LIPOSA pharmacopuncture, a new herbal formula, affects localized adiposity by regulating lipid metabolism *in vivo*

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Abstract. Localized adiposity is a serious aesthetic problem and a well-known health risk factor. There is a growing interest in minimally invasive treatment options for excessive fat accumulation, such as pharmacopuncture. LIPOSA is a newly developed pharmacopuncture formula from three natural herbs: The tuber of *Pinellia ternata* (Thunb.) Breitenb., the whole plant of *Taraxacum platycarpum* Dahlst. and the root of *Astragalus membranaceus* Bunge. The present study investigated the effects of pharmacopuncture treatment with LIPOSA on localized adiposity. Male C57BL/6J mice were fed high fat diet for 8 weeks to induce obesity. Then, 100 μl LIPOSA was injected into the left-side inguinal fat pad at various concentrations, including 13.35, 26.7 and 53.4 mg/ml. Normal saline was injected into the right-side inguinal fat pad of each mouse as a control. The treatment was performed three times per week for 2 weeks. The weight and histological changes were analyzed in the inguinal fat pad of the obese mice. The expression levels of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), autophagy-related gene (ATG)5, ATG7 and LC3-II, as lipophagy-related factors, were evaluated to confirm the lipid-catabolic effects of LIPOSA. LIPOSA pharmacopuncture markedly decreased the weight of the fat tissue and the size of the adipocytes in the inguinal region of the mouse models of obesity in a dose-dependent manner. The expression levels of ATGL, HSL, ATG5, ATG7 and LC3-II were significantly increased by the LIPOSA treatments. In addition, LIPOSA pharmacopuncture was found to decrease the expression levels of ACC, PPAR-γ and PEPCK. The results indicated that subcutaneous injection of LIPOSA can degrade local fat and induce lipophagic and lipase activation effects. In addition, lipid metabolism related to fat accumulation was regulated by the LIPOSA treatment. The present study suggests that LIPOSA pharmacopuncture can be a non-surgical alternative in the treatment of localized adiposity.

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Introduction

Excessive fat accumulation in obesity is a serious aesthetic problem (1). The regional distribution of local fat is a well-known risk factor of obesity rather than body mass index (BMI) (2). Abdominal adiposity, with subcutaneous or visceral fat deposition, is implicated in several medical conditions such as metabolic syndrome, cardiovascular disease and lower quality of life (3). Aesthetic treatment for localized adiposity is performed mainly for abdominal subcutaneous adipose tissue (4). Liposuction, suction-assisted lipectomy, is the most commonly used technique of plastic surgery procedures in North America (5). Although fat tissue can be extracted by liposuction effectively, complications may cause contour deformities, embolism and even death (5). Injections of phosphatidylcholine (PC) and sodium deoxycholate (DC) have been widely used as a minimally invasive treatment for localized adiposity (6). However, PC and DC injection may have substantial side effects including fibrosis and necrosis of the tissue (7). Pharmacopuncture, a new acupuncture technique with the injection of a herbs extract at the acupuncture point, could be a non-surgical alternative in the treatment of localized adiposity (8).

LIPOSA, consisting of the tuber of *Pinellia ternata* (Thunb.) Breitenb., the whole plant of *Taraxacum platycarpum* Dahlst. and the root of *Astragalus membranaceus* Bunge, is a newly developed formula of pharmacopuncture treatment for localized adiposity. *P. ternata*, *T. platycarpum* and *A. membranaceus* have been used for treating metabolic disorders including obesity as traditional Korean medicines (9-11). In the theories of traditional Korean medicine, obesity can be caused by 'phlegm dampness', 'deficiency of Qi' and 'pathologic dampness-heat' (12). *P. ternata* and *T. platycarpum* are known to be effective herbs for dispelling 'phlegm dampness' and 'dampness-heat', respectively (12). *A. membranaceus* is one of the most frequently prescribed 'Qi tonifying' herbs in traditional Korean medicine (12).

P. ternata, *T. platycarpum* and *A. membranaceus* can be used for the treatment of obesity, however, the effects of pharmacopuncture with these herbs on localized adiposity have not been studied yet. In this study, we investigated the efficacy of LIPOSA pharmacopuncture on localized adiposity by analyzing the fat pad weight and histological changes of the fat tissues in obese mice. To confirm the underlying mechanism of then LIPOSA pharmacopuncture on the inhibition of local fat, lipolytic enzymes including adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) and lipophagic molecules including LC3-II, autophagy-related gene (ATG) 5 and ATG7 were investigated in high fat diet (HFD)-induced obese mice. Moreover, lipogenesis-related factors such as acetyl-CoA carboxylase (ACC) and phosphoenolpyruvate carboxykinase (PEPCK) and peroxisome proliferator-activated receptors (PPAR)-γ as an adipogenetic biomarker were evaluated in the inguinal fat tissues of the obese mice.

Materials and methods

Preparation of LIPOSA pharmacopuncture. LIPOSA pharmacopuncture compose *A. membranaceus*, *T. platycarpum* and *P. ternata*. *A. membranaceus* and *T. platycarpum* were extracted with 20-folds of distilled water at 100˚C for 2 h by refluxing. *P. ternata* was extracted with 15-folds of distilled water at RT for 2 h. Each extract was filtered by 3 μm paper filers, respectively, and then mixed. Mixed extracts were evaporated and freeze-dried. The yields of mixed extracts were 8.65%. Dried extracts were diluted in normal saline and compensated the pH range from 6.8 to 7.2 by 1N NaOH solution.

Animal treatment. Male C57BL/6J mice (5 weeks old) were purchased from Raonbio Inc. Mice were housed under temperature- and humidity-controlled facility. After 1 week of housing, all mice were fed high-fat diet (HFD) containing 60% fat for 8 weeks to induce obesity. To monitor and compare any adverse effects of LIPOSA pharmacopuncture with normal, 8 normal mice were fed standard diet. Body weight was measured once a week until the end of the animal experiments. Following 8 weeks of HFD, mice (n=8) were divided into 3 groups in accordance with the weight of the mice, which was LIPOSA 13.35, 26.7, 53.4 mg/ml. We conducted previous experiments to determine the effective doses of LIPOSA. Effective dose ranges of *Pinellia ternate*, *Taraxacum platycarpum* and *Astragalus membranaceus* were 5-10, 2-4 and 15-20 mg/ml, respectively. Therefore, a tentative dose (26.7 mg/ml) with half dose (13.35 mg/ml) and double dose (53.4 mg/ml) of LIPOSA were tested to establish the actual therapeutic dose of LIPOSA in this study. Obese mice were used as self-control, vehicle (normal saline) was injected in the right inguinal fat pad and LIPOSA were injected in the left inguinal fat pad. The samples were injected 100 μl each, 3 times a week for 2 weeks. Body weight and food intake were monitored every week. No significance was observed in body weight of LIPOSA-treated groups, suggesting that LIPOSA had inhibitory effects against localized fat accumulation rather than body weight reduction (Fig. S1). During the treatment of LIPOSA, no significant differences of daily food intake were shown in LIPOSA-treated mice (Table SI). In addition, there was no sign of toxicity in all LIPOSA-treated mice. All animal procedures were approved by Committee on Care and Use of Laboratory Animals of the Kyung Hee University (KHUASP(SE)-18-070; Seoul, Korea).

Measurement of inguinal fat weight. At the end of the 10 weeks, all animals under anesthesia were scanned from total-body scanner (InAlyzer dual X-ray absorptiometry; Medikors). Dual energy X-ray absorptiometry (DXA) measures one time with low energy and one time with high energy to separate the images into tissues in gram units by separating them into fat and lean before analysis. Fat distribution mice was visualized by body composition view by a mapping image processed by a software in the device. Red, blue and white color indicates the fat tissue, lean tissue and bone tissue, respectively. After then, all mice were sacrificed under anesthesia with 1% avertin (cat. no. T4,840-2; Sigma-Aldrich; Merck KGaA). Blood samples were collected by orbital puncture. Mice are euthanized by cervical dislocation. Inguinal fat pad was collected from the thigh and weight was measured. The regions to determine the weight of inguinal fat pad were from knee to tail based on line of ventral spine. The inguinal fat pad weight by LIPOSA treatment was calculated by relative intensity that the saline-treated fat weight was converted to 1.

Histology. After 10 weeks of feeding experimental diets, inguinal fat pad was collected from the C57BL/6 mice. Inguinal fat tissues were fixed in 10% neutralized formalin. The dehydrated fat tissue was then embedded in paraffin wax. Histological sections of 5 μm thickness were stained with hematoxylin and eosin (H&E). Adipocyte size was evaluated in 6 mice from each group and 6 random fields (magnification, x400) per mice. To measure cross-sectional adipocyte area, micrographs were taken using a light microscope (Nikon) and analyzed by using the ImageJ software.

Western blot analysis. Proteins were extracted by homogenizing inguinal fat tissues in the tissue protein extraction reagent (T-PER) including protease inhibitor cocktail. The homogenate was extracted in ice for 2 h and centrifuged at 17,000 rpm for 30 min. Supernatant was collected and quantified using Bradford assay. 20 μg of protein samples were electrophoresed in 10% SDS-PAGE gels for 2 h at 100 V. Proteins were transferred onto methanol-activated polyvinylidene difluoride (PVDF) membrane using trans-blot turbo transfer system (Bio-Rad Laboratories, Inc.). The membranes were blocked with 3% BSA and washed with TBS-T buffer for 3 times. The membranes were blotted with primary antibodies (1:1,000 dilution) for overnight at 4˚C and HRP conjugated secondary antibodies (1:3,000 dilution) were incubated for 1 h at RT. The target protein bands were detected by chemi-imaging system, Davinch-Chemi™.

Measurement of serum toxicity. Blood samples were separated by centrifuging the blood at 17,000 rpm for 20 min to determine the serum toxicity by enzyme-linked immunsorbent assay (ELISA). Serum biochemical indicators including blood urea nitrogen (BUN), creatinine, aspartate transaminase (AST) and alanine transaminase (ALT) were analyzed by Mouse Blood Urea Nitrogen ELISA kit (cat. no. MBS2611085; Mybiosource),

Mouse Serum Creatinine ELISA kit (cat. no. MBS3807501; Mybiosource). Mouse Aspartate Aminotransferase ELISA kit (cat. no. MBS450720; Mybiosource) and Mouse Alanine Aminotransferase ELISA kit (cat. no. MBS264717; Mybiosource), respectively. Based on standard curve, serum BUN, creatinine, AST and ALT was calculated.

Statistical analysis. Significance between vehicle and LIPOSA was determined by paired Student's t-test. Serum BUN, creatinine, AST and ALT measurements and body weight differences were analyzed using one-way ANOVA and Tukey's multiple comparisons test. In all analyses, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of LIPOSA pharmacopuncture on the inguinal fat weight in obese mice. The left inguinal fat administered with LIPOSA was significantly reduced compared to the vehicle side (Fig. 1A). Radiography of fat displayed by red color showed that LIPOSA treatment remarkedly decreased the fat deposition in inguinal fat pad (Fig. 1B). LIPOSA-treated groups exhibited decreases of inguinal fat weight ratio. LIPOSA treatments with 13.35, 26.7 and 53.4 mg/ml concentrations reduced the inguinal fat pad weight by 14.5, 21.2 and 19.0% in HFD-induced obese mice, respectively (Fig. 1C). Based on the DXA scan to measure the exact weight of inguinal fat, the ratio of fat weight administered with LIPOSA were significantly lower than that with vehicle. Subcutaneous injection with 13.35, 26.7 and 53.4 mg/ml of LIPOSA significantly attenuated the inguinal fat pads weight by 26.2, 19.6, and 21.8%, respectively (Fig. 1D).

Effects of LIPOSA pharmacopuncture on histological changes of inguinal fat tissues in obese mice. As shown in H&E staining, fat diameter of in inguinal fat pad was markedly reduced by LIPOSA treatment compared to the vehicle side-fat pad (Fig. 2A). LIPOSA pharmacopuncture injection dose-dependently decreased the inguinal fat adipocyte size about 48.5, 50.9 and 61.4% (Fig. 2B and C).

Effects of LIPOSA pharmacopuncture on expressions of lipophagy-related factors in inguinal fat tissues in obese mice. The protein levels of LC3-II were significantly increased by about 2.9-, 2.8- and 4.1-fold in the LIPOSA 13.35, 26.7 and 53.4 mg/ml-treated fat tissues compared to self-control, right side. Compared with saline-injected right side, the expressions of ATG5 were markedly upregulated to 3.1-, 3.0- and 4.6-folds in all three doses of LIPOSA group. Also, the ATG7 expressions were significantly increased by about 7.5-, 8.9- and 10.3-folds in the 13.35, 26.7 and 53.4 mg/ml of LIPOSA-treated fat tissues (Fig. 3).

Effects of LIPOSA pharmacopuncture on the expressions of lipolytic enzymes in inguinal fat tissues in obese mice. The levels of phosphorylated ATGL were increased about 1.5-, 1.8- and 1.5-folds in the inguinal fat tissues with LIPOSA 13.35 mg/ml, LIPOSA 26.7 mg/ml and LIPOSA 53.4 mg/ml compared to vehicle side-fat tissues. In similar, compared with right side, the levels of phosphorylated HSL were elevated about 2.1-, 2.6- and 2.6-folds in the LIPOSA 13.35 mg/ml, LIPOSA 26.7 mg/ml and LIPOSA 53.4 mg/ml-treated inguinal fat tissues (Fig. 4).

Effects of LIPOSA pharmacopuncture on the expressions of ACC, PEPCK and PPAR-γ in inguinal fat tissues in obese mice. In Fig. 5, the expression of phosphorylated ACC was significantly downregulated 69.5, 70.0 and 66.1%, respectively, in 13.35, 26.7 and 53.4 mg/ml of LIPOSA-treated left inguinal fat pads. In addition, the levels of PEPCK were remarkedly attenuated by 27.4, 21.3 and 54.8%, respectively, in the LIPOSA 13.35, LIPOSA 26.7 and LIPOSA 53.4 groups. PPAR- γ expressions were lowly expressed by 38.7, 53.3 and 61.6% compared to right inguinal fat pads in dose-dependent manner.

Effects of LIPOSA pharmacopuncture on serum toxicity in obese mice. We evaluated levels of injury marker of liver and kidney to assess potential toxic effect of LIPOSA (Table I). The levels of BUN were 25.80±2.17, 30.60±4.67 and 29.00±2.92 by LIPOSA injection. Creatinine levels in serum were 0.27±0.05, 0.25±0.04 and 0.29±0.03 by injection of LIPOSA 13.35, 26.7 and 53.4 mg/ml, respectively. The serum levels of AST were 187.50±40.83, 230.75±20.56, 173.25±44.30 and ALT were 33.00±7.66, 33.00±4.97, 29.00±5.23 in each LIPOSA treated groups. There was no significant difference of serum BUN, creatinine, AST and ALT levels in all three LIPOSA-treated groups. We conducted the additional evaluation of serum BUN, creatinine, AST (GOT) and ALT (GPT) in normal mice. Also, the levels of blood urea nitrogen (BUN), creatinine, AST and ALT were not changed by LIPOSA injection within normal range. We suggested that no toxicities in the serum levels of BUN, creatinine, AST and ALT by LIPOSA 13.35, 26.7 and 53.4 mg/ml injection.

Discussion

Lipolytic stimulation of fat cells by lipolytic injection or liposuction has been known to reduce localized body fat, that is intended to slim down specific body parts (13). Adipose cells, known as adipocytes, are the cells of adipose tissues that synthesize, store and release fat into the blood (14). In the condition of obesity by excessive fat intake, the size of adipocytes is about 10 times bigger than the original size (15). Enlargement of adipocytes in specific regions forms 'love handles' known as fat deposits (16). In this study, the potential of LIPOSA pharmacopuncture as a localized lipolytic material was investigated by comparing sample-injected left side and saline-injected right side for self-control. Subcutaneous injection with LIPOSA pharmacopuncture decreased the accumulation of inguinal fat tissues. As shown in the x-ray images, the red-indicated fat deposition was remarkedly reduced in the LIPOSA-treated site compared to the saline-treated site. In addition, the diameter of the adipocytes in the inguinal fat tissues was significantly decreased by the LIPOSA injection at all concentrations. Those results suggested that LIPOSA pharmacopuncture has a role as a lipolytic material in localized fat depositions.

'Lipolysis signaling' is defined as the hydrolysis of triacylglycerols stored in lipid droplets in the fat. There are

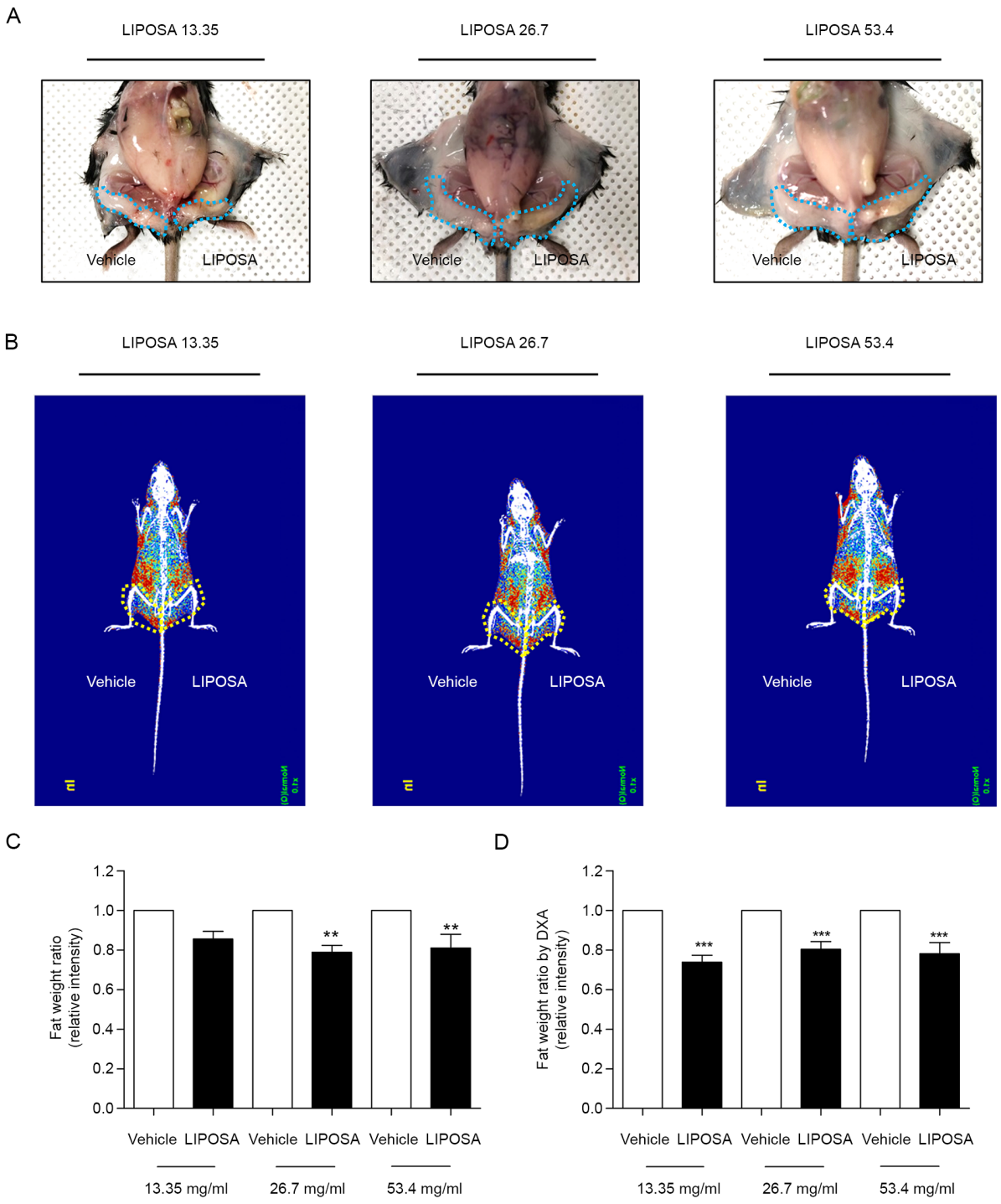

Figure 1. LIPOSA pharmacopuncture reduces the inguinal fat ratio. (A) Representative morphological images of inguinal fat tissues. Blue dots from knee to tail indicate the designated regions of inguinal fat. (B) Representative mapping images of body composition of mice constructed using DXA software. Yellow dots from knee to tail indicate the designated regions of inguinal fat. Red, fat tissue; blue, lean tissue; white, bone tissue. (C) Relative weight ratio of inguinal fat collected from knee to tail based on line of ventral spine at the end of the experiment. Relative ratio of fat weight after LIPOSA treatment was calculated when the saline-treated fat weight was normalized to 1. (D) Relative ratio of fat weight measured by targeting regions of interest (the designated regions of inguinal fat pad) indicated by yellow dots in DXA software. The saline-treated fat weight was normalized to 1 and the inguinal fat pad weight after LIPOSA treatment was calculated as the relative intensity. Quantitative data are presented as the mean ± standard error of the mean. **P<0.01 and ***P<0.001 compared with each saline-treated side (vehicle).

several potential mechanisms related to lipolytic stimulation, which induces fat cell destruction (17). The neutral lipids in a lipid droplet breakdown into free fatty acids by lysosomal degradation autophagy, called lipophagy (18). The events of the lipophagic process are coordinated by components of the autophagic machinery, ATGs, with the generation of LC3-II. ATG7 has been reported to regulate ATG5, leading to the conjugation of LC3 to a lipid. LC3-II activation in the lipid droplet forms the autophagosomes following the degradation of lipid stores (19). LC3 is reported to regulate ATGL-mediated lipid mobilization, although the contribution of lipases and lipophagy to lipolytic process has not

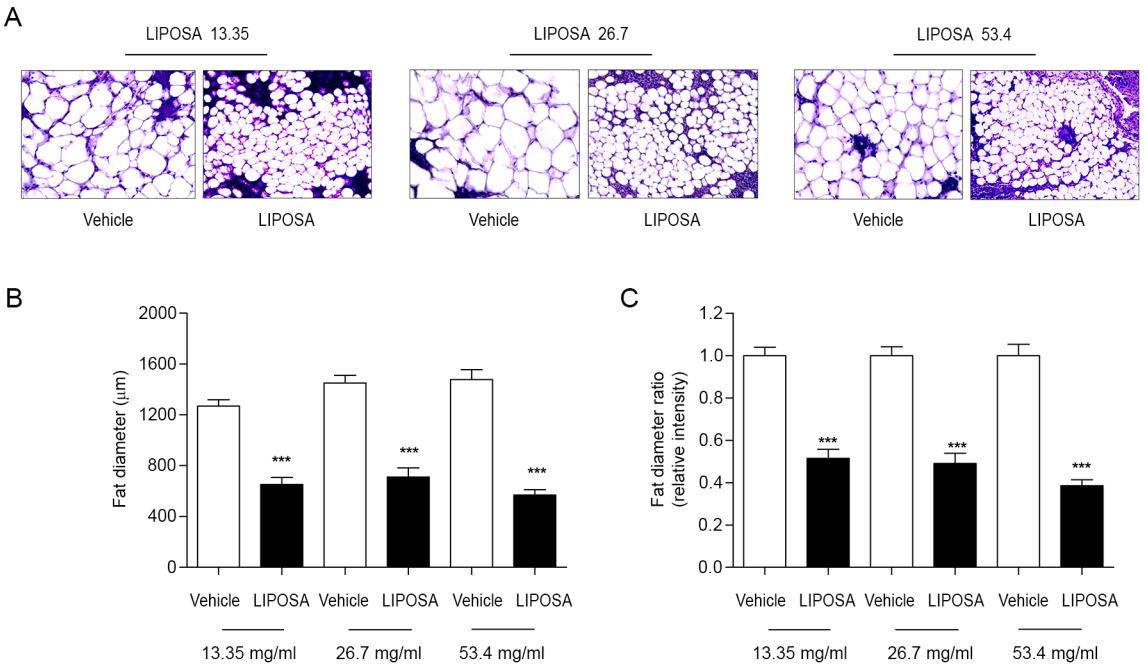

Figure 2. LIPOSA pharmacopuncture decreases the fat diameter of inguinal fat tissues. (A) Representative histological images of inguinal fat tissues. Sections were stained by H&E and viewed under a microscope (magnification, x400). (B) Value of fat diameter in inguinal fat quantified using ImageJ software. (C) Relative value of fat diameter in inguinal fat measured compared with vehicle. The fat diameter in the saline-treated side was normalized to 1 and that in the LIPOSA-treated side was calculated as the relative intensity. Quantitative data are shown as the mean ± standard error of the mean. ***P<0.001 compared with each saline-treated side (vehicle). H&E, hematoxylin and eosin.

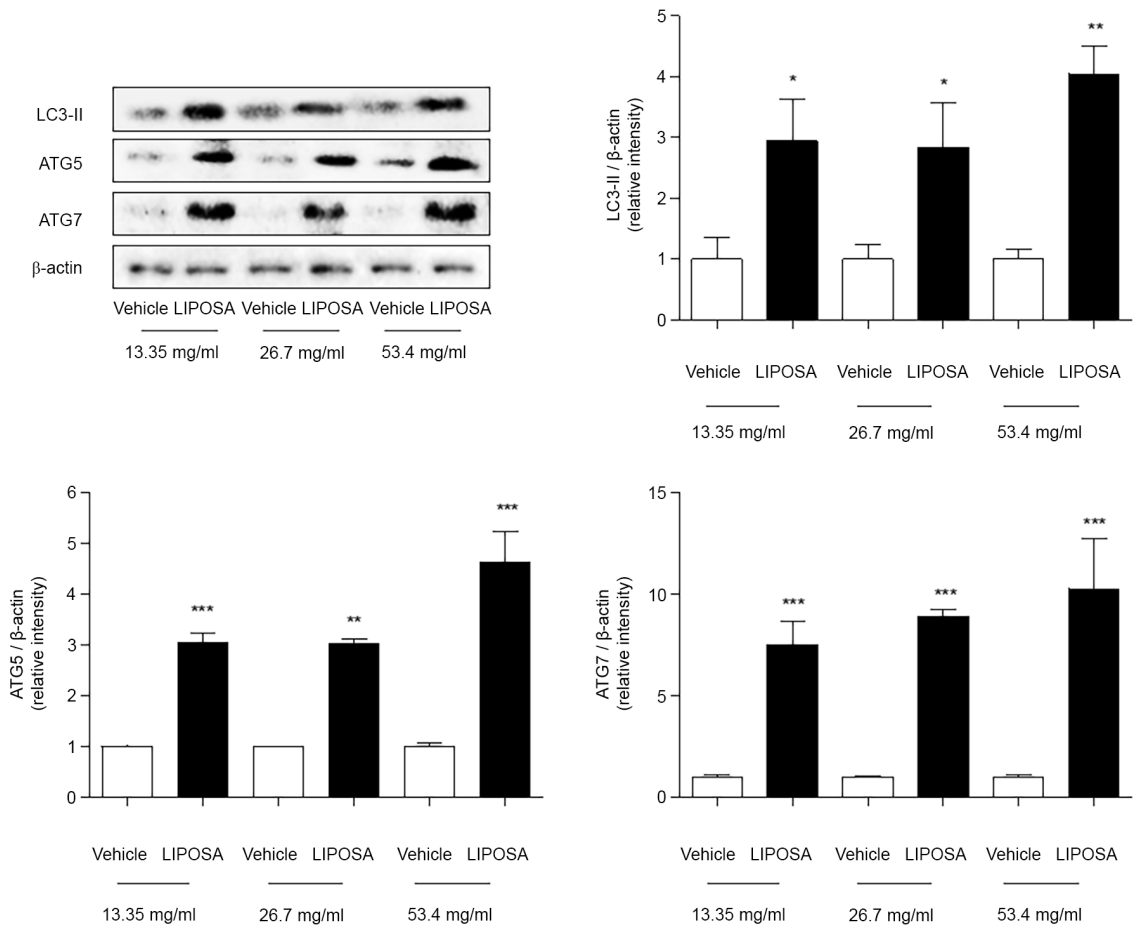

Figure 3. LIPOSA pharmacopuncture increases the expression of lipophagy-related factors in inguinal fat tissues in obese mice. Expression levels of LC3-II, ATG5 and ATG7 were analyzed by western blotting. Quantitative data are shown as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001 compared with each saline-treated side (vehicle). ATG, autophagy-related gene.

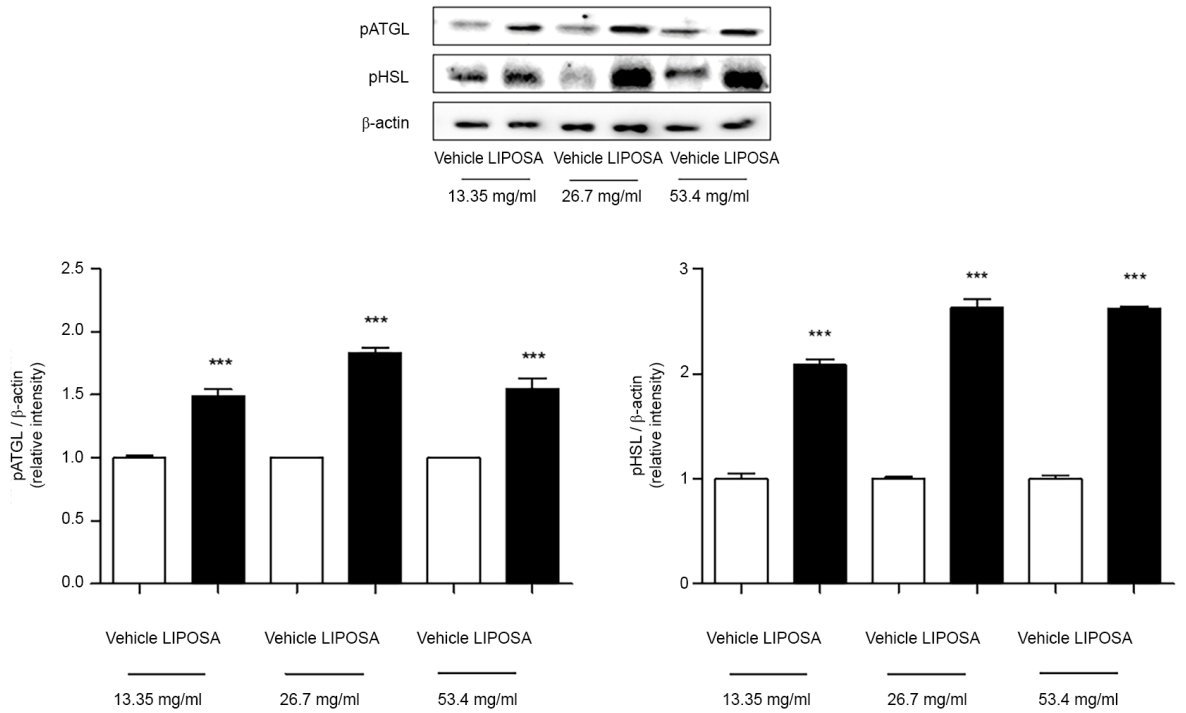

Figure 4. LIPOSA pharmacopuncture elevates the expression levels of lipolytic enzymes in inguinal fat tissues in obese mice. Levels of pATGL/ATGL and pHSL/HSL were analyzed by western blotting. Quantitative data are shown as the mean ± standard error of the mean. ***P<0.001 compared with each saline-treated side (vehicle). ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; p, phosphorylated.

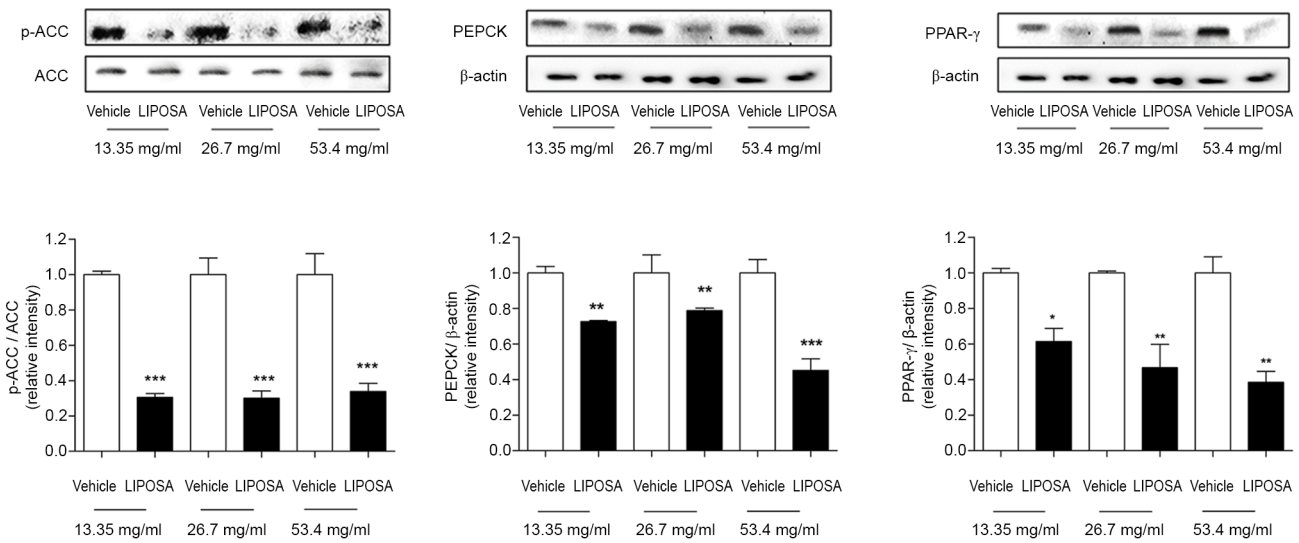

Figure 5. LIPOSA pharmacopuncture decreases the expression levels of ACC, PEPCK and PPAR-γ in inguinal fat tissues in obese mice. Expression levels of p-ACC/ACC, PEPCK and PPAR-γ were analyzed by western blotting. Quantitative data are shown as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001 compared with each saline-treated side (vehicle). ACC, acetyl-CoA carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PPAR-γ, peroxisome proliferator-activated receptors-γ; p, phosphorylated.

been clearly known (20). Lipolysis by neutral lipases such as ATGL and HSL releases fatty acids and glycerol on triglycerides (21). The triglycerides can be disrupted by lipolytic drugs without an issue of body energy deposition (22). In this study, subcutaneous injection with LIPOSA markedly increased the expressions of lipophagic factors including ATG7, ATG5 and LC3-II, and lipases including ATGL and HSL. Taken together, LIPOSA pharmacopuncture regulated the lipolytic process, especially lipophagy and lipase activation. Based on the results from the fat weight and adipocyte diameter, we expected that LIPOSA pharmacopuncture degraded fat cells containing triglycerides and reduced the enlargement of lipid droplets through lipolysis and lipophagy, which are therapeutic strategies to inhibit localized body fat (Fig. 6).

Apart from lipolysis, molecules were investigated that were involved in lipid accumulation. In addition to the stimulation of lipolysis, strategies to regulate the lipid

Table I. Changes in concentrations of serum BUN, creatinine, AST and ALT of mice after 2 weeks of LIPOSA pharmacopuncture injection.

Group	BUN	Creatinine	AST (GOT)	ALT (GPT)
Normal mice	36.67±0.58	0.26±0.04	143±35.16	41.33±10.26
13.35 mg/ml LIPOSA	25.80±2.17	0.27±0.05	187.50±40.83	33.00±7.66
26.7 mg/ml LIPOSA	30.60±4.67	0.25±0.04	230.75±20.56	33.00±4.97
53.4 mg/ml LIPOSA	29.00±2.92	0.29±0.03	173.25±44.30	29.00±5.23

Data are presented as the mean ± standard error of the mean. BUN, blood urea nitrogen; ALT, alanine aminotransferase; GOT, glutamate oxaloacetate transaminase; ALT, alanine aminotransferase; GPT, glutamic pyruvate transaminase.

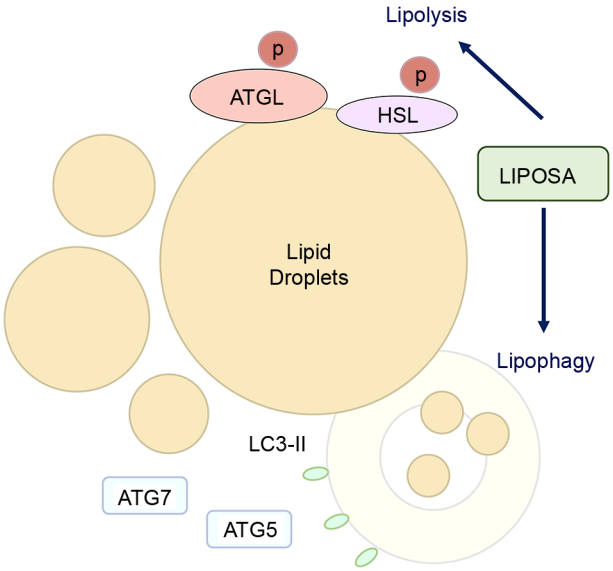

Figure 6. Schematic diagram of the effect of LIPOSA pharmacopuncture on localized fat. LIPOSA injection effectively inhibits the accumulation of localized adiposity by regulating lipophagy and lipolysis-related mechanisms.

metabolism by inhibiting the differentiation of adipocytes (adipogenesis), release of glucose (gluconeogenesis) in adipose tissue or synthesis of fatty acids (lipogenesis) can be targets for obesity and obesity-related diseases (23). The phosphorylation of ACC, a lipogenesis enzyme, has been known to induce the synthesis of fatty acids (24). PPAR-γ is highly expressed in white adipose tissues and associated with lipid metabolism (25). Activation of PPAR-γ mediates the differentiation of preadipocytes into mature adipocytes (25). In terms of PEPCK, it acts as a regulatory enzyme of gluconeogenesis in adipose tissues (26). An increase in PEPCK activity leads to adipocyte hypertrophy by free fatty acid re-esterification, resulting in fat accumulation (26). LIPOSA pharmacopuncture was found to decrease the expressions of ACC, PPAR-γ and PEPCK. Along with the lipolytic effects, LIPOSA pharmacopuncture might inhibit fat accumulation by regulating lipid metabolism.

Taken together, LIPOSA pharmacopunture breaks down localized areas of fat by its lipolytic property. Triglyceride accumulation in the inguinal fat pad was inhibited by LIPOSA subcutaneous injection with its promotive effects on lipophagy and lipase activation. In addition, lipid metabolism related to fat accumulation including adipogenesis, gluconeogenesis and lipogenesis was regulated by the LIPOSA treatment. LIPOSA pharmacopuncture might reduce localized body fat as a lipolytic injection.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL, MHK and WMY designed the study and drafted the manuscript. SCJ, LYC and YKN performed the experiments and analyzed the data. MHK and WMY assessed the authenticity of all the raw data. YWM revised and edited the manuscript, supervised the project and obtained the research grants for the current study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Committee on Care and Use of Laboratory Animals of the Kyung Hee University [approval no. KHUASP(SE)-18-070; Seoul, South Korea].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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