Preventive effects of *Salacia reticulata* on non-alcoholic fatty liver disease (NAFLD)/ non-alcoholic steatohepatitis (NASH) in monosodium glutamate treated mice

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Abstract

There has been a rapid increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). NASH is associated with possible progression to liver cirrhosis and cancer. Monosodium glutamate (MSG) treated ICR mice (ICR-MSG) are known to develop obesity, diabetes mellitus, and also NASH-like histopathological changes that are similar to those found in humans. In Ayurvedic medicine, *Salacia reticulata* has traditionally been used to treat diabetes mellitus and rheumatism. In the present study, we studied the preventive effects of *S. reticulata* on NAFLD/NASH in ICR-MSG mice.

ICR-MSG mice were given *S. reticulata* extract (SRE) for 8 weeks. Body weight and food intake were determined over time, and at the completion of treatment, blood was drawn and the liver was dissected for determination of biochemical parameters and histopathological examination of the liver cells.

Compared with the Normal group, the MSG-Control group showed significant obesity, hyperinsulinemia, abnormal lipid metabolism, marked fatty liver. In contrast, significant suppression of body weight gain and improvement of hyperinsulinemia and abnormal lipid metabolism were observed in the groups treated with SRE. Furthermore, SRE was found to suppress liver hypertrophy and to reduce hepatic triglyceride and total cholesterol levels. Histopathological examination revealed that SRE prevents vacuolar degeneration of hepatocyte by lipid accumulation and suppresses infiltration of inflammatory cells. The above results demonstrated SRE’s ability to improve obesity and insulin resistance and to suppress development of fatty liver and infiltration of inflammatory cells, suggesting that SRE is a promising agent for the prevention and treatment of NAFLD/NASH.

**Key words** *Salacia reticulata*, NAFLD, NASH, vacuolar degeneration of hepatocyte by lipid accumulation, liver hypertrophy, infiltration of inflammatory cells, obesity.

Introduction

In recent years, the incidence of obesity has increased due to westernization of dietary habits and use of mechanical transportation. As a consequence, there has been a rise in the prevalence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).\textsuperscript{3} NASH is a condition seen in patients with no history of alcohol intake in which the liver nevertheless shows histopathology similar to that encountered in patients with alcoholic liver injury. NASH shows gradual
progression, and 5 to 20% of the patients are reported to
develop liver cirrhosis in 5 to 10 years\textsuperscript{2,3} and the 5-year
incidence of liver cancer in patients with cirrhosis is re-
ported to be 15 to 20%\textsuperscript{4-5}. In Japan, as NASH is ex-
pected to become a far more serious condition than viral
hepatitis in the future, there are growing calls for its pre-
vention. The mechanism of pathogenesis of NASH has
yet to be elucidated, and few drugs are available for its
treatment.

Previously, we reported that the ICR-MSG mice,
which are created by administration of monosodium
glutamate (MSG) to newborn ICR mice, develop severe
obesity, insulin resistance, diabetes mellitus and lipid
abnormalities, and show changes in the liver that are
similar to those found in the livers of humans with
NASH, i.e. fatty liver, infiltration of inflammatory cells,
fibrosis, and liver cancer.\textsuperscript{6,7} The basal metabolism
of MSG mice can lowered due to hypofunction of the au-
nomous nervous system and the brown adipose tissue.\textsuperscript{8-10}
The causes of obesity in the MSG mouse mirrors the
lack of physical activity.

\textit{Salacia reticulata} is a climbing plant from the
Hippocrateaceae family and is found in Sri Lanka and
other areas of Southeast Asia. The root and stem of this
plant have been used traditionally for the prevention of
diabetes mellitus and rheumatism in Ayurvedic medi-
cine. To date, \textit{S. reticulata} has been reported to be ef-
efective in suppressing postprandial hyperglycemia and
to have antiobesity, antidiabetic, hepatoprotective, and
antioxidant effects.\textsuperscript{11-16} In addition to these effects, we
have also confirmed that \textit{S. reticulata} improves glucose
and lipid metabolism, suppresses hypertension, prevents
peripheral neuropathy, and inhibits adipocyte differenti-
tation.\textsuperscript{17,18} \textit{S. reticulata} is expected to be efficacious in
the treatment of not only individual metabolic disorders
but of the overall symptoms of metabolic syndrome re-
sulting from obesity. However, the effect of \textit{S. reticulata}
on NAFLD/NASH has not yet been studied.

In the present study, the objective was to establish
if \textit{S. reticulata} could prevent NAFLD/NASH in the
ICR-MSG mouse, an animal model of NAFLD/NASH.

\textbf{Method}

\textbf{Animals:} Male ICR-MSG mice were supplied by the
Institute for Animal Reproduction (Ibaraki); they were
prepared by injecting one subcutaneous dose of 4 mg
MSG into newborn ICR mice. Animals in the Normal
group were given a subcutaneous injection of physio-
logical saline solution. In both groups, the animals were
purchased at the age of 11 weeks and acclimatized for
1 week before the experiment was started at the age of
12 weeks. They were given powdered standard diet
(MF; Oriental Yeast Co., Ltd., Chiba) and tap water ad
libitum and housed at a temperature of 23 ± 1\textdegree C, rela-
tive humidity of 55 ± 5\%, with a 12-hour light cycle
(8:00 to 20:00). This study was approved by the experi-
mental animal ethics committee of the vivarium of
Musashino University.

\textbf{Experimental materials:} The \textit{S. reticulata} extract (SRE)
identified by SRILANKA INDIGENOUS MEDICINE
EXPORTERS & MANUFACTURERS ASSOCIATION,
was provided by Kothalahim Japan Co., Ltd., (Tokyo).
The powdered \textit{S. reticulata} was treated by hot water,
and the solvent was separated, concentrated, and fil-
tered. After sterilized, the remaining solution was spray-
dried to obtain the SRE. The test drug was thoroughly
blended with powdered standard diet MF to make uni-
form mixtures containing 0.25\% or 0.5\% of SRE. The
chromatogram shown in Figure 1 was obtained from the
extract powder with branched cyclodextrin using three-
dimensional high performance liquid chromatography
(HPLC). In this study, only SRE was investigated due to
the absence of an appropriate control drug for NASH in-
vestigation.

\textbf{Mode of administration:} After acclimation of 1 week,
the ICR-MSG mice were allocated to three groups such
that there was no difference in body weight among the
groups. The groups were: MSG-Control group (newborn
ICR mice were treated with MSG, n=9); MSG-SRE
0.25\% group (MSG-Control mice were given feed con-
taining 0.25\% SRE, n=9); MSG-SRE 0.5\% group
(MSG-Control mice were given feed containing 0.5\%
SRE, n=9). A Normal group (n=8) was also included in
the study. The study was performed with the above 4
groups.

The animals in the Normal and MSG-Control groups
were given powdered standard diet MF and the animals
in the MSG-SRE 0.25\% and MSG-SRE 0.5\% groups
were given test feed containing SRE at the respective concentrations from a feeder ad libitum for 8 weeks. These dosages of SRE were equivalent to 0.5 and 1.0% of the mixture of SRE and cyclodextrin used in the previous study.\textsuperscript{18}

**Body weight change and food intake:** Body weight was measured every week from the start of the experiment (animals were 12 weeks old) to its completion (20 weeks old). The mean daily individual food intake was calculated by deducting the weight of the remaining amount and the spilled amount from the weight of the filled feed box, and then dividing by the number of animals.

**Glucose tolerance test:** At week 8 (mice were 20 weeks old), glucose at a dose of 2 g/kg was given orally (\textit{p.o.}) to the mice after a 24-hour fast. Blood samples were drawn from the orbital venous plexus using a heparinized glass capillary immediately before glucose administration (0 min), and 30, 60, 120, and 180 minutes after glucose administration and plasma was obtained by centrifugation. The plasma samples were stored at -30°C until use.

**Determination of plasma parameters:** Blood was drawn from the vena cava inferior of the mice under ether anesthesia at the end of the experiment (8 weeks after start of drug administration) using a heparinized syringe. The collected blood samples were centrifuged to obtain plasma for further tests. Plasma glucose was determined using Glucose CII Test Wako (Wako Pure Chemicals Industries Ltd., Osaka). Plasma cholesterol was determined using Cholesterol E-Test Wako (Wako Pure Chemicals). Plasma triglyceride was determined using Triglyceride E-Test Wako (Wako Pure Chemicals). Plasma high-density lipoprotein cholesterol (HDL) was determined using L-type Wako-HDL-C:M (Wako Pure Chemicals). Plasma low-density lipoprotein cholesterol (LDL) was determined using L-type Wako-LDL-C:M (Wako Pure Chemicals). Aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were determined using transaminase CII-Test Wako (Wako Pure Chemicals). Plasma insulin was determined using an insulin determination kit (ELISA method, Shibayagi Co., Ltd. Gunma).

**Determination of fat content in the liver:** Total lipids in the liver were extracted as described by Freedman \textit{et al.}\textsuperscript{19} Briefly, mouse liver was homogenized in 1M...
NaCl. Liver tissue homogenate was extracted with chloroform/methanol (2:1) and 1M NaCl. The organic phase was collected, dried, and resuspended in Triton X-100/methanol (2:1). Triglyceride (TG) and total cholesterol (TC) were measured using biochemical test kits (Wako).

**Histopathological examination of the liver:** After the blood was drawn at the completion of the experiment, the mice were autopsied to collect the liver and mesenteric fat and to determine their weights. The collected livers of all groups were fixed with 10% phosphate-buffered formalin solution and 4-μm thick serial sections were cut and stained with hematoxylin and eosin. The degree of histopathology was scored 0 for normal, 1 for slight, 2 for moderate, and 3 for severe.

**Statistical analysis:** The statistical significance between the Normal and MSG-Control group was determined using Student’s t test. As a multiple comparison test between the MSG-Control mice and each SRE treatment group, ANOVA was used and post-hoc analysis was performed with Dunnett’s test. A difference was considered significant when \( p < 0.05 \).

**Results**

**Effects on changes in body weight and food intake:** Figure 2 shows the changes in body weight from the start of test drug administration to week 8. Compared to the Normal group, the MSG-Control group showed significantly higher body weight gain throughout the entire treatment period, which demonstrated that the animals in the MSG-Control group were obese. Significant suppression of body weight gain as compared to the MSG-Control group was observed in the animals treated with 0.25% and 0.5% SRE.

A significant decrease in food intake during the treatment period was observed in the MSG-Control group compared to the Normal group (Table 1). There was no difference in the amount of food intake between the MSG-Control and SRE treatment group.

**Effect on glucose tolerance:** Changes in blood glucose levels after oral glucose tolerance test (OGTT) are presented in Figure 3. Compared to the Normal group, the blood glucose levels were significantly increased in
the MSG-Control group at 30, 60, and 180 minutes after the glucose challenge indicating abnormal glucose tolerance in this group. In the MSG-SRE 0.25% group, blood glucose levels were significantly lower compared to those in the MSG-Control group at 30 and 180 minutes after glucose administration, though a significant difference was not observed in the 0.5% SRE treatment group.

**Effect on plasma parameters:** Biochemical parameters of plasma at the completion of the experiment are presented in Table 2. Compared to the Normal group, significant increases in TC, insulin and ALT levels were observed in the MSG-Control group, which indicated hypercholesterolemia and hyperinsulinemia. In contrast, significant suppression of TC and insulin levels were found in the SRE 0.5% group. A significant increase in HDL level was found in the SRE 0.25% group. No significant differences among the groups were found with regard to glucose, TG, LDL and AST levels.

**Effects on liver and mesenteric fat weight and fat content in the liver:** In the MSG-Control group, the liver weight increased significantly in comparison to the Normal group, indicating liver hypertrophy (Figure 4a). Administration of SRE caused a dose-dependent decrease in liver weight; a significant suppression of liver hypertrophy was observed in the MSG-SRE 0.5% group as compared with the MSG-Control group. Mesenteric fat weight increased significantly in the MSG-Control group (1.7 ± 0.3 g) compared with the Normal group (0.3 ± 0.2 g). However, SRE had no influence on mesen-

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>MSG-Control</th>
<th>MSG-SRE 0.25%</th>
<th>MSG-SRE 0.5%</th>
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<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>113.4 ± 10.2</td>
<td>117.7 ± 8.3</td>
<td>97.6 ± 9.3</td>
<td>121.6 ± 9.6</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>152.2 ± 5.9</td>
<td>216.1 ± 11.8*</td>
<td>203.0 ± 10.7</td>
<td>168.1 ± 9.3*</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>173.0 ± 20.2</td>
<td>199.7 ± 23.0</td>
<td>209.7 ± 15.6</td>
<td>235.8 ± 15.2</td>
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<tr>
<td>HDL (mg/dL)</td>
<td>110.6 ± 12.1</td>
<td>124.0 ± 6.2</td>
<td>150.3 ± 5.4*</td>
<td>134.7 ± 9.3</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>15.3 ± 1.7</td>
<td>19.6 ± 1.3</td>
<td>21.3 ± 2.7</td>
<td>16.0 ± 1.2</td>
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<tr>
<td>AST (Karmen Unit)</td>
<td>67.9 ± 8.1</td>
<td>123.9 ± 39.3</td>
<td>134.4 ± 38.4</td>
<td>95.0 ± 16.5</td>
</tr>
<tr>
<td>ALT (Karmen Unit)</td>
<td>14.5 ± 1.2</td>
<td>35.7 ± 6.7*</td>
<td>42.9 ± 14.4</td>
<td>42.3 ± 10.9</td>
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<tr>
<td>insulin (ng/mL)</td>
<td>7.0 ± 1.2</td>
<td>198.2 ± 104.8*</td>
<td>52.9 ± 22.8</td>
<td>31.9 ± 12.4*</td>
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</table>

TC: total cholesterol, TG: triglyceride, HDL: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, AST: aspartate aminotransferase, ALT: alanine aminotransferase (ALT) Data represent the mean ± S.E. of 8 to 9 animals. *: p<0.05, Significant differences from the Normal group by student’s t test. #: p<0.05 Significant differences from the MSG-Control group by Dunnett’s test.

![Figure 4](image_url)  
**Figure 4** Effect of SRE on Liver Weight and Fat Content in the Liver of ICR-MSG mice.  
a) Liver weight, b) Hepatic TG level, c) Hepatic TC level  
*: p<0.05, Significant differences from the Normal group by student’s t test. #: p<0.05 Significant differences from the MSG-Control group by Dunnett’s test.
teric fat (SRE 0.25% treatment: 1.5±0.3 g, SRE 0.5% treatment: 1.5±0.3 g). Liver TG levels and TC levels in the MSG-Control group were significantly higher compared to the Normal group, which indicated that the animals in the MSG-Control group had developed fatty liver (Figures 4b, c). The SRE treatment groups showed a dose-dependent decrease in liver TG and TC levels and the difference was significant in the 0.5% SRE treatment group.

**Effect on liver histopathology:** HE-stained sections of the liver collected at dissection are shown in Figure 5.
and scores of vacuolar degeneration of hepatocyte by lipid accumulation and infiltration of inflammatory cells are shown in Table 3.

**Table 3** Table of scores of vacuolar degeneration of hepatocyte by lipid accumulation and infiltration of inflammatory cells in the liver.

a) Evaluation of vacuolar degeneration of hepatocyte by lipid accumulation

<table>
<thead>
<tr>
<th>Animal No.</th>
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<td>5</td>
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<td>9</td>
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b) Evaluation of infiltration of inflammatory cells

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<th>Animal No.</th>
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<th>MSG-SRE 0.5%</th>
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*Severity: Each abnormality was divided into three grades from + to ++, mild, moderate and severe, qualitatively.*

Comparing to the Normal group, the mice in the MSG-Control group showed a significant increase in vacuolar degeneration of hepatocyte by lipid accumulation and infiltration of inflammatory cells such as neutrophils, lymphocytes and macrophage. Treatment with SRE resulted in a dose-dependent suppression of vacuolar degeneration of hepatocyte by lipid accumulation and reduction in infiltration of inflammatory cells.

**Discussion**

NAFLD and NASH are known to occur with certain drugs and during pregnancy, but more often they develop in association with metabolic syndrome as a consequence of obesity. Since NASH progresses to fibrosis and liver cancer, the disease is a matter of great urgency; however, little basic research has been done regarding the pathology of NAFLD/NASH and there are virtually no effective therapeutic agents available. In this study, we investigated the effects of *S. reticulata* in preventing the development of NAFLD/NASH using ICR-MSG mice.

As previously reported by Nagata *et al.* and Nakanishi *et al.*, ICR-MSG mice at the age of 20 weeks were observed to be obese and to have insulin resistance, enlarged liver, fatty liver, and infiltration of inflammatory cells. Compared to the MSG-Control mice showing these pathologic conditions, a significant suppression of body weight gain was observed in animals treated with SRE at concentrations of 0.25% and 0.5% starting at week 6. Since administration of SRE was found to have no influence on the amount of feed consumed, the suppression of body weight gain was not attributable to a decrease in food intake. In previous studies using TSOD mice, a multifactorial genetic disease animal model of obese type 2 diabetes mellitus, it was confirmed that SRE prevented obesity when its administration was started at an early age when obesity had not yet developed. Moreover, we found that SRE had an anti-obesity effect in TSOD mice in which obesity had already developed (unpublished data). In the present study, SRE was found to suppress body weight gain in 12-week-old ICR-MSG mice that were already obese. The result suggests not only that SRE is effective in preventing obesity but that it can be used in already obese patients to suppress body weight gain. The group receiving SRE also showed significant improvement in abnormal glucose tolerance and hyperinsulinemia. These results coincide with the results of studies conducted in TSOD mice and other animal models and suggest that SRE is efficacious in improving insulin resistance. Since insulin resistance is one of the central pathologies in the development of diabetes mellitus and various other complications affecting the liver and kidneys, the ability of SRE to improve insulin resistance is
regarded as highly useful. SRE was also effective in improving abnormal lipid metabolism. SRE caused a significant decrease in TC levels and a significant increase in HDL levels in the ICR-MSG mice that had hypercholesterolemia. The ability to suppress blood TC levels and increase HDL levels is useful in the management of arteriosclerosis, which is a serious cardiovascular condition.

SRE was found to have marked effects against pathologic liver conditions. It was confirmed on histopathological examination that SRE dose-dependently suppressed enlargement of the liver and increased TG and TC levels found in the ICR-MSG mice, and also suppressed vacuolar degeneration of hepatocyte by lipid accumulation. Huan et al. reported that S. oblonga, which belongs to the same genus as S. reticulata, reduces the hepatic TG and NEFA levels in Zucker diabetic obese rat and these effects were attributed to its capacity to induce expression of peroxisomal proliferator-activated receptor (PPAR)α, acyl-CoA oxidase (ACO), and carnitine palmitoyl transferase (CPT) genes involved in fatty acid oxidation.\(^{20}\) The above results suggest that the ability of SRE to reduce fat content in the liver observed in the present study may involve promotion of the fatty acid oxidation system in the liver by SRE. In addition, SRE caused a dose-dependent suppression of infiltration of inflammatory cells such as neutrophils, lymphocytes and macrophages into the liver tissue, which is characteristic of NASH. The so-called “second hit theory” of NASH pathogenesis asserts that, after the first hit of fat accumulation in the liver, a second hit involving infiltration of inflammatory cells and oxidative stress contributes to disease progression. The suppression of both fatty liver and infiltration of inflammatory cells by SRE observed in the present study means that the “second hit” leading to progression of NASH was prevented by SRE. Yoshikawa et al. reported that SRE inhibited CCl4-induced lipid peroxidation, which suggests that SRE had antioxidative activity.\(^ {19}\) Therefore, SRE is expected to suppress progression from NAFLD to NASH, which can eventually lead to cirrhosis and liver cancer if left unchecked. Because ICR-MSG mice also develop liver cirrhosis and liver cancer at a high rate, further investigation of the effects of SRE should be performed using this model at a later disease stage when cirrhosis and liver cancer have occurred. In the present study, SRE did not influence the AST and ATL levels, which are markers of liver function. Fibrates which are clinically used to correct lipid abnormalities are known to act as ligands to PPARα, induce expression of genes involved in fatty acid oxidation and thus lead to an improvement of the fat content values in the blood and liver. However, in rodents, they are reported to cause hepatocyte enlargement and increases in AST and ALT levels.\(^ {22,23}\) In the present study, MSG led to a significant increase in ALT levels, however, SRE did not prevent the increase. While it is known that increased ALT levels are attributable to liver cell necrosis/apoptosis associated with degeneration, it has been shown that hepatic disorders caused by MSG do not induce extensive liver cell necrosis. Nor did histopathological examinations reveal apparent liver cell necrosis/apoptosis. These facts suggest that while MSG may induce severe degeneration of liver cells, it only causes very mild apoptosis. The results of this study suggest that SRE has a preventive effect on fat accumulation but almost no influence on liver cell apoptosis. Since no adverse drug reactions such as increased AST and ALT levels were observed with SRE in the present study, the test drug was considered to be useful for the treatment of fatty liver without liver damage, which is a concern with fibrates.

We have reported that SRE has an antiobesity effect and that suppression of adipocyte differentiation from precursor cells is involved in its mechanism of action.\(^ {19}\) While enlargement of adipocytes is reported to induce insulin resistance and production of various inflammatory cytokines, leading to the occurrence of insulin resistance and inflammation in the liver and muscles, SRE in this study did not show any suppression of the mesenteric fat weight. This suggests that SRE acts mainly upon the liver resulting in improved insulin resistance, suppression of fat accumulation and reduction in inflammatory cell accumulation. Further studies are needed to investigate in more detail the direct action of SRE on the liver, and the effect of SRE on muscle that contributes to insulin resistance.

**Conclusion**

As described above, SRE had the ability to prevent
obesity and improve insulin resistance and abnormal lipid metabolism in ICR-MSG mice. Furthermore, since SRE suppressed liver hypertrophy, decreased the hepatic TG and TC levels, and suppressed vacuolar degeneration of hepatocyte by lipid accumulation of the hepatocytes and inflammation in the liver, it is expected to be a promising candidate not only for the management of metabolic syndrome but also for the prevention and treatment of NAFLD and its progressive form, NASH.

References