})</td>
<td>Radix</td>
<td>AS-2001-01-Ra</td>
<td>4</td>
</tr>
<tr>
<td>Angelica dahurica (\text{Radix})</td>
<td>Radix</td>
<td>AD-2001-01-Ra</td>
<td>4</td>
</tr>
<tr>
<td>Aconitum carmichaeli (\text{Radix})</td>
<td>Radix</td>
<td>AC-2001-01-Ra</td>
<td>4</td>
</tr>
<tr>
<td>Glycyrrhiza uralensis (\text{Radix})</td>
<td>Radix</td>
<td>GR-2001-01-Ra</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total amount</strong></td>
<td></td>
<td></td>
<td><strong>64</strong></td>
</tr>
</tbody>
</table>
relative humidity at 55 ± 10%, 12 hrs light/dark and fed commercial pellets (Samyang Feed Ltd., Korea) and tap water ad libitum.

24 rats were divided into 4 groups of 6. Hyperlipidemia was induced by feeding the animals a high-cholesterol diet for 4 weeks. The high-cholesterol diet was made at our laboratory and contained 1% cholesterol, 0.25% cholic acid and 2.55% olive oil.

Animals in the treatment group were given the high-cholesterol diet ad libitum for 4 weeks, whereas rats in the normal group were given a commercial diet (Samyang feed, Korea). 2 weeks after being given the high-cholesterol diet, the rats in the treatment group were orally administrated TBE, (200 mg/10 kg) or Lipidil, (3.33 mg/10 kg); the rats in control group were given 10 mg/kg of distilled water. Every week during the experimental period, the animals were fasted for 4 hrs and blood was collected from the orbital vein under ether anesthesia. On the last day, the animals were fasted for 12 hrs and whole blood samples were collected from an abdominal aorta, and the liver, spleen and thymus were removed, weighted and fixed in phosphate buffered formalin. After the blood had clotted for 1 hr it was centrifuged at 3000 rpm for 15 min to separate a serum.

3. Hematological examinations

Under ether anesthesia, whole blood was collected from the abdominal vein and transferred to vials treated with ethylenediamine tetraacetic acid (EDTA).

Hematological parameters, red blood cell (RBC), white blood cell (WBC), hematocrit (HCT), hemoglobin (HB) and platelet (PLT) was performed using an automated blood analyzer, Hemavet (CDC Technologies, Inc., Oxford, CT).

4. Serum biochemical analysis

The levels of serum aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol (CHO), high-density lipoprotein cholesterol (HDL), and triglyceride (TG) were determined using Olympus Optical Reply (Olympus Ltd., Japan).

5. Histopathological observations

For the histomorphological evaluation, a portion of liver tissue was removed and fixed in 10% phosphate buffered formalin. The paraplast-embedded liver section (4 mm in thickness) was stained with hematoxylin & eosin for histopathological examination.

6. RT/PCR for analysis diacylglycerol acyltransferase (DGAT) and acyl-coenzyme A:cholesterol acyltransferase (ACAT) gene expression

Total cellular RNA was isolated by the TRIzol reagent (Gibco, MD) according to the manufacturer’s instructions. The mRNA levels were fixed quantity at 260 nm by spectrophotometer (Cary50, Varian, U.S.A.). Total RNA was extracted from homogenized liver samples of SD female rats.

The RNA (1 µg) was reverse-transcribed (RT) into first strand cDNA in an RT mixture containing 2 µl 10mM dNTPs mix, 1 µl oligo-dT primer (20 pmol/µl), 2 µl 100mM DTT, 4 µl 5 xRT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM MgCl2, RNase inhibitor 20U), 1 µl M-MLV RT (200 U/µl; Promega, U.S.A.) and 2 µl DDW. The RT mixture was incubated at 42°C for 60 min, heated to 72°C for 10 min to inactivate the reverse transcriptase activity, and chilled to 4°C for 5 min. A portion of the RT product (1 µl) was then subjected to the polymerase chain reaction (PCR) in a DNA thermal cycler (TaKaRa, Tokyo).

To determine the expression pattern of DGAT and ACAT mRNA in rat liver, 1 µl of cDNA was amplified by a thermal cycler using the primers (Table 2). The PCR mixture was made as follows: 1.5 units of Taq
DNA polymerase (Bioneer, Korea), 3 μL of 10 mM dNTPs, 3 μL of 10 × PCR buffer, 1 μL of 10 pmol sense and antisense primers, and 3 μL of cDNA in 19.7 μL of ultra-distilled water.

PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95 °C for 10 min; then 35 cycles at 95 °C for 1 min (denaturing), at 60 °C for 40 s (annealing), and at 72 °C for 10 min. The PCR products were run on a 1% agarose gel in 0.5 × TBE buffer.

7. Statistical analysis
The results were expressed as the mean ± standard deviation (S.D.). Statistical analysis of the data was carried out by Student’s t-test. The difference from the respective control data at the levels of p<0.05 was regarded as statistically significant.

Table 2. Oligonucleotide sequences of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Sense</td>
<td>5'-GTG GGG CGC CCC AGG CAC CA-3'</td>
<td>539 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'</td>
<td></td>
</tr>
<tr>
<td>DGAT</td>
<td>Sense</td>
<td>5'-GAA TAT CCC GTG GCA CAA GT-3'</td>
<td>255 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-CAC AGC TGC ATT GCC ATA GT-3'</td>
<td></td>
</tr>
<tr>
<td>ACAT</td>
<td>Sense</td>
<td>5'-CCT CCC GGT TCA TTC TGA TA-3'</td>
<td>370 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-ACA CCT GGC AAG ATG GAG TT-3'</td>
<td></td>
</tr>
</tbody>
</table>

Results

1. Clinical signs and changes in body weight
During the entire experimental period, no rats died. The rats' gain in body weight for those administered TBE and Lipidil was less than that of the normal and control groups. Especially, the body weight gain of rats in the group administrated TBE was significantly lighter than that of rats in the control group at 4 weeks (p<0.01), 2 weeks after administration (Fig. 1).

2. Gross findings and organ weights
At autopsy after the termination of treatment, hypertrophy of liver was observed in rats of all groups given a high-cholesterol diet (Fig. 2).

In the control group, absolute liver weight increased significantly (p<0.01) as compared with the normal group, but not significantly in the groups administrated TBE or Lipidil. Absolute liver weight in the group administrated TBE was significantly lighter than that in the control group (p<0.01), but not in the group administrated Lipidil (Fig. 3).

Relative liver weight increased significantly (p<0.001) in all groups that were given a high-cholesterol diet. Relative liver weight in the group administrated TBE decreased significantly (p<0.05) compared with the control group (Fig. 4).

3. Hematological parameters
As shown in Table 3, RBC counts increased signifi-
Significantly in the group administered Lipidil compared with the control group ($p<0.05$). PLT counts decreased in rats of all groups given a high-cholesterol diet.

4. Histopathological observations

After hyperlipidemia was induced by a high-cholesterol diet for 4 weeks, macrovacuolar cytoplasmic
alterations of hepatocytes were detected in the periportal area. These alterations were observed in all groups given a high-cholesterol diet, most significantly in the control. Macrovacuolar and microvacuolar in the group administrated TBE were least among the groups given a high-cholesterol diet. An increase in the number of apoptoses was markedly observed in the control group (Fig. 5).

5. Serum biochemical analysis
AST and ALT activities did not show significant differences through the experimental period (Fig. 6, 7).

In the group administered TBE, at 3 weeks and 4 weeks (1 week and 2 weeks after administration), TG levels decreased significantly as compared with the control group \(p<0.01, p<0.05\). Also, in the group administrated Lipidil, TG levels at 4 weeks (2 weeks after administration) decreased significantly as compared with the control group (Fig. 8).

Cholesterol levels decreased at 3 weeks and 4 weeks (1 week and 2 weeks after TBE administration), as compared with the control group (Fig. 9).

HDL levels decreased at 3 weeks and 4 weeks (1 week and 2 weeks after TBE administration), as compared with the control group (Fig. 10).

6. Gene expression of DGAT, ACAT
DGAT gene expression decreased slightly in the group administered TBE, whereas it increased in the group administered Lipidil. In contrast, ACAT gene
expression decreased in the group administered TBE, but increased in the group administered Lipidil.

**Discussion**

Atherosclerotic vascular disease, particularly coronary heart disease, is known to be one of the leading causes of morbidity and mortality worldwide. High levels of low-density lipoprotein (LDL) and low levels of high-density lipoprotein (HDL) cholesterol are acknowledged as important coronary risk factors. Dietary control remains the sheet anchor of treatment for these conditions, but in many cases it becomes necessary to add a drug. As none among the available agents fulfill the requirements of the desired drug, there is a need to explore the possibility of introducing effective, safe and inexpensive alternatives.

Vascular diseases continue to take an awful toll on our society, so the recent mass of evidence from angiographic and clinical trials that coronary events in patients with symptomatic vascular disease can be reduced through lowering LDL may well be the most significant advance yet in cardiovascular therapeutics.

Reduction in serum cholesterol levels by various interventions, including diet, exercise, changes in lifestyle or pharmacological approaches, is associated with a significant reduction in cardiovascular mortality and morbidity. Recently, the effectiveness of a new class of powerful hypolipidemic agents has been tested.
in several large clinical trials\textsuperscript{4}. However, many promising agents have serious side effects, especially on adrenal function. In many cultures of the world, herbal remedies are increasingly being employed in an attempt to achieve the same purpose, but with less toxicity\textsuperscript{5}.

Fatty acids are an important and efficient energy source for many living cells.

In animals, most fatty acids are obtained from the diet. For example, in the average U.S. diet, between 30\% and 40\% of calories ingested are provided by fat\textsuperscript{12}.

Oxidation of fatty acids is our major metabolic source of energy to support our biological survival. Dietary fatty acids are esterified to form triglyceride (TG) and cholesterol, which is stored by fat cells in lipid droplets\textsuperscript{11,13}. Cholesterol, included in steroid lineage, is mainly synthesized in the liver by internalizing lipoprotein and concentrated primarily in the brain and spinal cord\textsuperscript{14}. Upon demand, intracellular triglyceride and cholesterol are hydrolyzed by the action of a hormone sensitive lipase to release free fatty acids in the form of lipoprotein particles and oxidased to generate energy, which is mainly controlled by the liver. Lipoproteins, known to transport cholesterol and triglycerol, solubilize hydrophobic lipids and signals to find target cells.

These are as follows: chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL)\textsuperscript{15-18}.

Numerous population studies have linked the elevated concentration of total cholesterol or LDL-cholesterol in plasma with an increased incidence of atherosclerotic events\textsuperscript{7}. A direct relationship between an elevated concentration of LDL in plasma and the development and progression of coronary heart disease has been reported. A reduction of plasma LDL levels has been suggested as an important means for prevention of coronary heart disease development\textsuperscript{8}. On the contrary, HDL accepts or stimulates the removal of cholesterol from peripheral cells\textsuperscript{19}, protects against coronary heart disease by preventing the oxidation of LDL or neutralizing the atherogenic effects of oxidized LDL in the artery wall\textsuperscript{17}, and protects by inhibiting the formation of platelet aggregates at sites of endothelial injury\textsuperscript{20}, or by inhibiting production of monocyte adhesion molecules by endothelial cells in the first stage of atherogenesis\textsuperscript{12,21}.

In addition, many of these enzymes are related with the absorption and storage of metabolic fatty acid. Diacylglycerol acyltransferase (DGAT)\textsuperscript{22,23} catalyzes the final acylation of the TG pathway, which is unique to TG synthesis. Cholesterol acyltransferase (ACAT)\textsuperscript{24}, responsible for the esterification of cholesterol, is the primary enzyme in the intestinal mucosal cholesterol absorption and it synthesizes the cholesterol esters that flow into very low density lipoproteins (VLDL), and to store in fatty cells. Inhibitors of these enzymes can lower plasma cholesterol and triglyceride levels by inhibiting absorption and storage of metabolic fatty acid; consequently, reduced VLDL production in the liver could directly block atherosclerotic lesion formation, reducing the possibility of vascular attacks\textsuperscript{25}.

The present studies demonstrate that TBE can reduce the rise in plasma cholesterol and TG levels induced by a high-cholesterol diet and also reverse pre-established hypercholesterolemia and hypertriglyceremia. In the TBE group, total cholesterol levels decreased(Fig. 9), TG levels decreased(Fig. 8), but HDL-cholesterol levels also decreased(Fig. 10). These show the decrease of other lipoprotein fractions including LDL and IDL. In the analysis of absolute and relative liver weight, TBE significantly inhibited the weight gain induced by a high-cholesterol diet as compared with the control group. We could suggest that the accumulation of fatty acid in the hepatocytes may be blocked by TBE. In the histological observations, lipid droplet and apoptotic
change in the TBE treated group were less compared with the control group.

In the serum biochemical analysis, a difference of serum AST and ALT changes among groups was not observed, but TG and total cholesterol levels were less and HDL level decreased compared with the control group. In the gene expression related with TG and cholesterol metabolism, DGAT decreased slightly but ACAT decreased more as compared with the control and Lipidil groups. This suggests that TBE inhibits preferentially the expression of ACAT but not DGAT.

From this study, we can suggest that TBE extract possesses a hypolipidemic effect by lowering serum total cholesterol and TG levels. The possible mechanism for prevention of hyperlipidemia may be related to lowering ACAT gene expression; TBE ingestion decreased the intestinal absorption and storage of exogenous and endogenous cholesterol. The implication is that TongBiEum (TBE) could be used for patients with hyperlipidemia.

**Conclusion**

This study was aimed to elucidate the effects of TBE on hyperlipidemia and the results were as follows.

1. TBE treatment significantly inhibited the liver weight gain induced by a high-cholesterol diet compared with the control group.
2. TBE treatment inhibited lipid droplet accumulation and apoptotic change in the liver compared with the control group.
3. TBE treatment significantly inhibited the increasing of serum TG and total cholesterol levels induced by high-cholesterol diet compared with the control group but did not affect HDL-cholesterol levels.
4. TBE treatment suppressed ACAT gene expression compared with the control group, but did not affect DGAT.

From this study, we can infer that TBE possesses hypolipidemic effect by inhibiting the intestinal absorption and storage of exogenous and endogenous cholesterol.

**References**