
Original

Efficient Reduction of Inguinal Subcutaneous Adipose Tissue in Mice by Combination of External Application of Caffeine and Oral Administration of Olive Leaf Extract

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Abstract: Excess accumulation of subcutaneous fat causes numerous diseases. The combined effect of caffeine liniment and orally administered olive leaf extract on subcutaneous fat accumulation was studied.

C57BL/6J male mice were evenly divided into 1) control group, 2) caffeine liniment group, 3) oral olive group and 4) caffeine liniment plus oral olive group. Inguinal subcutaneous fat accumulation was measured by computed tomography (CT)-based analysis at 4 and 8 weeks of the experiment.

Inguinal subcutaneous fat accumulation in the caffeine liniment plus oral olive group, but not caffeine liniment group nor oral olive group, showed a significant reduction when compared with the control group at the 8 weeks.

These results indicated that the combination of caffeine liniment and oral olive is more effective than the external or internal agents alone in controlling subcutaneous obesity. These results are expected to improve the therapeutic outcomes of subcutaneous obesity.

Key words: inguinal subcutaneous fat; caffeine; olive; obesity; liniment

Introduction

The prevalence of overweight and obesity in developed and a part of developing countries has increased significantly during recent years¹⁾. Obesity is associated with an increased risk of atherosclerotic cardiovascular disease and mortality²⁾. Excess accumulation of visceral fat, correlates with various diabetogenic, atherogenic, prothrombotic and pro-inflammatory metabolic abnormalities, that increase the risk of atherosclerotic cardiovascular disease³⁻⁵⁾. Therefore, visceral obesity may have a key role in the development of multiple risk factors. In addition, obese individuals whose body mass index (BMI) above 30 (kg/m²) may have health problems caused by an excessive amount of subcutaneous fat, including osteoarthritis owing to knee overload⁶⁾, and obstructive sleep apnea syndrome (OSAS)⁶⁾. Insulin resistance is caused by high serum free fatty acids, 75% of which are released from subcutaneous fat. In fact, glucose infusion rates are significantly correlated with cross-sectional area of subcutaneous fat⁷⁾.

Therefore, to prevent the diseases caused by obesity, we must control both visceral and subcutaneous fat.

Obesity is caused by energy imbalance, which may be corrected by exercise and calorie restriction. Exercise or calorie restriction produces catecholamines that promote lipolysis^{8, 9)}. Catecholamines have a greater effect on visceral fat than on subcutaneous fat¹⁰⁾, thus they preferentially reduce visceral fat. Although exercise and calorie restrictions are the major therapies for obesity, they are less effective on subcutaneous obesity. Thus, it is necessary to develop new approaches that affect subcutaneous fat in preference to visceral fat.

Caffeine is a well-known chemical compound that increases lipolysis. It is an analog of adenosine that promotes thermogenesis through the central nervous system, and lipolysis of adipocytes directly via hormone-sensitive lipase. Using this characteristic, Velasco *et al.* reported that the diameter of subcutaneous adipocytes was decreased by 17% using external application of caffeine-containing cream¹¹⁾. This indicated the usefulness of external application

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of lipolytic compounds such as caffeine to restrict subcutaneous obesity. Thus we decided to use caffeine for external agent.

However, to date, there have been no studies evaluating the effects of external agents on the reduction process in whole subcutaneous fat tissue. Although external application of caffeine promotes lipolysis of local subcutaneous fat, free fatty acids released from local subcutaneous fat tissue may return to the fat tissue if there are no changes in total energy balance.

Thus, we explored effective fat reduction methods using a combination of ingested food components that promote whole body fat metabolism, and external application of caffeine as liniment. Olive ingredients are reported to improve cardiac and hepatic metabolism in high carbohydrate-, high fat-fed rats, and to have high availability and efficiency in humans^{12, 13}). Thus, we decided to use olive leaf extracts for internal agent. Oleuropein which is the major ingredient of olive leaf extract is absorbed by the small intestine, and then promotes the secretion of adrenaline, and finally upregulates thermogenesis in brown adipose tissue (BAT)¹⁴).

Materials and Methods

Animal; Six-week-old male C57BL/6J mice (SLC Japan, Shizuoka, Japan), diet-induceral obese model mouse, were maintained at a controlled temperature of $23 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ humidity with a 12-h light/dark cycle. Mice were allowed free access to water and standard CRF-1 laboratory diet (Oriental Yeast, Tokyo, Japan) for 1 week before the start of the experiment.

Acclimatized seven-week-old mice were divided into four groups of 8 mice: 1). Control group (30 wt% high-fat diet and external application of 70% ethanol (EtOH)); 2). Caffeine group (30 wt% high-fat diet and external application of liniment composed of 0.375 wt% caffeine-70% EtOH); 3). Olive group, (oral administration of 30 wt% high-fat diet containing 0.0625 wt% olive leaf extract); and 4). Caffeine plus Olive group (30 wt% high-fat diet containing 0.0625 wt% olive leaf extract and external application of the liniment containing 0.375 wt% caffeine-70% EtOH). The composition of the 30 wt% high-fat diet and that of the 30 wt% high-fat diet containing 0.0625 wt% olive leaf extract is shown in Table 1. In both diets, the composition of vitamins and minerals were as listed for AIN93G¹⁵). AIN93G is the growth diet for rodents recommended

Table 1. 30 wt% high-fat diet (A) and 30 wt% high-fat diet containing 0.0625 wt% olive leaf extract (B)

	A	B
Milk casein	25.000%	25.000%
α corn starch	14.869%	14.807%
Sucrose	20.000%	20.000%
Beef fat	14.000%	14.000%
Lard	14.000%	14.000%
Soy fat	2.000%	2.000%
Cellulose powder	5.000%	5.000%
Mineral mix AIN93G	3.500%	3.500%
Vitamin mix AIN93G	1.000%	1.000%
Choline bitartrate	0.250%	0.250%
Tert-butyl hydroquinone	0.006%	0.006%
L-cystine	0.375%	0.375%
Olive leaf extract	—	0.0625%
Total	100.000%	100.000%

by American Institute of Nutrition. Liniment containing caffeine and 70% EtOH solution was prepared every month and stored at 4°C . All mice were shaved on the lower back and 100 μl of external caffeine preparation was applied to mice in three groups by micropipette. Shaving was performed under sevoflurane anesthesia, and shaving frequency was limited to three-six times during the experimental period. Body mass and the amount of food left in cages were weighed once per week. Interscapular BAT and liver was eviscerated at the time of death, and weight was measured. All animal protocols were approved by the Ethics Committee of Kagawa Nutrition University.

Chemicals and Diets; Anhydrous caffeine was purchased from Wako Pure Chemical Industries (Osaka, Japan) and was dissolved in 70% EtOH completely for external application. Olive leaf extract was purchased from Eisai Food & Chemical (Tokyo, Japan), which contains 35% oleuropein.

Analysis of Inguinal Subcutaneous Fat Mass; We used X-ray computed tomography (CT) to evaluate mouse subcutaneous fat mass by targeting the inguinal subcutaneous fat tissue, as it was a separate tissue and was the largest among subcutaneous fat tissues. For CT analysis of body fat composition, visceral fat was mainly measured between L2 and L4¹⁶). But optimum measurement area for inguinal subcutaneous fat was unknown. Therefore we assessed the correlation of inguinal subcutaneous fat volume and CT analysis area, and concluded that L3 and L6 was the most suitable (data not shown). Mice were anesthetized with intraperitoneal injections of pentobarbital sodium (Nembutal; Dainippon Pharmaceutical, Osaka, Japan) and then scanned using a LaTheta (LCT-100) experimental animal

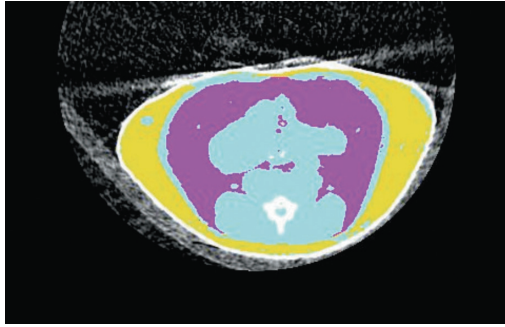


Fig. 1. Representative CT images of control mouse at L5 level

For CT analysis of inguinal subcutaneous fat composition, mice were scanned. Contiguous 1-mm slice images between L3 and L6 were summed for quantitative assessment. The pink and yellow areas represent the visceral and inguinal subcutaneous fat, respectively.

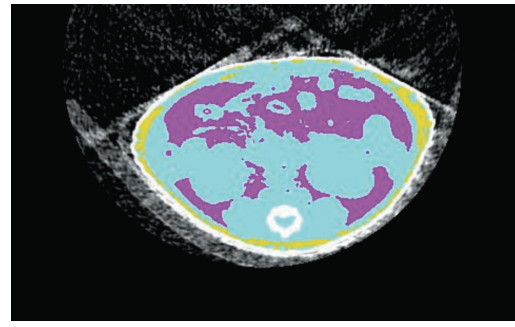


Fig. 2. Representative CT images of control mouse at L3 level

Contiguous 1-mm slice images between L2 and L4 were summed for quantitative assessment. The pink and yellow areas represent the visceral and inguinal subcutaneous fat, respectively.

Table 2. Total food intake, final body weight, subcutaneous fat weight and visceral fat weight in C57BL/6J mice fed high-fat diet in control, caffeine, olive and caffeine plus olive groups

	Total Food Intake (g)			Final Body Weight (g)			Subcutaneous Fat Weight (g)			Visceral Fat Weight (g)		
	Mean	SE	% of Control	Mean	SE	% of Control	Mean	SE	% of Control	Mean	SE	% of Control
Control	140.6	1.6	100.0	29.1	0.7	100.0	0.720	0.100	100.0	0.942	0.173	100.0
Caffeine	129.7*	2.8	92.3	28.4	0.7	97.8	0.603	0.082	83.8	0.683	0.101	72.5
Olive	139.5	2.9	99.2	29.7	0.7	102.2	0.719	0.049	99.9	0.979	0.090	103.9
Caffeine + Olive	129.3	5.0	92.0	25.9**	0.5	88.9	0.366*	0.042	50.9	0.418	0.093	44.3

*, $p < 0.05$ vs. value in controls

**, $p < 0.01$ vs. value in controls

CT system (Aloka, Tokyo, Japan). Contiguous 1-mm slice images (areas) between L3 and L6 were summed for quantitative assessment of the fat mass using LaTheta software (version 1.60) (Fig. 1).

Analysis of Visceral Fat Mass; Mice were anesthetized using the same method of the analysis of Subcutaneous Fat Mass and were then scanned using a LaTheta (LCT-100) experimental animal CT system. Contiguous 1-mm slice images (areas) between L2 and L4 were summed for quantitative assessment of fat mass using LaTheta software (version 1.60) (Fig. 2).

Serum analysis; After CT analysis of the final week, mice were killed. Blood was collected by cardiac puncture, and isolated serum was stored at -80°C until use. Total cholesterol (T-cho) and triglyceride (TG) concentrations were measured using Fuji DRI-CHEM 4000 kits (TSCH-PIII and TG-PIII, respectively; Fuji Film, Tokyo, Japan).

Statistical analysis; Each result were expressed as a mean $\pm$ standard error (SE). Statistical tests were performed using the Dunnett's multiple-comparison test for comparing with the control group in body weight, liver weight, serum triglyceride concentration and serum total cholesterol

concentration. Statistical tests were performed using the Steel multiple-comparison test for comparing with the control group in total food intake, subcutaneous fat weight, visceral fat weight, and interscapular BAT weight. P-values were shown in the section of result. The significant level was set at 0.05. The StatLight 2000 procedure was applied for statistical analysis.

Results

Total food intake in the caffeine plus olive group was not significantly different when compared with the control group. While the olive group did not show significant difference with the control group, the caffeine group showed significant difference with the control group ($p = 0.02$) (Table 2). The time courses of body weight increase in each group are shown in Fig. 3. Body weight in the caffeine plus olive group was significantly lower than that in control mice at 4 to 8 weeks (4w, $p = 0.04$; 5w, $p = 0.008$; 6w, $p = 0.004$; 7w, $p = 0.003$; 8w, $p = 0.004$). Body weight in both the caffeine group and olive group was not significantly different from those in control group. Final body weight in the caffeine plus olive group was 88.9% of that in the control group,

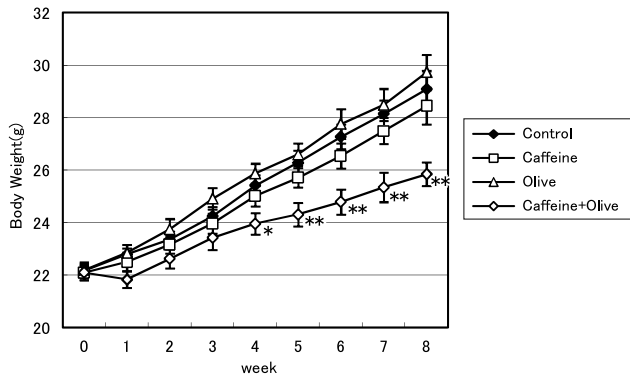


Fig. 3. Time courses of body weight increases in each group

C57BL/6J mice received high-fat diet (control group), external caffeine application (caffeine group), oral olive leaf extract administration (olive group), external caffeine and oral olive leaf extract administration (caffeine plus olive group) for 8 weeks. Values represent means $\pm$ SE of eight mice for each group.

*, $p < 0.05$, **, $p < 0.01$, vs. values in controls.

while those in the caffeine group and olive group were 97.8% and 102.2%, respectively, of that in the control mice (Table 2).

The amounts of inguinal subcutaneous fat are shown in Fig. 4. At 4 weeks, the amount of subcutaneous fat in mice in the caffeine group, olive group, and caffeine plus olive group was 86.8%, 91.8% and 64.5%, respectively, of that in the control mice, but these differences were not significant. At 8 weeks, these values were 83.8%, 99.9% and 50.9%, respectively, of that in the control mice, and the change was significant only in the caffeine plus olive group ($p = 0.02$).

The amounts of visceral fat are shown in Fig. 5. At 4 weeks, the amounts of visceral fat in mice in the caffeine group, the olive group and the caffeine plus olive group were 75.2%, 95.1% and 52.0%, respectively, of that in the control mice. At 8 weeks, the amounts of visceral fat in those groups were 72.5%, 103.9% and 44.3%, respectively, of that in the control mice. However, these values were not significantly different from those in controls during the experimental period.

Table 3 shows the liver weight, interscapular BAT weight, and serum TG and T-cho concentrations. Liver weight (g) was lower in the caffeine plus olive group than that in the control group ($p = 0.02$), but the liver weight based on body weight (%) was not different from that in the control group. Interscapular BAT weight (g) and serum TG concentration (mg/dl) of the three groups were not significantly different from those in the control

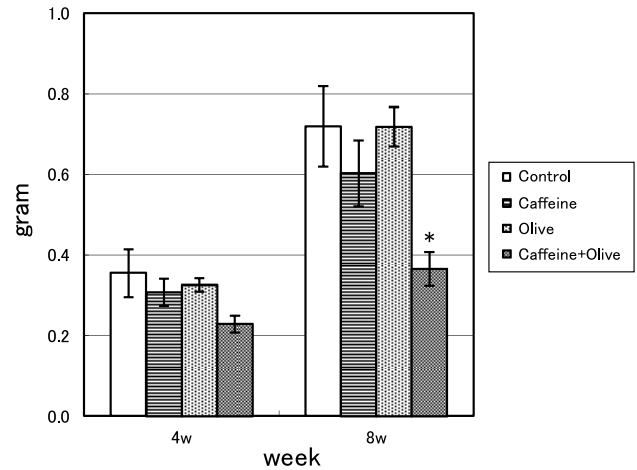


Fig. 4. CT-based subcutaneous fat composition analysis at 4 and 8 weeks

CT-estimated amounts of subcutaneous fat in the abdominal area of L3-L6.

*, $p < 0.05$, vs. values in controls.

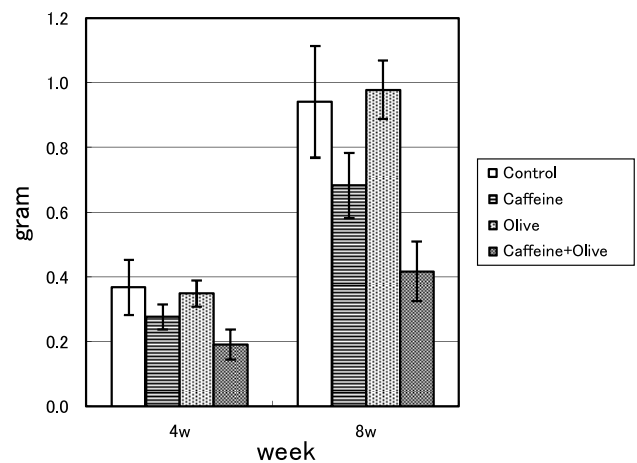


Fig. 5. CT-based visceral fat composition analysis at 4 and 8 weeks

CT-estimated amounts of visceral fat in the abdominal area of L2-L4.

group. On the other hand, serum T-cho concentrations (mg/dl) in the caffeine plus olive group mice were significantly lower than those in the control group ($p = 0.04$). Serum T-cho concentrations in the caffeine group, olive group, and caffeine plus olive group were 99.8%, 96.4% and 89.7%, respectively, of that in the control mice.

Discussion

We demonstrated in this study that only in the caffeine plus olive group, both body weight ($p = 0.004$) and accumulation of inguinal subcutaneous fat ($p = 0.02$) were significantly different from

Table 3. Final liver weight, interscapular BAT weight, serum TG levels and serum T-cho levels in C57BL/6J mice fed high-fat diet in control, caffeine, olive and caffeine plus olive groups

	Liver Weight (g)			Interscapular BAT Weight (g)			Serum TG levels (mg/dl)			Serum T-cho levels (mg/dl)		
	Mean	SE	% of Control	Mean	SE	% of Control	Mean	SE	% of Control	Mean	SE	% of Control
Control	0.938	0.020	100.0	0.062	0.005	100.0	72.6	4.5	100.0	123.6	3.0	100.0
Caffeine	0.920	0.002	98.1	0.056	0.002	90.7	70.4	5.0	96.9	123.4	3.2	99.8
Olive	0.961	0.004	102.4	0.060	0.004	96.0	87.8	4.6	120.8	119.1	3.9	96.4
Caffeine + Olive	0.866*	0.002	92.3	0.051	0.002	81.7	64.9	8.2	89.3	110.9*	3.7	89.7

*; $p < 0.05$ vs. value in controls

**; $p < 0.01$ vs. value in controls

control group without any changes of total food intake.

In preliminary research, we orally administered olive leaf extract to mice. The concentration of olive leaf extract was 0.125wt%, 0.25wt%, and 0.5wt% in the same high-fat diet, and internal olive leaf extract restricted inguinal subcutaneous fat accumulation significantly only when the concentration exceeded 0.25wt% (data not shown). With regard to oleuropein, Oi-kano *et al.* reported that 44 mg/kg, but not 22 mg/kg, orally administered oleuropein was effective in restricting visceral fat accumulation¹⁴). In our study, olive group mice were fed 22 mg/kg oleuropein, and we found that the oral administration of 22 mg/kg oleuropein was effective when combined with externally applied caffeine. Actually, the amount of subcutaneous fat in mice in the caffeine plus olive group were significantly lower than those in the olive group ($p=0.03$, by the Steel Dwass multiple-comparison test). This indicates that the effects of oleuropein or other olive leaf ingredients on subcutaneous fat restriction were enhanced by caffeine liniment.

On the other hand, the amount of subcutaneous fat in mice in the caffeine plus olive group were not significantly lower than those in the caffeine group ($p=0.12$, by the Steel Dwass multiple-comparison test). So we could not demonstrate the effect of external application of caffeine on subcutaneous fat restriction was enhanced by oral olive leaf extract. In this study, caffeine concentration in the liniment applied to the skin was 0.375 wt% in 70% EtOH. As the molecular weight of caffeine is low, it is mostly absorbed through skin¹⁷). If 100% of the applied caffeine is absorbed, 100 μ l of 0.375 wt% caffeine liniment corresponds to 15 mg/kg/day caffeine administration, based on the average weight of mice. Caffeine has relatively high toxicity. According to material safety data sheet (MSDS), half of people who ingest more than 6.5 mg/kg caffeine experience acute symptoms within an hour,

and all people who ingest more than 17 mg/kg caffeine experience acute symptoms within three hours. The lethal dose 50% (LD50) of caffeine is 200 mg/kg (mouse: 127 mg/kg). It is indicated that ineffective and effective dose of caffeine for subcutaneous fat were close, and actually, the caffeine group in this study reduced subcutaneous fat unexpectedly. As to body weight, the caffeine plus olive group was significantly lower than that in control ($p=0.01$, by the Tukey multiple-comparison test), caffeine ($p=0.04$, by the Tukey multiple-comparison test), and olive ($p=0.001$, by the Tukey multiple-comparison test) at 8 week. It might demonstrate that the effect of caffeine liniment was enhanced by the combination use with orally administered olive leaf extract. Further investigation is necessary to seek the optimal caffeine liniment concentration.

The mechanism of this efficiency was not fully understood. As visceral fat accumulation also tended to be restricted in the caffeine plus olive group, caffeine applied to mouse skin was absorbed and was distributed throughout the body via blood flow. Therefore, the combination of caffeine and olive leaf extract might enhance lipolysis and thermogenesis in adipocytes, as well as restrict lipogenesis in hepatocytes. As a result, visceral fat tissues and serum lipid levels might decrease.

We were unable to identify changes in the weight of isolated interscapular BAT tissue. Further investigation is necessary to assess serum adrenaline levels and changes in gene expression of uncoupling protein-1 (UCP-1) in interscapular BAT tissue. Finally, the observed effects presented here must be confirmed in human visceral and local subcutaneous fat.

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