Preventive Effects of Bofutsushosan on Obesity and Various Metabolic Disorders

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Visceral fat accumulation has been reported as the most important risk factor for the development of various metabolic disorders. In this study, the preventive effects of Bofutsushosan, a Japanese Kampo preparation, on obesity and various metabolic disorders were investigated focusing on visceral fat accumulation using Tsumura, Suzuki, Obese, Diabetes (TSOD) mice, which showed significant accumulation of visceral fat, and developed metabolic disorders including glucose intolerance, hyperlipidemia, hypertension and hyperinsulinemia. At 2 months after initiation of the study, the control TSOD mice developed various metabolic disorders such as marked obesity and visceral fat accumulation, increases in the levels of blood glucose, insulin, total-cholesterol (TC) and triglyceride (TG), and abnormal glucose tolerance, hypertension and peripheral neuropathy as distinct from the control Tsumura, Suzuki, Non-Obesity (TSNO) mice, which do not develop obesity and various metabolic disorders. In the TSOD mice treated with Bofutsushosan, body weight gain and visceral/subcutaneous fat accumulation were significantly suppressed. Biochemical parameters in plasma (glucose, TC, insulin and tumor necrosis factor-α (TNF-α) level) were significantly suppressed, and abnormal glucose tolerance, elevation of blood pressure and peripheral neuropathy accompanying progression of metabolic disorders were also significantly suppressed. On the other hand, in TSNO mice, Bofutsushosan showed no noteworthy impacts on most parameters except for an improvement of the lipid plasma level. The above results suggested that Bofutsushosan could be effective in preventing obesity and various metabolic disorders.

Key words Bofutsushosan; Tsumura, Suzuki, Obese, Diabetes (TSOD) mouse; obesity; various metabolic disorder; visceral fat accumulation; glucose intolerance; hyperlipidemia; hyperinsulinemia; hypertension; peripheral neuropathy.

MATERIALS AND METHODS

Experimental Animals We used male TSOD mice aged 3 weeks. As a control animal, male TSNO (Tsumura, Suzuki, Non-Obesity) mice aged 3 weeks were used as these animals do not develop obesity and various metabolic disorders. All mice were obtained from the Institute for Animal Reproduction (Ibaragi Prefecture). The animals were housed in an animal room under the following conditions: room temperature of 23 ± 2 °C, relative humidity of 55 ± 10% and 12 h of light per day. In the 1-week acclimation period, the animals were given ad libitum powder feed MF (Oriental Yeast Co., Ltd.) and water. Following this period, the powder feed was switched to the study feed containing the test drug, and the animals were kept for further 8 weeks. We certify that all ap-

Table 1. The Ingredients of Bofutsushosan Formula

<table>
<thead>
<tr>
<th>Crude drugs</th>
<th>Weight ratio (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scutellaria baicalensis GEORGI</td>
<td>2.0</td>
</tr>
<tr>
<td>Glycyrrhiza uralensis FISCH.</td>
<td>2.0</td>
</tr>
<tr>
<td>Platycodon grandiflorum A. DC.</td>
<td>2.0</td>
</tr>
<tr>
<td>Gypsum Fibrosum</td>
<td>2.0</td>
</tr>
<tr>
<td>Atractylodes japonica KOEDZUMI</td>
<td>2.0</td>
</tr>
<tr>
<td>Rheum palmatum L.</td>
<td>1.5</td>
</tr>
<tr>
<td>Schizonepeta tenuifolia BRIO.</td>
<td>1.2</td>
</tr>
<tr>
<td>Gardenia jasminoides ELLIS</td>
<td>1.2</td>
</tr>
<tr>
<td>Paeonia lactiflora PALL.</td>
<td>1.2</td>
</tr>
<tr>
<td>Cnidium officinale MAX.</td>
<td>1.2</td>
</tr>
<tr>
<td>Angelica acutiloba KITAGARA</td>
<td>1.2</td>
</tr>
<tr>
<td>Mentha arvensis L. var. piperatae MALINVAUD</td>
<td>1.2</td>
</tr>
<tr>
<td>Ledebouriella seseloides WOLF.</td>
<td>1.2</td>
</tr>
<tr>
<td>Ephedra sinica STAT.</td>
<td>1.2</td>
</tr>
<tr>
<td>Forsythia suspensa Vahl.</td>
<td>1.2</td>
</tr>
<tr>
<td>Zingiber officinale ROSC.</td>
<td>0.3</td>
</tr>
<tr>
<td>Talcum Crystallinum</td>
<td>3.0</td>
</tr>
<tr>
<td>Natrium Sulphate</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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Applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

**Test Drug** The Bofutsushosan extract powder (hereinafter referred to as Bofutsushosan) was supplied by Tsumura & Co. Table 1 shows the ingredients of the Bofutsushosan preparation used in this study. Figure 1 shows the three-dimensional HPLC chart for Bofutsushosan. The study feed was prepared by mixing the powder feed MF with Bofutsushosan at a concentration of 1.0% or 3.0%.

**Mode of Administration** The TSOD mice and TSNO mice were grouped. After the acclimation period, the control group was given the powder feed MF *ad libitum* continuously and the study drug group was given *ad libitum* the study feed containing 1.0% Bofutsushosan (Bofutsushosan low-dose group) or 3.0% Bofutsushosan (Bofutsushosan high-dose group).

**Body Weight Change and Feed Intake** Body weight was measured every week from the start of the experiment (age 4 weeks) to the completion of the experiment (age 12 weeks). Feed intake was measured from 1 week after initiation of the experiment. The feed box was filled every week, and the mean daily individual feed intake was calculated by deducting the weight of the remaining amount and the spilled amount from the weight of the filled feed box, then dividing by the number of days and the number of animals.

**Changes in Amounts of Visceral and Subcutaneous Fat** At 0 week (age 4 weeks), 4 weeks (age 8 weeks) and 8 weeks (age 12 weeks) after initiation of the study drug, each mouse was anesthetized with Nembutal (50 mg/kg, i.p.) and fixed in an experimental X-ray CT instrument (LaTheta, Aloka Co., Ltd., Tokyo) to determine the amount of visceral and subcutaneous fat by scanning from the ensiform cartilage to the sacral bone at intervals of 1.5 mm.

**Blood Biochemical Tests** At 8 weeks after starting treatment with the study drug (age 12 weeks), blood samples were taken from the orbital venous plexus under non-fasting and unanesthetized conditions. The collected blood samples were centrifuged to obtain plasma samples. Blood glucose, total cholesterol (TC), triglyceride (TG) and insulin were measured using commercial assay kit according to our previous method. Tumor necrosis factor-α (TNF-α) were measured using TNF-α ELISA kit (COSMO BIO CO., LTD., Tokyo).

**Glucose-Tolerance Test** At 8 weeks after treatment initiation, the mice were made to fast for one night and then received oral glucose (2 g/kg). Blood samples were collected from the orbital venous plexus immediately before glucose loading (0 min) and at 30, 60, 120 and 180 min after glucose loading and centrifuged to obtain plasma samples, with which the glucose level was determined.

**Blood Pressure** At 8 weeks after treatment initiation, each mouse was fixed in a positioner THC-2 (Softron, Co., Ltd., Tokyo) while body temperature was maintained at 37.5 °C, and blood pressure was determined non-invasively by inserting the tail up to its base into the tail cuff of a non-invasive blood pressure meter BP-98A (Softron, Co., Ltd.).

**Pain Test** The foot pinch method as described by Suzuki *et al.* was used as a pain test. For this test, the proximal part of the tarsus in the metatarsal region of hind limb was pinched with an artery clip (BHO20R, pressure: 300 g, Bulldog Clamp, Johns Hopkins, Tokyo), and the time until the mouse bites the clip was determined as the latent reaction time. The mean latent reaction time was calculated from the values for both hind limbs and this was regarded as the latent reaction time for each individual mouse.

**Statistical Processing** In each experiment, significant differences between the groups were examined using Dun-
RESULTS

Growth Curve and Feed Intake Figure 2 shows the growth curve up to 8 weeks after initiation of the study drug treatment (age 12 weeks).

The TSOD control group showed a larger body weight gain than the TSNO control group throughout the entire experiment period. Among the TSOD mice, the body weight gain was dose-dependently suppressed in the groups treated with Bofutsushosan compared with the control group from 1 week after treatment initiation. At 8 weeks after initiation of the study drug treatment (age 12 weeks), body weight suppression was 4 and 10% in the low- and high-dose group TSOD mice, respectively, compared with the control TSOD mice, with a significant difference noted in the high-dose group. In the TSNO mice, all groups showed similar body weight changes with no intergroup differences.

Bofutsushosan did not affect the food intake in TSOD mice group (5.02, 5.18, 4.86 g/d/body in control, 1%, 3%, respectively) and in TSNO mice group (4.18, 4.60, 4.32 g/d/body in control, 1%, 3%, respectively) at age 6 weeks.

Changes in the Amount of Visceral and Subcutaneous Fat Figure 3A, 3B shows the CT imaging results for the TSNO control, TSOD control and TSOD high-dose group at 8 weeks (age 12 weeks) after initiation of treatment with Bofutsushosan. The amount of both visceral and subcutaneous fat accumulation in TSOD control was obviously higher than in the TSNO control. In the TSOD high-dose group, Bofutsushosan suppressed both visceral and subcutaneous fat accumulation significantly in comparison with the TSOD control.

Figure 3B shows changes in the amounts of visceral and subcutaneous fat in each group of TSOD mice and TSNO mice. The amount of both visceral and subcutaneous fat in the TSOD control group was significantly higher than in the TSNO control group. Intergroup comparisons in the TSOD mice groups showed that the amount of visceral and subcutaneous fat was significantly suppressed in the Bofutsushosan treated group compared with the TSOD control group. On the other hand, in the TSNO mice, there were no marked differences in visceral and subcutaneous fat accumulation between the control group and either group treated with Bofutsushosan.

Blood Biochemical Tests Table 2 shows the levels of blood glucose, insulin, TNF-α, TC and TG in each group of TSOD mice and TSNO mice determined at the completion of the experiment (age 12 weeks). The levels of insulin, TNF-α, TC and TG were significantly higher in the TSOD control group than in the TSNO control group. The blood glucose level tended to be higher in the TSOD control group than in the TSNO control group.

Intergroup comparisons in the TSOD mice groups showed
that the levels of blood glucose, insulin, TNF-α and TC were reduced in a dose-dependent manner in the Bofutsushosan treated group in comparison with the control group, and were significantly lower in the high-dose group compared with the levels in the TSOD control group. The TG level tended to be lower in both the low-dose and high-dose groups compared with the TSOD control group.

In the TSNO mice, the level of TC was significantly lower in the TSNO high-dose group than in the control group. There were no marked differences in the levels of glucose, TG, insulin and TNF-α between the control group and either group treated with Bofutsushosan.

**Glucose-Tolerance Test** The TSOD mice and TSNO mice were made to fast for one night and then each animal was administered oral glucose 2 g/kg at the completion of the experiment (age 12 weeks). Figure 4 shows the time-course changes in the blood glucose level in each group of TSOD mice and TSNO mice.

The blood glucose level after glucose loading was significantly higher in the TSOD control group than in the TSNO control group, indicating the presence of abnormal glucose tolerance in the TSOD control group.

From intergroup comparisons in the TSOD mice, compared with the control group, the blood glucose level was significantly lower in the low-dose group at 30 min after glucose loading, and significantly lower in the high-dose group at 30, 120 and 180 min after glucose loading.

On the other hand, in the TSNO mice, there were no marked differences in blood glucose levels after glucose loading between the control group and either group treated with Bofutsushosan.

**Blood Pressure** Table 3 shows systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) values in the TSOD mice and TSNO mice at the completion of the experiment (age 12 weeks).

The SBP, DBP and MBP values in the TSOD control group were significantly higher than those in the TSNO control group.

Intergroup comparisons in the TSOD mice showed that the SBP values were significantly lower in the groups treated with Bofutsushosan compared with the control group, showing an apparent dose–response relationship, with the values in the high-dose group comparable with those in the TSNO control group. The DBP and MBP values were similarly suppressed in Bofutsushosan treated group compared with the control group, and a significant difference was seen between the high-dose group and the TSOD control group.

In the TSNO mice, there were no marked differences in SBP, DBP and MBP values between the control group and either group treated with Bofutsushosan.

**Pain Test** Figure 5 shows the latent reaction time in the foot pinch test for the TSOD mice and TSNO mice at the completion of the treatment (age 12 weeks). In this pain test, the latent reaction time was significantly longer in the TSOD control group than in the TSNO control group.

Intergroup comparisons in the TSOD mice showed that the latent reaction time significantly decreased in the both low and high-dose groups compared with the control group. The latent reaction time after the treatment was on a par with that seen in the TSNO control group.

On the other hand, in the TSNO mice, there were no marked differences in latent reaction time between the control group and either group treated with Bofutsushosan.

**DISCUSSION**

Visceral fat accumulation has been shown to play a critical role in the development of cardiovascular disease as well as the development of obesity-related disorders such as diabetes mellitus, hyperlipidemia and hypertension and the so-called metabolic syndrome. Therefore, it is important to suppress visceral fat accumulation in order to prevent metabolic disorders from developing. In this study, we examined the obesity and metabolic disorders-preventing effect of Bofutsushosan, which has been shown to be an effective treatment against obesity, focusing on suppression of visceral fat accumula-
The TSOD mice were established as an animal model of spontaneous obesity and type 2 diabetes, having the polygenic pattern of inheritance for diabetes and obesity. In our experiments, the TSOD mice showed significant accumulation of visceral fat, and developed metabolic disorders including glucose intolerance, hyperlipidemia, hypertension, hyperinsulinemia and peripheral neuropathy. This was in sharp contrast to the TSNO control group. The TSNO mice were established as a non-disease model that did not develop obesity and diabetes. From the above results, we speculated that TSOD mice would develop obesity and various metabolic disorders, as has been shown to occur in studies in other animal models which we have previously reported and would be suitable for use in the evaluation of the drug effects on obesity and metabolic disorders. At the age of 4 weeks, body weight, and the amounts of visceral and subcutaneous fat were comparable between TSOD mice and TSNO mice, suggesting that obesity and metabolic disorders had not yet developed at this age. Accordingly, we investigated the preventive effects of Bofutsushosan on obesity and various metabolic disorders using animals aged 4 weeks.

Intergroup comparisons of body weight gain in the TSOD mice showed dose-dependent suppression from the early stage of Bofutsushosan treatment, with a significant difference between the high-dose group and the control group. On analysis of the amount of body fat using X-ray CT examination, it was found that Bofutsushosan significantly suppressed visceral and subcutaneous fat accumulation from 4 weeks after starting treatment, and this effect was dose-dependent, as were the results of body weight gain. It is known that ephedrine contained in Ephedra sinica STAFF enhances release of norepinephrine from the sympathetic nerves to active BAT and skeletal muscles. Licoricidin contained in Glycyrrhiza uralensis FISCH., d-pinorestinol contained in Forsythia suspensa VAHL. and Schizonepeta tenuifolia BRIG. have an inhibitory effect on phosphodiesterase, resulting in weight loss and visceral and subcutaneous fat suppression in rodents and humans. Although Bofutsushosan contains sennoside A in its Rheum palmatum component, which is a purgative medicine, no diarrhea was observed over the course of the experiment in the Bofutsushosan-treated TSOD group. Mild diarrhea was noted only in the TSNO high-dose group. Moreover, there were no intergroup differences in food intake in either the TSOD mice or TSNO mice. From these results, it was apparent that neither reduced food intake nor impaired nutrient absorption was a cause of body weight suppression and visceral/subcutaneous fat suppression.

In the present study, Bofutsushosan reduced insulin levels and improved abnormal glucose tolerance dose-dependently in the TSOD mice groups. It has been reported that Bofutsushosan causes a decrease in visceral fat and an improvement in insulin resistance in both clinical practice and animal tests. It is known that visceral fat accumulation induces insulin resistance, and elevation of blood glucose level leads to excessive secretion of insulin, resulting in hyperinsulinemia. Since the TSOD mice used in this study developed hyperinsulinemia, it is reasonable to assume that Bofutsushosan treatment induced suppression of visceral fat accumulation and prevented aggravation of insulin resistance, resulting in decreased insulin levels and prevention of abnormal glucose tolerance. In the past 10 years, the mechanism of insulin resistance has been investigated, and it has been suggested that TNF-α, which is secreted from hypertrophied adipocytes, is a mediator of insulin resistance in obesity. It is possible that Bofutsushosan prevents insulin resistance by reducing the levels of TNF-α.

Insulin resistance is a widespread pathology associated with the diabetes mellitus and hypertension. Bofutsushosan lowered the levels of glucose, SBP, DBP and MBP in the TSOD mice and significantly lowered these parameters in the high-dose group approximately to the levels in the TSNO control group. Bofutsushosan significantly lowered the level of TC and tended to lower the TG levels in both the TSOD and TSNO mouse groups. Saito et al. reported that Bofutsushosan inhibited pancreatic lipase in vitro (IC50: 20—30 mg/ml) and suppressed the elevation of plasma TG after oral administration of lipid emulsion. Morimoto et al. reported that Bofutsushosan inhibited TG synthesis in the liver. For these reasons, it is thought that Bofutsushosan decreased the plasma lipid level not only by direct suppression of lipid absorption seen in the TSNO mice but also by indirect suppression through suppression of visceral fat accumulation seen in the TSOD mice.

Patients with advanced diabetes develop peripheral neu-
ropathy as a complication, and it has been reported that TSOD mice develop similar impairments of hind limb movement with increasing age. Diabetic neuropathy is thought to occur from direct hyperglycemia-induced damage to the neural parenchyma by the polyol pathway, non-enzymatic glycation of proteins, oxidative stress and the effects of protein kinase C and TNF-α. In the pain test, the prolonged latent reaction time was shortened by Bofutsushosan treatment. It is suggested that Bofutsushosan, mainly by preventing visceral fat accumulation, may exert a preventive effect on the development of peripheral neuropathy by indirectly suppressing glucose levels and TNF-α.

In the TSNO mice used in this study as a non-disease model, no marked changes were observed in any parameters except for improvement in the lipid plasma level and mild diarrhea that was noted only in the high-dose group. Kampo medicine has been used by dialectic order in medical treatment, which means a tailor-made medicine. This experimental result suggested that Bofutsushosan was beneficial for visceral fat accumulative obesity such as TSOD mice, but wasn’t suitable for normal such as TSNO mice because the mild diarrhea was noted in the high-dose group. These results show that Bofutsushosan exerts a preventive effect on each of the disease states associated with metabolic disorders (obesity, visceral fat accumulation, hyperglycemia, abnormal lipid metabolism, hypertension and peripheral neuropathy) but has no direct influences on the healthy body.

CONCLUSION

The above results suggest that Bofutsushosan may prevent various disorders such as abnormal lipid metabolism, hyperglycemia, hyperinsulinemia, hypertension and peripheral neuropathy mainly by preventing visceral fat accumulation and improving lipid metabolism. Thus, Bofutsushosan is considered to be a valuable drug for the treatment of obesity and various metabolic disorders.

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REFERENCES