A high-cholesterol diet enriched with polyphenols from Oriental plums (*Prunus salicina*) improves cognitive function and lowers brain cholesterol levels and neurodegenerative-related protein expression in mice

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Abstract

Ageing accompanied by a decline in cognitive performance may be a result of the long-term effects of oxidative stress on neurologic processes. It has been shown that high-cholesterol contents in the blood and brain may lead to the deposition of the β-amyloid (Aβ) protein in the brain, which damages brain cells. The present study was designed to observe the effect of polyphenol-rich Oriental plums on cognitive function and cerebral neurodegeneration-related protein expression in mice that were fed a high-cholesterol diet for 5 months. The study consisted of four groups: the control (Ctrl) group, which was fed the American Institute of Nutrition (AIN)-93M diet; the high cholesterol (HC) group, which was fed the AIN-93M diet with 5% cholesterol; the high cholesterol + low Oriental plum (HOP) group, which was fed the AIN-93M diet with 5% cholesterol and 2% Oriental plum powder; and the high cholesterol + high Oriental plum (LOP) group, which was fed the AIN-93M diet with 5% cholesterol and 5% Oriental plum powder. Measurements of cognitive function were assessed using the Morris water maze, and the mRNA expression of cholesterol hydroxylase (Cyp46), Aβ and β-secretase 1 (BACE1) were analysed. The results showed that cholesterol concentrations in both the blood and the brain were significantly higher in the HC group than in the Ctrl and HOP groups at the end of the trial. The high-cholesterol diet per se produced significant cognitive deficits, which were accompanied by a significantly increased mRNA expression of Cyp46, BACE1, Aβ and 24-hydroxycholesterol in the brain cortex and hippocampus. However, all of these variables were non-significantly increased in the HOP group as compared to the Ctrl group. In conclusion, incorporating polyphenol-enriched Oriental plum into a high-cholesterol diet can ameliorate some of the symptoms of neurodegenerative conditions.

Key words: Alzheimer’s disease; Cholesterol; Oriental plums; β-Amyloid; 24-Hydroxycholesterol; Neurodegeneration

As the proportion of the elderly population in the world increases, so will the prevalence of cognitive impairment as a result of normal ageing and neurodegenerative conditions such as Alzheimer’s disease (AD)(¹) which is one of the most common forms of neurodegenerative disease. Patients who suffer from cognitive deficits exhibit memory loss, delusions and problems carrying out activities associated with normal daily life. The pathologic characteristics of AD include the presence and accumulation of β-amyloid (Aβ), neurofibrillary tangles, inflammation, oxidative stress and cell loss in the brain(²). There are two subtypes of AD. One is caused by genetics (early-onset AD), and the other, more common, subtype is called late-onset AD. Factors that cause late-onset AD include ageing, diabetes mellitus, hypercholesterolaemia and oxidative stress(³).

Studies have examined the relationship between increased plasma cholesterol concentrations and AD(⁴). They have also indicated that the cholesterol level in the brain is one of the factors that regulates amyloid precursor protein (APP) processing and Aβ formation(⁵). The accumulation of cholesterol in hippocampal neurons, which results in accelerated cleavage of the APP into amyloidogenic components, has been observed in cell culture results(⁶). In the brain, cholesterol almost exclusively originates from in situ synthesis, whereas circulating cholesterol is normally prevented from entering the central nervous system by the blood–brain barrier(⁷). Because cholesterol cannot be eliminated from the central nervous system and may be toxic to neurons when it is present in excess, it is converted to 24-hydroxycholesterol (24-OHC) by 24-hydroxylase (also called Cyp46) before being transported...
Effect of polyphenols from Oriental plums on cholesterol

of the brain. This mechanism constitutes the major pathway of cholesterol homeostasis in the brain. Epidemiological studies have indicated that 24-OHC concentrations in cerebrospinal fluid are increased in AD patients.

The Oriental plum (*Prunus salicina* Lindley), also called the Chinese plum or Japanese plum, is rich in polyphenols and anthocyanins. It has been shown that consuming polyphenol-rich foods may prevent the onset of AD. In addition, the accumulation of Aβ in the brain was reduced and cognitive degradation was slowed in streptozotocin-induced diabetic rats that were fed a powered plum diet. It has also been shown that blood cholesterol concentrations in rats can be reduced in a dose–response manner by consuming a polyphenol-rich diet. The purpose of the present study was therefore to examine the effects of consuming Oriental plums on cognitive performance and cerebral neurodegeneration-related protein expression in C57BL/6 mice that were fed a cholesterol-enriched diet.

### Materials and methods

#### Animals and diets

The present experiment was approved by the Institutional Animal Care and Use Committee or Panel (IACUC/IACUP) of Taipei Medical University. A total of sixty 4-week-old male C57BL/6-strain mice were purchased from BioLASCO. Before the experiment began, the mice had access to food and water *ad libitum* and were housed at 23 ± 2°C. After being allowed to acclimatise to these conditions for 2 weeks, the mice were randomly divided into four groups of twenty mice each and kept on a 12 h light–12 h dark cycle. Each group received one of the following treatments for 20 weeks (5 months): the control (Ctrl) group was fed the American Institute of Nutrition (AIN)-93M diet; the high cholesterol (HC) group was fed the AIN-93M diet with 5% cholesterol; the high cholesterol + low Oriental plum (LOP) group was fed the AIN-93M diet with 5% cholesterol and 2% Oriental plum powder; and the high cholesterol + high Oriental plum (HOP) group was fed the AIN-93M diet with 5% cholesterol and 5% Oriental plum powder. Oriental plums (*P. salicina*) were purchased from a local market. Dried plum powder was produced by freeze-drying and then grinding the plums. The experimental diets consisting of placing the mice in the water for 60 s. Performances of the submerged platform were removed. The retention test consisted of placing the mice in the water for 60 s. Performances

### Table 1. Diet composition of each group

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Ctrl</th>
<th>HC</th>
<th>LOP</th>
<th>HOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize starch</td>
<td>46.8</td>
<td>44.6</td>
<td>42.6</td>
<td>39.2</td>
</tr>
<tr>
<td>Dextrinised maize starch</td>
<td>15.6</td>
<td>14.8</td>
<td>14.8</td>
<td>15.0</td>
</tr>
<tr>
<td>Casein</td>
<td>14.1</td>
<td>13.4</td>
<td>13.4</td>
<td>13.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
<td>9.6</td>
<td>9.6</td>
<td>9.7</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>4.0</td>
<td>3.8</td>
<td>3.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>3.5</td>
<td>3.3</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.0</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Plumb powder</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Ctrl, control; HC, high cholesterol; LOP, high cholesterol + low Oriental plum; HOP, high cholesterol + high Oriental plum.
were evaluated and analysed with image tracking software (FG34PATH; HaSoTec), which measured the number of crossings over the original platform location, the latency of arrival at the original platform location and the time spent in the target quadrant.

**Serum cholesterol concentrations**

Serum was separated from blood samples by centrifugation at 3000g for 10 min at 4°C. Concentrations of total cholesterol were determined by the colorimetric enzymatic method using an autoanalyzer (Roche Modular P800).

**Serum thiobarbituric acid-reactive substances**

A method modified from that described by Yagi\(^{(15)}\) was used. Serum (100 μl) was reacted with 800 μl of 0.22% H$_2$SO$_4$, 100 μl of 10% phototungstic acid and 200 μl of a 0.61% thiobarbituric acid solution. The samples were heated to 95°C for 1 h before the addition of 400 μl of 1-butanol. The samples were then vigorously stirred for 2 min and centrifuged at 800g for 15 min. The absorbance of the organic phase was measured at excitation of 515 nm and emission of 555 nm, and the samples were compared to a blank.

**Brain cholesterol and 24-hydroxycholesterol concentrations**

After the Morris water maze test, the mice were killed. The cortices and hippocampi of the mice were excised and minced. Cholesterol was extracted using the procedure described by Folch et al.\(^{(36)}\). Total cholesterol was determined using a commercial kit (Roche). Brain 24-OHC content was determined using a 24(S)-hydroxycholesterol ELISA kit (Enzo Life Sciences). The resulting yellow colour was read at 450 nm. The signal was inversely proportional to the concentration of 24-OHC in the sample.

**Cyp46 mRNA analysis**

Total RNA was isolated from the cortical and hippocampal samples using TRIzol reagent according to the manufacturer’s protocol (Life Technologies). Complementary DNA was synthesised by reverse transcription using 3 μg of total RNA. Complementary DNA was synthesised using 1 μg of oligo dT, 1 μl of an RNase inhibitor, 2 μl of 10×-dNTP mixture, 4 μl of 5× reaction buffer and 1 μl of Moloney murine leukaemia virus (MMLV) (RT) in an 8-μl reaction mixture. After allowing the oligo dT to anneal further at 65°C for 5 min, reverse transcription was carried out at 45°C for 60 min. The enzyme, RT, was heat-inactivated at 70°C for 5 min. To quantify the mouse Cyp46 mRNA, 1–3 μg of complementary DNA was used as a template for a real-time PCR analysis based on SYBR Green I with the LightCycler\(^{(9)}\) Carousel-Based System (Roche Diagnostics). A 285-bp PCR product containing Cyp46 was amplified using specific primers (forward primer: 5’-AAC TTT GTC ACC TTC TTC ATT GC-3’; reverse primer: 5’-CCA TCA CTG TGA ATG CCA GA-3’). Amplification and detection were done on a LightCycler 480 System (Roche Diagnostics) under the following conditions: an initial 5 min denaturation step at 94°C, followed by thirty-five cycles of denaturation at 94°C for 10 s, annealing at 60°C for 5 s and extension at 72°C for 8 s. The specificity of the amplified PCR product was inspected by performing a melting curve analysis on the LightCycler 480 System. The gene expression of Cyp46 was calculated based on crossing point values obtained by the second derivative maximum procedure (with β-actin as the housekeeping gene).

**β-Secretase 1 and β-amyloid protein analysis**

Proteins were extracted from cortical and hippocampal samples using radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM-Tris—HCl at pH 7.6, 150 mM-NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100) with protease and a phosphatase inhibitor cocktail (Thermo Fisher Scientific). Extracted proteins were separated by 12% SDS-PAGE. Following electrophoresis, the separated proteins in

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**Fig. 1.** Serum cholesterol concentrations at 1 month and 5 months during the experimental period. Values are means (n 20 at 0 months; n 15 at 5 months), with their standard errors represented by vertical bars. * Mean values were significantly different (P<0.029; Duncan post hoc analysis). Ctrl, control; HC, high cholesterol; LOP, high cholesterol + low Oriental plum; HOP, high cholesterol + high Oriental plum. To convert cholesterol from mg/dl to mmol/l, multiply by 0.0258.

**Fig. 2.** Cholesterol concentrations in the hippocampus (■) and cortex (□) at the end of month 5. Values are means (n 6–8 per group), with their standard errors represented by vertical bars. a,b Mean values with unlike letters were significantly different from the matched tissue (P<0.008; Duncan post hoc analysis). Ctrl, control; HC, high cholesterol; LOP, high cholesterol + low Oriental plum; HOP, high cholesterol + high Oriental plum. To convert cholesterol from mg/dl to mmol/l, multiply by 0.0258.
the gel were transferred onto polyvinylidene difluoride membranes (Millipore) at 80 mA for 2 h. Once the transfer was complete, the membranes were immediately incubated in blocking buffer for 1 h. To detect the target proteins, blotted membranes were incubated overnight at 4°C with primary antibodies against Ab1-42 (mice anti-Ab; Millipore), BACE1 (rabbit anti-BACE; Abcam) and secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG (Sigma). Before quantifying the expression of the target proteins, a peroxidase-based detection of the blot was performed with a Western blotting luminal reagent and was visualised with a Biospectrum AC imaging system (UVP). Quantification of the target proteins was analysed using Image-Pro Plus software (Media Cybernetics).

Statistical analysis

Data are expressed as means with their standard errors. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc.) and SPSS version 18.0 (SPSS, Inc.). The correlation between serum and brain cholesterol concentrations was analysed by Pearson’s correlation coefficient analysis. A repeated measures ANOVA was performed on the Morris water maze test data. A one-way ANOVA followed by Duncan’s post hoc test was performed on the other analyses. Statistical significance was set at $P < 0.05$ for all of the tests.

Results

Body weight changes and serum cholesterol concentrations

Mean body weight among the groups did not differ significantly throughout the 5-month experiment period (averages of 23–36 g in each group, data not shown). Fig. 1 shows that the mice that consuming a 5% high-cholesterol diet (the HC group) exhibited a significantly higher concentration in serum cholesterol at the end of the experiment than the Ctrl and HOP groups did ($P=0.029$). No significant differences in cholesterol concentrations were found among the Ctrl, LOP and HOP groups after 5 months of treatment.

Cortical and hippocampal cholesterol concentrations

After five months of consuming a 5% high-cholesterol diet, the HC group had significantly higher cholesterol concentrations ($P=0.008$) in the cortex and hippocampus than the other groups did (Fig. 2). The cholesterol concentration in the cortex and hippocampus in the HOP group was not significantly different from that in the Ctrl group at the end of the experiment.

Correlation between serum and brain cholesterol concentrations in mice

Fig. 3 shows a significantly positive correlation between serum and cortical (Fig. 3(A), $r=0.73, P=0.0001$) and hippocampal (Fig. 3(B), $r=0.79, P=0.0001$) cholesterol concentrations in the mice from all of the groups.

Lipid peroxidation in serum

The results of measuring lipid peroxidation in the serum, which is expressed as thiobarbituric acid-reactive substances, showed that the thiobarbituric acid-reactive substances...
concentration in the serum of the HOP group was significantly lower ($P=0.051$) than that of the HC group and was not significantly different from that of the Ctrl group at the end of the experiment (Fig. 4).

**Cognition analysis with the Morris water maze**

Fig. 5(A) shows that after consuming a high-cholesterol diet for 5 months, on day 1 of the task, mice in the HC group had a longer escape latency than those in the Ctrl and HOP groups ($P=0.006$). On day 3, mice in the HOP, LOP and Ctrl groups spent significantly shorter time finding the target than those in the HC group did ($P=0.0012$). On the other hand, on day 4 (Fig. 5(B)), the time spent in the previously learned target quadrant by the HOP group tended to be closer to that of the Ctrl group and longer than those of the HC and LOP groups ($P=0.054$).

**Expression of Cyp46 mRNA and 24-hydroxycholesterol concentrations in the brain**

Cyp46 plays an important role in the elimination of brain cholesterol and the conversion of brain cholesterol into 24-OHC. Fig. 6(A) and (B) shows the expression of Cyp46 mRNA in the cortex and hippocampus at the end of the experiment. In the cortex, the expression of Cyp46 mRNA in the HOP and LOP groups was lower than that of the HC group ($P=0.05$). The concentration of 24-OHC in the cortex and hippocampus was also lower in the HOP group than that of the HC group ($P=0.05$) (Fig. 7).

**Expression of β-secretase 1 and β-amyloid proteins in the brains of mice**

BACE1 and $A\beta$ expressions in the brains of mice were shown in Figs. 8 and 9. In the cortex and hippocampus, the expression of both $BACE1$ and $A\beta$ in the HOP group was significantly lower than that of the HC group ($P=0.05$).

**Discussion**

In the present study, we investigated the protective effect of polyphenol-rich Oriental plums on cognitive function and cerebral neurodegeneration-related protein expression in mice that were fed a high-cholesterol diet. A loss of short-term memory is commonly observed among AD patients\(^2\). We found that mice that consumed a high-cholesterol diet...
for a fairly long experimental period (5 months) had elevated cholesterol concentrations in the serum and brain as well as elevated levels of Aβ in the brain when the diet was not supplemented with Oriental plum powder. The accumulation of Aβ is one of the pathological features of AD. Evidence from earlier studies has shown that a high-cholesterol diet induces oxidative stress and inflammatory response and might be related to neurodegeneration and cognitive deficits (17). Recent findings have indicated that consuming a diet high in cholesterol causes some cognitive decline in mice. In the present study, the addition of 2 and 5% Oriental plum powder to a high-cholesterol diet ameliorated the increased cholesterol concentrations in mice. In addition, we also found that there was a positive correlation between serum and brain cholesterol concentrations, which indicates that the blood–brain barrier function might be impaired under a long-term high plasma cholesterol concentration and that cholesterol levels in the brain are thus affected.

The pathogenesis of cognitive deficits caused by elevated cholesterol levels in the brain is still not completely understood (18). However, factors such as increased BACE1, 24-OHC and Aβ levels in the brain have been found to be associated with neurodegeneration (18). The Aβ protein is derived from cleaving APP by BACE1 through the β pathway. BACE1 is located in cholesterol-rich regions (i.e. in lipid rafts). Thus, elevated cholesterol concentrations may increase the size of lipid rafts and the activity of BACE1 (19). Some clinical observations provided in vivo and in vitro evidence indicating that cholesterol plays a role in APP processing and Aβ generation (20). It has also been shown that when rabbits were fed a diet enriched with cholesterol, they had increased levels of Aβ in the brain (21). In transgenic mice that expressed a mutant human APP, Aβ deposits increased in the brain along with plasma cholesterol concentrations (21). In the present study, expressions of BACE1 and Aβ in the HC group were significantly higher than those in the Ctrl and HOP groups, which suggests that cognitive declines in the HC group may have been caused by higher BACE1 and Aβ levels.

The conversion of cholesterol into 24-OHC by Cyp46 appears to be one of the most important mechanisms in the central nervous system for transporting cholesterol out of the brain (17). The present results showed that Cyp46 mRNA expression significantly increased in the cortices and hippocampi of the mice in the HC group. Its expression at transcriptional level was shown to be stable and not affected by steroid hormones or other factors, such as statins, cholesterol or oxysterols, but it can be regulated by oxidative stress (22). Thus, the present findings suggest that the increased Cyp46 mRNA expression in cholesterol-treated mice may be a consequence of oxidative stress, which is indicative of the cholesterol accumulation in the brain (23). In the cortex but not the hippocampus, the elevations were significantly blunted or ameliorated when compared with data from the LOP and HOP groups, which suggests that OP might act as

**Fig. 7.** 24-Hydroxycholesterol (24-OHC) contents in (A) the cortex and (B) the hippocampus in mice. Values are means (n=6–8 per group), with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (P<0.05). Ctrl, control; HC, high cholesterol; LOP, high cholesterol + low Oriental plum; HOP, high cholesterol + high Oriental plum. To convert 24-OHC from mg/dl to mmol/l, multiply by 0.0248.

**Fig. 8.** Expression of β-secretase 1 (BACE1) protein in (A) the cortex and (B) the hippocampus in mice. Values are means (n=3 per group) of the percent relative density (with the control set to 100%), with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (P<0.05; Duncan post hoc analysis). Ctrl, control; HC, high cholesterol; LOP, high cholesterol + low Oriental plum; HOP, high cholesterol + high Oriental plum.
an antioxidant to attenuate brain cholesterol oxidation. The increased Cyp46 mRNA expression might reflect an increase in its protein expression, which in turn might lead to the increased production of 24-OHC. Although we did not measure the protein level of Cyp46, this assumption is supported by the fact that significantly increased 24-OHC was found in the cortex and hippocampus in the HC group. It has been shown that the rate of transporting 24-OHC out of the brain is slower than that of forming 24-OHC from cholesterol; this might have caused too much 24-OHC and cholesterol in the brain in the present study. The raised 24-OHC might represent a higher catalytic efficiency of Cyp46.

It should be noted that this increase seemed to be higher in the hippocampus than in the cortex, even though both areas appeared to have a similar degree of Cyp46 mRNA expression. Because Cyp46 mRNA and protein expression is neuron-specific, we speculated that the difference in 24-OHC levels between the cortex and the hippocampus after cholesterol administration might be the result of other independent pathways, for example, the regulation of Cyp46 at the post-translation level. Further studies are warranted to elucidate this assumption. In addition, 24-OHC has been reported to prevent against the formation of amyloid, which confirms the present finding of lower levels of Aβ in the hippocampus than in the cortex in the HC group. Although a clinical study has shown that 24-OHC was higher in the frontal cortices of AD patients as compared to the controls, there is a lack of evidence to support a correlation between the levels of 24-OHC in the different subregions (i.e. the cortex and the hippocampus) and cognitive impairments. The increased levels of 24-OHC observed in patients with neurodegenerative disorders were more likely the consequence of blood–brain barrier damage or neuronal cell death rather than the metabolic inactivity of a large number of neuronal cells.

Polyphenols are natural antioxidants. It has been shown that polyphenols reduce serum cholesterol by inhibiting cholesterol absorption, increasing bile flow and inhibiting the activities of 3-hydroxy-3-methylglutaryl-CoA reductase and acyl-CoA:cholesterol acyltransferase. In addition, polyphenols are able to penetrate the blood–brain barrier and hence to protect neurons from oxidative stress. In the present study, those mice that consumed diets containing plum powder had lower peripheral plasma cholesterol and brain cholesterol, which may be the reason why they performed better in the cognitive tests than those that did not consume plum-containing diets. Furthermore, the present findings demonstrated that consuming a diet with 5% Oriental plum powder significantly decreased plasma and brain cholesterol concentrations, the mRNA expression of Cyp46, BACE1 protein, Aβ protein and 24-OHC in the cortex and the hippocampus and improved the cognitive function of mice that had cognitive declines as the result of a high-cholesterol diet. This suggests that a plum powder-containing diet may be beneficial for preventing hypercholesterolaemia-related cognitive impairment. Additionally, 2 and 5% Oriental plum powder-supplemented diets are equivalent to 32.4 and 81 g of plum powder for a 60 kg human per d, respectively, based on the body surface area formula. According to the United States Department of Agriculture food consumption database, the standard serving of dried plums is 42 g (five dried plums). Consequently, the dose of daily plum powder (approximately 1 or 2 servings) used in the present study was similar to the typical suggested adult intake. The larger dose (i.e. 5% plum powder supplementation) proved to be more effective in delaying hypercholesterolaemia-related cognitive declines. Accordingly, a higher-percentage plum powder diet is theoretically consumable and more practical for further clinical studies to determine whether the same significant effect is found in human subjects with hypercholesterolaemia.

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P.-H. K. contributed to the lab work. P.-H. K. and C.-I. L. drafted the manuscript. W.-C. C. provided advice on several aspects of the study. Y.-H. C. helped design the experiment, discussed the results and commented on the manuscript. S.-H. L. conducted the experiment, interpreted the data, supervised the research project and completed the manuscript.

The authors declare no conflicts of interest.
References


