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Mioga (*Zingiber mioga* Rosc.) Extract Prevents 3T3-L1 Differentiation into Adipocytes and Obesity in Mice

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We examined the effect of edible plant extracts on prevention of obesity. 3T3-L1 cell that differentiated into a mature adipocyte was used as a model for screening *in vitro*. After the cells had formed a confluent monolayer, they were treated with DEX-MIX and incubated in a medium containing edible plant extract for 12 days. The mioga (*Zingiber mioga* Rosc.) extract significantly suppressed the increase in glycerol-3-phosphate dehydrogenase activity and triglyceride accumulation in 3T3-L1 cells. The results of Oil-Red O staining supported these findings. We further investigated the effect of mioga extract in prevention of obesity in male ICR mice *in vivo*. Through oral administration of the extract (10 or 50 mg/mouse), increases in body weight and epididymal fat weight were prevented in the animals. These results indicate that mioga extract may be useful in preventing obesity.

Keywords: mioga, 3T3-L1, adipocyte, differentiation, mouse, obesity

Obesity is a serious problem for human health because it can often trigger chronic diseases such as hypertension, cardiovascular disease and diabetes mellitus. The physiological effects of spices and herbs demonstrated in studies on obesity using adipocytes or animals have been reported. For example, compounds in African nutmeg enhance insulin-mediated glucose uptake in 3T3-L1 adipocytes, and decrease plasma glucose levels in either *ob*/*ob* or *db*/*db* mice (Luo *et al*., 1999). Compounds in cinnamon have an insulin-like action in intact adipocytes (Imparl-Radosevich *et al*., 1998). Capsaicin from red pepper was found to reduce serum triglyceride levels and adipose tissue weight in rats (Kawada *et al*., 1986, 1988). The traditional herbal medicine bofu-tsusho-san has been found to activate brown adipose tissue thermogenesis. In mice treated with this herbal medicine, body weight and white adipose tissue weight decreased (Yoshida *et al*., 1995). Perilla oil, rich in (n-3) polyunsaturated fatty acids, has been found to suppress adipocyte differentiation in the late phase and prevents excessive growth of adipose tissue in rats. (Okuno *et al*., 1997). Therefore, edible herbal plants may be a rich source of anti-obesity compounds, as suggested by the reports mentioned above.

Inhibitors of differentiation into adipocyte are expected to be effective in preventing obesity, as suggested in a report on perilla oil (Okuno *et al*., 1997). 3T3-L1 fibroblasts established by Green and his colleagues from a mouse differentiate into adipocytes under the appropriate conditions (Green & Kehinde, 1973; Green & Meuth, 1974). The cultured 3T3-L1 adipocytes show many properties similar to those of normal adipocytes. During the process of differentiation, lipids are accumulated and the level of activity of glycerol-3-phosphate dehydrogenase, a key enzyme in lipogenesis, increases in the cells (Russell, 1981). Thus, this cell line is a convenient model system for research on adipocytes *in vitro*, and is widely used for anti-obesity research (Sul *et al*., 1998). Our objectives are to identify compounds effective in preventing obesity from edible plants such as spices and herbs, using 3T3-L1 cells in *in vitro* screening tests. In this study, we examined the anti-differentiation effect of extracts prepared from 15 kinds of edible plants. The anti-differentiation activity of mioga (*Zingiber mioga* Rosc.) phosphate-buffered saline (PBS) soluble extract was the most potent among the extracts examined and its effectiveness was found to be dose dependent. Mioga belongs to the *Zingiberaceae* family and is traditionally eaten in Japan as a spice, however, its physiological effects in relation to obesity are not known. We also investigated the obesity preventive effects of mioga extract *in vivo* in mice.

Materials and Methods

Materials Edible plants were purchased from a local market. The plants used in this study (scientific name) were the following: mioga (*Zingiber mioga* Rosc.), ginger (*Zingiber officinale* Rosc.), perilla (*Perilla frutescens* Britton *var. acuta* Kudo), yuzu-citrus (*Citrus junos* Tanaka), udo salad (*Aralia cordata* Thunb), welsh onion (*Allium schoenoprasum* L.), garlic chive (*Allium tuberosum* Rottl.), garlic (*Allium sativum* L. forma *pekinese* Makino), Japanese hornwort (*Cryptotaenia canadensis* DC.), celery (*Apium graveolens* L. var *dulce* DC.), parsley (*Petroselinum sativum* Hoffm.), radish (*Raphanus sativus* L. var. *acanthiformis* Makino), garland chrysanthemum (*Chrysanthemum coronarium* L. var. *spatiosum* Bailey), red pepper (*Capsicum annuum* L.), shishitou-sweet pepper (*Capsicum annuum* L. var *angulosum* Mill.). Phosphate buffered saline (PBS) soluble extract was prepared using an equal volume of PBS. After homogenizing, squeezing and centrifuging each extract, the supernatant was freeze-dried.

Cell culture The 3T3-L1 preadipocyte cell line was obtained from the Health Science Research Resources Bank (Osaka). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS);

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the medium was changed 3 times per week. The cells were cultured in 60 mm diameter culture dishes (Falcon 3002) at a cell density of 4×10^3 cells/ml. When the cells reached confluence after 5 or 6 days incubation, the medium was changed and 0.25 μM dexamethasone and 0.5 mM 3-isobutyl-1-methyl-xanthine (DEX-MIX) were added to the fresh medium. After DEX-MIX treatment for 48 h, the medium was changed and insulin (final concentration: 1 μg/ml) and edible plant PBS soluble extract (final concentration: 100, 200, 500 μg/ml) were added. The medium was changed 3 times per week and insulin and extract were added each time.

Measurement of 3T3-L1 differentiation Twelve days after the start of incubation in the presence of the extract, the 3T3-L1 cells were harvested by Hayashi's method with some modification (Hayashi *et al*., 1981). After removing the medium, the cells were washed twice with 5 ml of PBS. They were then collected in 1 ml of cold sonication buffer (25 mM Tris-HCl/1 mM EDTA, pH 7.5) using a cell scraper, and sonicated. For measurement of triglyceride (TG) concentration, 200 μl of the suspension was used. The TG concentration in the cells was measured using E-test WAKO (Wako Pure Chemical Industries Ltd., Osaka). The remaining portion of the suspension was centrifuged at $8000\times g$ for 20 min at 4°C, and the supernatant was stored at −80°C for use in the glycerol-3-phosphate dehydrogenase (GPDH) activity assay. This assay was carried out by measuring the decrease in absorbance of NADH at 340 nm at 25°C and the amount of activity per mg of protein was calculated. Protein concentration was determined using the DC-protein assay system (BIO-RAD, CA). The GPDH activity and the TG concentration per mg of protein in 3T3-L1 cells were each expressed as the ratio (%) of the control value. For observation of lipids in the cells, Oil-Red O staining was performed by the method described by Kuri-harcuch and Green (1978).

Animals Three-week-old male ICR mice were obtained from Charles River Japan Inc. (Yokohama). After one week of normal feeding, the animals were randomly assigned to an ex-

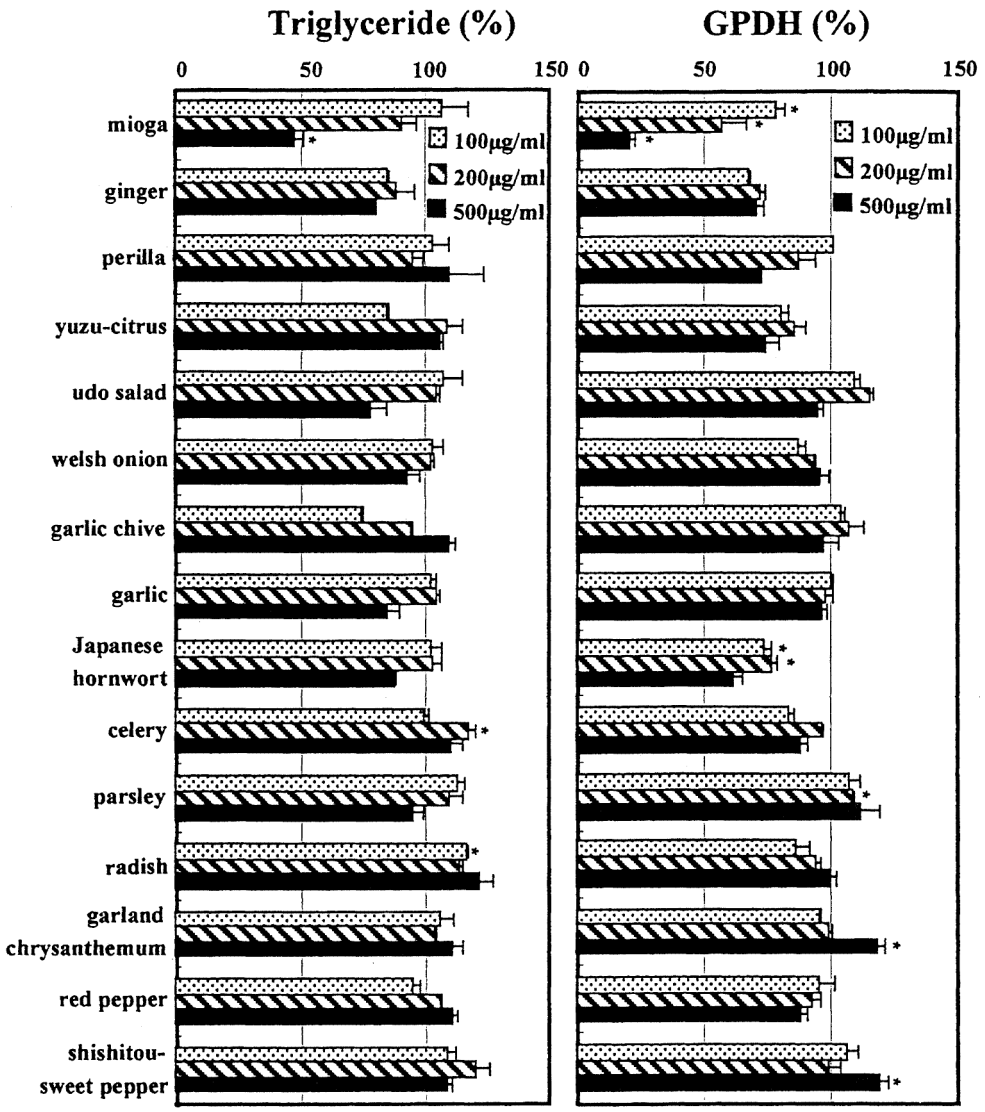

Fig. 1. Effects of edible plant PBS extracts on the differentiation of 3T3-L1 cells. 3T3-L1 cells were cultured in 60 mm diameter culture dishes and fresh medium was provided three times weekly. After six days of culture, the cells had formed a confluent monolayer and were then treated with DEX-MIX. Thereafter, they were incubated in a medium containing 1 μg/ml insulin and edible plant extract. After 12 days of incubation, GPDH activity and the triglyceride content, as indicators of 3T3-L1 cell differentiation, were measured. The results are shown as the ratio to the control and are expressed as means±SE, n=3. An asterisk indicates a significant difference ($^*p<0.01$) compared to the control as determined by Student's t-test.

perimental group (n=6). The mice were housed in a cage with free access to food (Type NMF, Oriental Yeast Co. Ltd., Tokyo) and water in a room kept at 25±3°C, with the lights on 12 h (8:00–20:00) a day. Powdered mioga extract was dissolved in 200 μl of PBS and administered orally to the mice (10 or 50 mg/mouse) every 2 days for 13 days; the mice in the control group were orally administered the same volume of PBS. The weight of food consumed by the mice in 3 cages per group was measured as food intake before oral administration, and body weight of the mice was measured daily. After sacrifice, the epididymal fat weight and liver weight were measured on day 7 and day 13 after the start of treatment. We followed our institute's guidelines on the care and use of laboratory animals.

Statistical methods Student's t-test was used to analyze the significance of differences between the control and experiment groups. Each experimental group was separately compared to the control, and results were expressed as means ±standard error. Data were analyzed by ANOVA to test the effect of the mioga extract on the TG content of 3T3-L1 cells from day 0 to day 12 and its effect on the body weight of the mice.

Results

The effects of extracts prepared from the 15 kinds of edible plants on the differentiation of 3T3-L1 adipocytes were examined. Figure 1 shows the data obtained in screening of the PBS soluble extracts from the plants. We measured the GPDH activity and the TG content of the 3T3-L1 cells as indicators of differentiation. The GPDH activity levels in the cells treated with parsley (200 μg/ml), garland chrysanthemum extract (500 μg/ml) or shishitou extract (500 μg/ml) were higher than the control level ($^{*}p$<0.01), but the changes in activity levels were not dose dependent. No significant effect on the TG accumulation in the cells treated with these extracts was observed. Celery extract (200 μg/ml) and radish extract (100 μg/ml) both accelerated TG accumulation but the effects were not dose dependent. Japanese hornwort extract at 200 or 500 μg/ml inhibited the GPDH activity ($^{*}p$<0.01) but TG accumulation was not suppressed, while the extract of mioga suppressed TG accumulation and suppressed the increase in GPDH activity in the 3T3-L1 cells in a dose-dependent manner. The GPDH activity and TG concentration in the cells treated with 500 μg/ml mioga extract were lower than the level in cells treated with the other extracts. The mioga extract did not damage the 3T3-L1 cells, because no abnormal morphology change was observed under light microscope.

In meaning the increase of GPDH activity in 3T3-L1 cells treated with mioga extract (500 μg/ml) (Fig. 2), the protein content was measured as an indicator of cell development. The differences in protein content between the control and mioga treated cells were small for 12 days. In contrast, GPDH in mioga treated cells was lower than that in control cells, although GPDH activity increased in both types of cells. The data shows that increase of GPDH in 3T3-L1 cell was suppressed by mioga treatment. The appearance of the Oil-Red O stained cells was consistent with the finding that the TG content of the mioga-treated cells

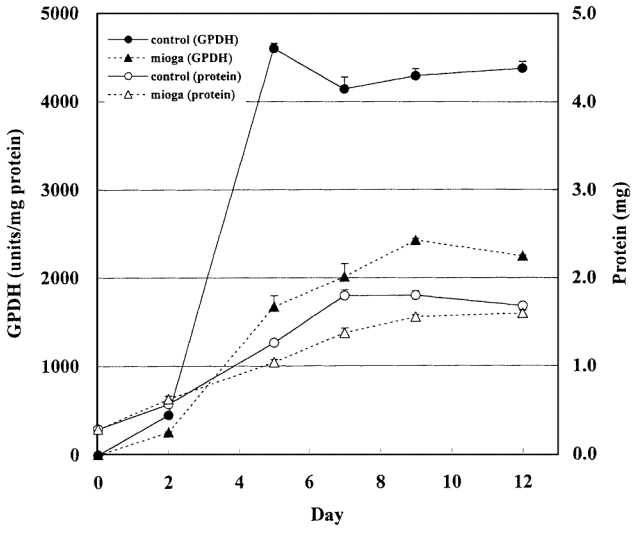

Fig. 2. Effect of mioga extract on activity of glycerol-3-phosphate dehydrogenase (GPDH) and protein content in 3T3-L1 cells. GPDH and protein, which reflect cell development, were measured during differentiation. The concentration of mioga extract was 500 μg/ml. Values are means±SE, n=3. ●, GPDH of control; ▲, GPDH of mioga treated cell; ○, protein of control; △, protein of mioga treated cells.

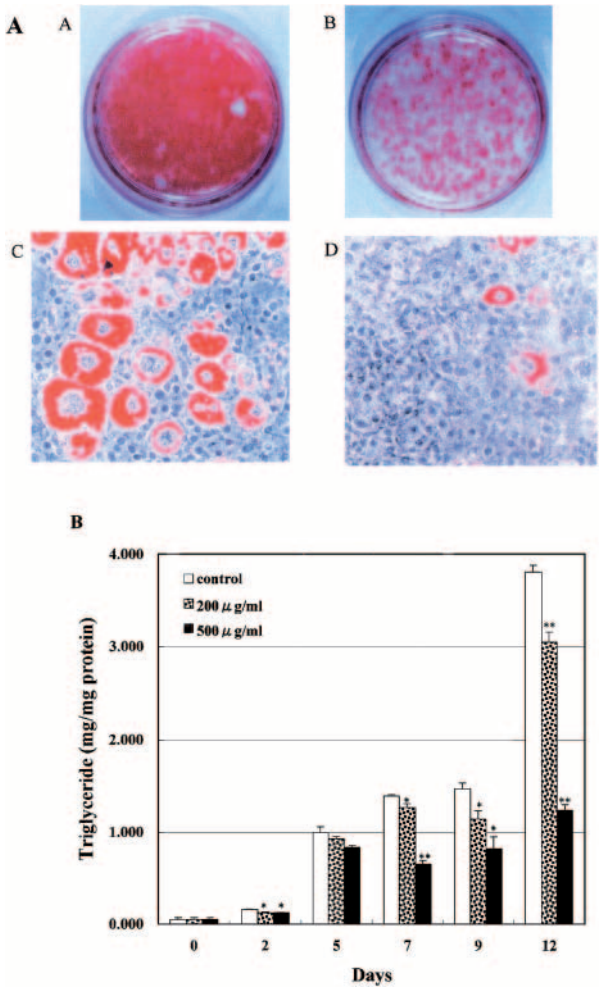

Fig. 3. (A) Effect of mioga extract on lipid accumulation in 3T3-L1 cells. After 12 days of treatment, the cells were fixed in 10% formaldehyde and stained with Oil Red O. A and C (×100 magnification, microscope observation): control, B and D (×100 magnification, microscope observation): medium containing the PBS soluble fraction of mioga (500 μg/ml) (B) Effect of mioga extract on the triglyceride content of 3T3-L1 cells during differentiation. Results are expressed as means±SE, n=3. Asterisk indicates a significant difference ($^{*}p$<0.05, $^{**}p$<0.01) compared to the control as determined by Student's t-test.

was lower than that of the control cells (Fig. 3A). There were fewer oil droplets in the 3T3-L1 cells treated with mioga extract and they were smaller than those in the control cells as determined by light microscope. These findings indicated that the mioga extract inhibited the differentiation of 3T3-L1 cells. We then measured the increase in TG content of these cells during differentiation (Fig. 3B). Cells treated with mioga extract were harvested at the time of every medium change, and the TG concentrations were found to increase during differentiation. On day 7 after the start of treatment and thereafter the TG content of the mioga-treated cells (200 μg/ml or 500 μg/ml) was lower than that in the control cells ($^{*}p<0.05$, $^{**}p<0.01$). Long-term treatment of the 3T3-L1 cells with the mioga extract suppressed TG accumulation significantly.

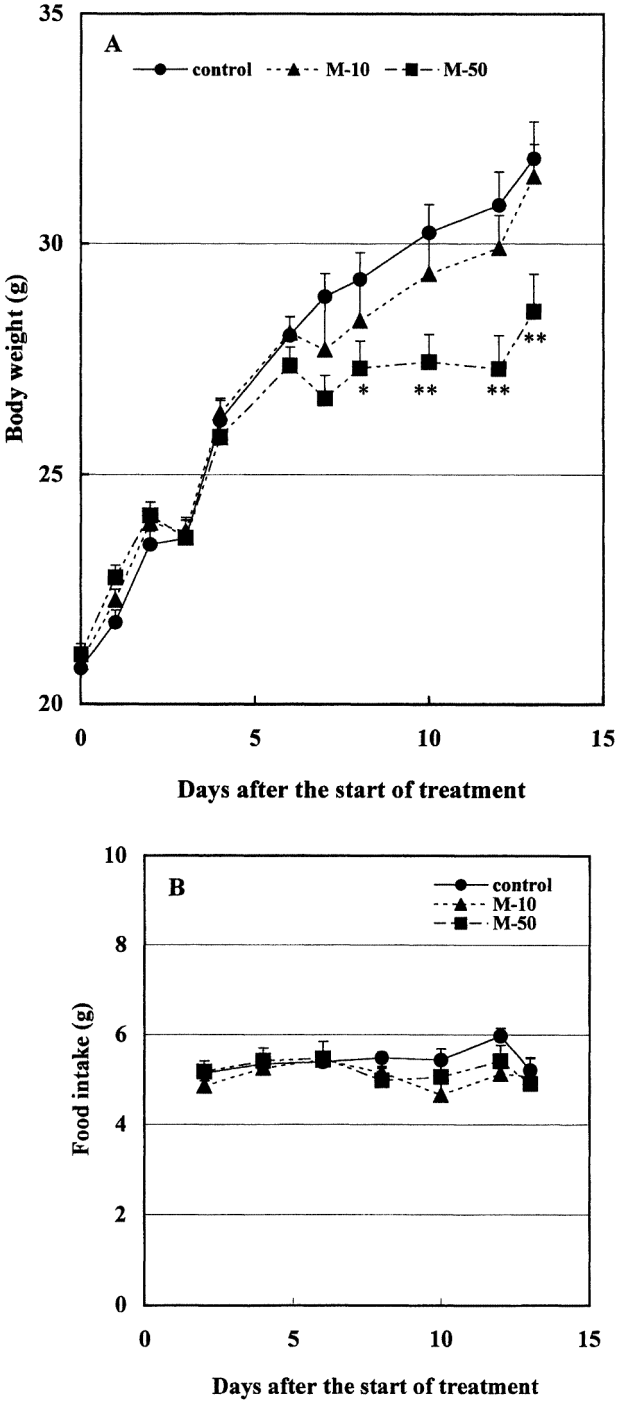

Fig. 4. (A) The body weight of mice orally administered 10 mg(M-10 group) or 50 mg (M-50 group) of mioga extract every two days was measured after the start of treatment. The values are means±SE, n=6. Asterisks indicate a significant difference ($^{*}p<0.05$, $^{**}p<0.01$) compared to the control group as determined by Student's t-test. (B) Food intake of mice orally administered 10 mg (M-10 group) or 50 mg (M-50 group) of mioga extract every two days. The values shown are the weight of food consumed/mouse/day and are means±SE.

We investigated the effectiveness of the mioga extract in the prevention of obesity in male ICR mice. The mice were divided into 3 groups: control (saline treatment), M-10 (mioga 10 mg/mouse) and M-50 (mioga 50 mg/mouse) groups. Figure 4A shows the time-course of changes in body weight during the 13-day treatment period. Food intake was measured before administration and is shown as food intake (g) per mouse per day (Fig. 4B). The body weight in all three groups increased continuously during the 13-day period (Fig. 4A), however, that of mice in the M-10 and M-50 groups was lower than the control group from day 7 to day 13. The body weight in the M-50 group was significantly lower than that in the control group ($^{*}p<0.05$ or $^{**}p<0.01$). These findings demonstrate that oral administration of mioga reduced the body weight gain in the ICR mice. On the other hand, there was no significant difference in food intake during tested time and the total food intake among the three groups (Fig. 4B).

On day 7 and day 13 after the start of treatment, mice were sacrificed and the epididymal fat pads and the liver were dissected and weighed. There was no significant difference among the groups in the ratio of liver weight to body weight and the livers seemed to be normal in appearance (data not shown). The fat results are shown in Fig. 5. As shown in Fig. 5A, the epididymal fat weight in the M-10 group was lower than that in the control group on day 7 ($^{*}p<0.05$), while there was no significant difference in the ratio of fat weight to body weight among the groups on day 7 (Fig. 5C). The fat weight in the mioga treatment groups, however, was less than that in the control group on day 13(Fig. 5B). The fat weight in the M-50 group was 34.3% less than in the control group, and the difference was significant ($^{**}p<0.01$). Also, the ratio of fat weight to body weight in the M-50 group on day 13 was significantly less than in the control group (Fig. 5D) ($^{**}p<0.01$). From these results it seems that the mioga extract was effective in suppressing fat accumulation in these mice.

Discussion

Not only the caloric intake from food, but also physiological functions of food factors can influence obesity. The effects of food factors, for example, vitamins and fatty acids, on adipose conversion have been reported (Kawada *et al*., 1996; Amri *et al*., 1991, 1994; Brodie *et al*., 1999), and it is known that spices and herbs have physiological activity and some are used as medicines. In this study, we examined PBS soluble extracts prepared from edible spices and herbal plants eaten in Japan to assess their effectiveness in inhibiting 3T3-L1 adipocyte differentiation. Among the extracts examined, only the mioga extract showed effectiveness in suppressing the increase in TG content and the level of GPDH activity in a dose dependent manner without cell damage. From data on GPDH and TG that are hallmarks of 3T3-L1 differentiation, mioga extract was shown to suppress the differentiation into mature adipocytes in 3T3-L1 cells. Mioga

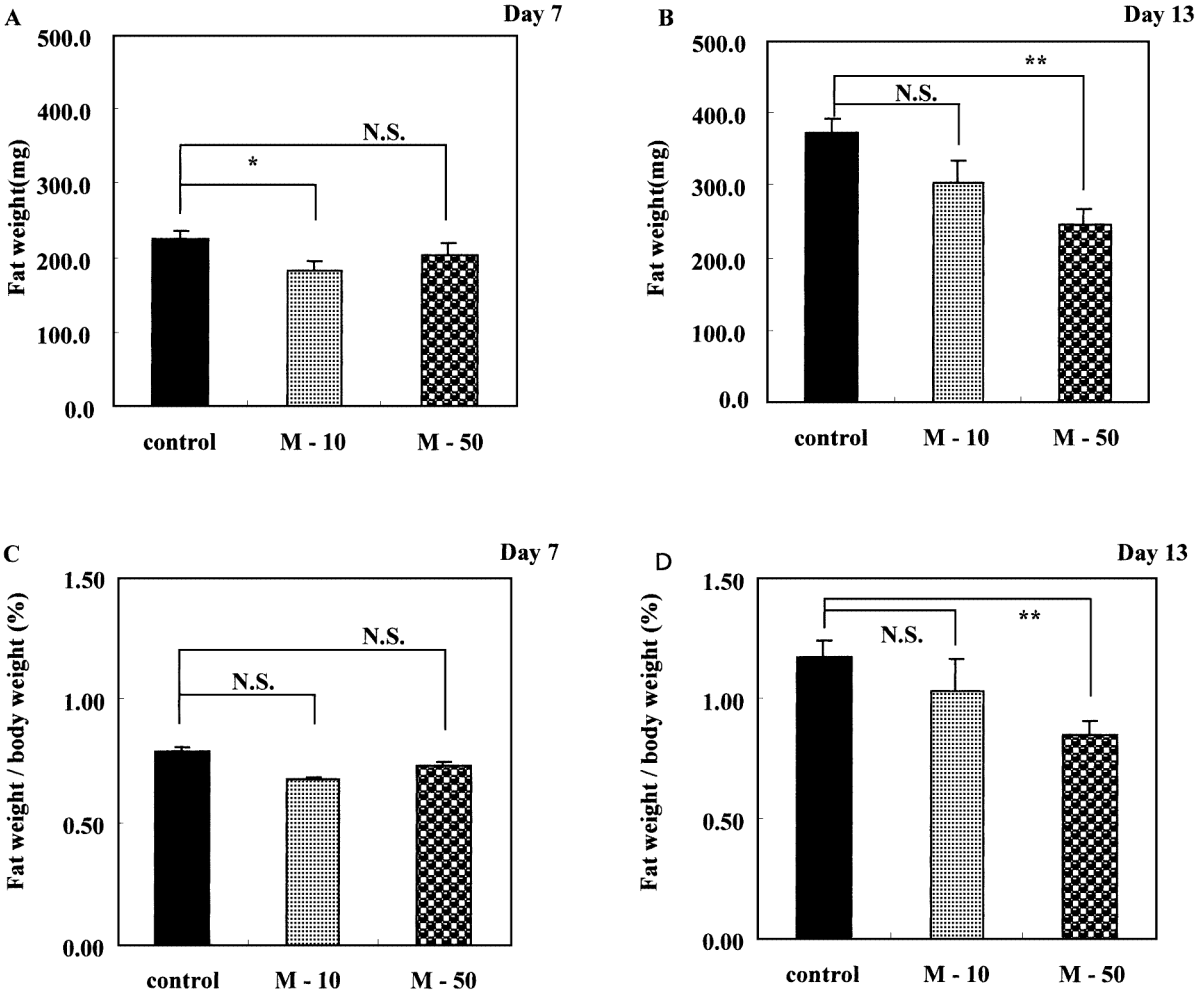

Fig. 5. The epididymal fat weight of mice administered 10 mg (M-10 group) or 50 mg (M-50 group) of mioga extract every two days on day 7 and day 13. The values are means±SE, n=6. The upper panels show the fat pad weight on day 7(A) and day 13(B), and the lower panels show the ratio of fat pad weight to body weight on day 7(C) and day 13(D). Asterisks indicate a significant difference ($^{*}p<0.05$, $^{**}p<0.01$) compared to the control group as determined by Student's t-test.

extract seemed to delay the differentiation, but not to block or damage the ability of differentiation in these cells (Fig. 2). Ethanol extracts of the tested plants in this study were examined to assess their anti-differentiation activity in 3T3-L1 cells (Iwashita *et al*., 1997, 1999), and among those tested, red pepper ethanol extract did inhibit the differentiation. The other extracts, including mioga ethanol extract, did not show this anti-differentiation activity. Red pepper studies related to obesity have reported (Kawada *et al*., 1986, 1988; Watanabe *et al*., 1987; Watanabe *et al*., 1999). In contrast, mioga is believed to cause an amnesic syndrome traditionally in Japan, but the physiological function of this plant is not clear. This is the first report concerning the actions of mioga related to obesity, and have selected mioga as the target for further investigations.

Mioga belongs to the *Zingberaceae* family and contains anthocyan. Ginger, a popular plant belonging to the ginger family, did not show any anti-differentiation activity in 3T3-L1 cells in this study. We prepared a mioga ethanol extract and a crude anthocyan fraction using a Sep-pak C18 cartridge; neither of these preparations showed any anti-differentiation activity (data not shown). Polyphenolic compounds, apple polyphenols and procyanidins have been shown to affect 3T3-L1 differentiation into adipocyte, lipolysis and obesity in rats (Shoji *et al*., 2000; Ardevol *et al*., 2000; Han *et al*., 1999). Treatment of the mioga extract with polyvinylpolypyrrolidone, which adsorbs polyphenolic compounds, did not disturb the activity (data not shown). From these results, the active compounds in mioga extract are thought to be of mioga origin, hydrophilic and not a polyphenolic compound such as anthocyan. Because the active compounds in mioga are not yet known, investigations of the active components are currently underway. Two active fractions have now been obtained by precipitation with 70% ethanol and gel filtration. These fractions reacted by phenol-sulfuric acid method and showed a low content of protein by Lowry's method. Maldi TOF-MS about these fractions was performed with Reflex II (Bruker Daltonik Gmbh, Bremen, Germany) as follows: laser: nitrogen, positive-ion mode, matrix: 2% 2,5-dihydroxy benzoic acid/20% ethanol. They were believed to contain high molecular weight components (about 84 kDa and 96 kDa) by TOF-MS analysis. We are studying the active component in mioga and will report on its characteristics in future.

It is possible that both lipogenesis and lipolysis occur in the adipocytes, and it is assumed that the mioga extract suppresses fat synthesis or enhances lipolysis in adipocytes. An experiment

was performed examining glycerol and fatty acid release from mature 3T3-L1 adipocytes to learn the mechanism of action of the extract. However, when mature 3T3-L1 adipocytes were treated with mioga extract, glycerol and fatty acid release indicative of lipolysis was not observed (data not shown). The extract was suggested to suppress lipogenesis in 3T3-L1 cells. (Watanabe *et al.,* 1998, 1999) reported that inhibitors of acetyl-CoA carboxylase, a key enzyme involved in fatty acid biosynthesis, are present in red pepper and green tea. The inhibition of acetyl-CoA carboxylase by mioga extract was examined according to Watanabe's method, but no inhibition of acetyl-CoA carboxylase was observed (data not shown). The mechanism of action of the extract in suppressing fat accumulation is assumed to involve inhibition of other factor which play a role in lipogenesis, such as an insulin-related action or inhibition of other enzymes.

The TG content of the 3T3-L1 cells increased beginning on the day treatment with mioga extract was began (day 0) (Fig. 3B). On day 7 and thereafter, significant differences between the control cells and the cells treated with the extract were evident. The TG content was low and the fat droplet size was small in cells treated with extract for 12 days (500 μg/ml), although, fat was synthesized in these cells (Fig. 3A). In an *in vitro* test, the mioga extract suppressed the increase of TG content in 3T3-L1 cells time dependently. Treatment, longer than 7 days was effective in suppressing fat accumulation in these cells. In addition, in an *in vivo* test, mioga extracts suppressed the increase of body weight gain and epididymal fat weight in mice (Fig. 4A and 5). Earlier reports on obesity showed fat weight, liver weight and their percentages to body weight in the results of an *in vivo* test (Niiho *et al*., 1993; Cha & Jones, 1998; Kabir *et al*.,1998). Perilla oil suppressed the increase of epididymal fat weight and the ratio of epididymal fat weight to body weight, although, liver weight and its ratio to body weight were not affected by this oil (Okuno *et al*., 1997). In this study, the extent of increase in epididymal fat weight from day 7 to day 13 in the control group was 148.6 mg, whereas that in the administered group (M-10, M-50), was 118.6 mg and 40.7 mg respectively. The percentage (fat mass (g)/body mass (g)) in the control group was higher than that of these two groups, while the liver weight ratio to body weight of the administered group was similar to the control group. These findings indicated that mioga extract might inhibit obesity *in vitro* and *in vivo*.

In summary, it was demonstrated that mioga extract is effective in inhibiting fat accumulation in 3T3-L1 adipocytes and causes a decrease in body weight gain and a decrease in fat mass in ICR mice. Our results suggest that mioga extract may be an effective crude drug for prevention of obesity.

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