Effects of Kampo Formulas on Differentiation and Proinflammatory Cytokines Expression of 3T3-L1 Cells

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Effects of Kampo formulas which have been long used clinically for obese patients remain unclear at the cellular level. The objective of this study is to investigate the effects of 8 commonly-used Kampos on the differentiation and proinflammatory cytokines expression of 3T3-L1 cells. 3T3-L1 preadipocytes were induced differentiation being treated with test 8 Kampos through the induction period. Oil red O staining and mRNAs expressions of peroxisome proliferator-active receptor-γ （PPARγ） and adiponectin were examined to evaluate effects of the Kampos on the differentiation. Tumor necrosis factor-alpha （TNF-α）, interleukin-6, and monocyte chemoattractant protein-1 （MCP-1） mRNAs were examined to investigate the anti-inflammatory effects of the Kampos. PPARγ and/or adiponectin, as well as MCP-1 and/or TNF-α mRNAs were significantly decreased by Boiogito and a few other Kampos when compared with the control. These results at the cellular level are compatible with the clinical beneficial effects of these Kampos.

Key words: 3T3-L1, differentiation, inflammation, PPARγ, MCP-1

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INTRODUCTION

The Kampo formulas, Japanese traditional medicines （Kampos）, as well as the traditional Chinese medicines, which are less toxic and free from adverse effects compared to general synthetic drugs, have contributed to the prevention and treatment of various diseases, especially for the lifestyle-related chronic diseases such as obesity and metabolic syndrome [1, 2]. Obesity is currently and will continue to be significant public health concern worldwide which leads to a number of pathologies including type 2 diabetes mellitus （T2DM）, cardiovascular diseases （CVD）, and renal diseases, and is considered a risk factor for some cancers [3, 4].

Among the commonly-used Kampos, we selected in the present study eight that have been used as clinically effective ones for obesity, T2DM, and the associated complications: Boiogito （BOT）, Bofutsushosan （BTS）, Daisaikoto （DST）, Hachimijiogan （HJG）, Goshajinkigan （GJG）, Choijokito （CJT）, Byakkokaninjinto （BNT）, and Seishinrenshiin （SRI）.

BOT, BTS, and DST, are well known anti-obesity agents, suppress weight gain, and show a preventive effect on abnormal lipid metabolism, hypertension, and nonalcoholic fatty liver disease （NAFLD） [5-7]. Previous studies reported the anti-obesity effects of these Kampos in Tsumura Suzuki Obese Diabetes （TSOD） mice, a model which is known to develop disease state similar to human metabolic disorders. Administration of BOT, BTS, or DST in TSOD mice, the body weight gain and visceral/subcutaneous fat accumulation were significantly suppressed, and the abnormal glucose tolerance and elevation of blood pressure were also significantly suppressed [5, 8, 9]. In addition, BTS was shown in a clinical trial to reduce body weight and visceral fat, and

improve impaired glucose tolerance [10].

HJG and GJG are used clinically to improve diabetic nephropathy and neuropathy, and recent animal experiments suggested that these two Kampos not only decreased serum glucose level but also regulated lipid metabolism [11-13]. It is suggested that GJG administration can improve the glucose utilization and insulin resistance in STZ-induced diabetic rats [13].

CJT, BNT, and SRI are clinically used for the treatment of diabetes and its complications in Japan and China. SRI and BNT are especially for the symptoms of dry mouth, polydipsia, and fatigue in diabetic patients. However, there is little study of these Kampos on obesity or diabetes *per se*.

The above herbal preparations are widely used as therapeutic agents for obesity, T2DM and its complications in Japan and in China. Most of the findings of these Kampos, however, were obtained from animal studies, while *in vitro* researches have been limited and their effects at the cellular level remain unclear. Adipose tissue growth, hypertrophy and/or hyperplasia, plays an important role in the development of obesity [14, 15]. A state of low-grade chronic inflammation in the adipose tissue in obesity promotes the development of insulin resistance both locally and systemically which contributes to the development of T2DM and CVD [16, 17]. However, there is only limited number of *in vitro* studies on the effects of these Kampos on the adipocyte differentiation and/or expression of proinflammatory cytokines/factors.

In this study, we investigated the effects of these 8 Kampos on the differentiation of preadipocytes into adipocytes and on the expression of proinflammatory cytokines using 3T3-L1 cell which is known as a well-established preadipose cell line for the study of adipogenesis and obesity-related characteristics including inflammation [18].

MATERIALS AND METHODS

Kampo medicines and test sample preparation

The extract powders of the following 8 Kampo medicines were provided by Tsumura & Co. (Japan): BOT, BTS, DST, HJG, GJG, CJT, BNT, and SRI (Table 1). Each Kampo formulation was dissolved

at 500 μg/ml in phosphate-buffered saline (PBS, NISSUI, Japan) as stock solution with sonication at 37°C for 60 min.

Cell culture and induction of differentiation

3T3-L1 preadipocytes, from the American Type Culture Collection (Rockville, MD, USA), were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Wako, Japan), supplemented with 10% fetal bovine serum (FBS, Corning, USA) and 1% penicillin-streptomycin (Wako, Japan) in 5% CO_2 at 37°C in humid conditions. The cells were grown in 6-well plates at 6.0×10^4/well to full confluence for 48 h and cultured in differentiation medium containing 0.25 μM dexamethasone (Nacalai Tesque, Japan), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, USA), 10 μg/ml insulin (Sigma, USA), and 100 μg/ml test Kampos for 48 h, and replaced the medium with 10 μg/ml insulin and 100 μg/ml test Kampos for another 48 h. Then the cells were cultured in the culture medium without insulin, and the test Kampos at 100 μg/ml were added each time when the medium was exchanged every 48 h in total 14 days for differentiation induction before further analyses. Using the same 3T3L1 cell line, Ikarashi *et al.* examined 50 Kampos' effects on intracellular lipid droplet at a concentration of 100 μg/mL without any cytotoxicity [19]. Yamakawa *et al.* reported the effect of BOT and BTS on adipogenesis in cultured rat white adipocytes in 1-100 μg/mL without cytotoxicity [20]. Whereas the effect of Kampos have been suggested in the clinical and animal studies, papers using cell lines reported remain few. We therefore aimed in this first study using the reported concentration of 100 μg/mL to show whether these clinically effective 8 Kampos also have effects *in vitro* on adipocytes. Cells induced to differentiation by adding PBS instead of test Kampos were regarded as the control group.

Oil red O staining

As an index of 3T3-L1 cell differentiation, lipid accumulation was measured using oil red O staining because lipid droplets produced in the adipocyte cytoplasm are selectively stained with oil red O. Oil red O stock solution was prepared with 150 mg oil red O powder in 50 ml 100% isopropanol (Wako,

Table 1. The ingredients of each Kampo formulation

Kampo formulation	Crude drugs
Boiogito（BOT）	Sinomenium stem and Rhizome; Astragalus Root; Atractylodes Lancea Rhizome; Glycyrrhiza; Ginger; Jujube
Bofutsushosan（BTS）	Scutellaria Root; Glycyrrhiza; Platycodon Root; Gypsum; Atractylodes Rhizome; Rhubarb; Schizonepeta Spike; Gardenia Fruit; Peony Root; Cnidium Rhizome; Japanese Angelica Root; Mentha Herb; Saposhnikovia root and Rhizome; Ephedra Herb; Forsythia Fruit; Ginger; Aluminum Silicate Hydrate with Silicon Dioxide; Anhydrous Mirabilite
Daisaikoto（DST）	Bupleurum Root; Pinellia Tuber; Scutellaria Root; Peony root; Jujube; Immature Orange; Rhubarb; Ginger
Hachimijiogan（HJG）	Rehmannia Root; Cornus Fruit; Dioscorea Rhizome; Alisma Rhizome; Poria Sclerotium; Moutan Bark; Cinnamon Bark; Processed Aconite Root
Goshajinkigan（GJG）	Rehmannia Root; Achyranthes Root; Cornus Fruit; Dioscorea Rhizome; Plantago Seed; Alisma Rhizome; Poria Sclerotium; Moutan Bark; Cinnamon Bark; Processed Aconite Root
Choijokito（CJT）	Rhubarb; Anhydrous Mirabilite; Glycyrrhiza
Byakkokaninjinto（BNT）	Gypsum; Anemarrhena Rhizome; Glycyrrhiza; Brown Rice; Ginseng
Seishinrenshiin（SRI）	Ophiopogon Root; Poria Sclerotium; Nelumbo Seed; Scutellaria Root; Plantago Seed; Ginseng; Astragalus Root; Lycium Bark; Glycyrrhiza

Table 2. Primer pairs used in real-time PCR

Gene		Primer
GAPDH	Sense	5'AACTTTGGCATTGTGGAAGG3'
	Antisense	5'ACACATTGGGGGTAGGAACA3'
PPARγ	Sense	5'GTCTGTGGGGATAAAGCATC3'
	Antisense	5'CTGATGGCATTGTGAGACAT3'
Adiponectin	Sense	5'ATGGCAGAGATGGCACTCCT3'
	Antisense	5'CCTTCAGCTCCTGTCATTCCA3'
MCP-1	Sense	5'CCACTCACCTGCTGCTACTCAT3'
	Antisense	5'TGGTGATCCTCTTGTAGCTCTCC3'
TNF-α	Sense	5'AATGAGGCTGGATAAGAT3'
	Antisense	5'AGAGGTTCAGTGATGTAG3'
IL-6	Sense	5'ACAACCACGGCCTTCCCTACTT3'
	Antisense	5'CACGATTTCCCAGAGAACATGTG3'

Japan） overnight shake at 60℃. Following medium removal, 3T3-L1 cells were washed with PBS twice and fixed with 10% formalin solution for 10 min at room temperature. Subsequently, washed with PBS twice and treated with 60% isopropanol for 1 min, the cells were stained with filtered oil red O stock solution diluted to 60% in distilled water （DW） for 30 min, then treated with 60% isopropanol for 1 min again. Finally, after PBS washes twice, cells were observed under a microscope.

Extraction and measurement of total RNA

Total RNA was isolated from cultured 3T3-L1 adipocytes using Trizol reagent （Ambion, USA）, according to the manufacturer's recommended protocol. Samples were then extracted with chloroform （Wako, Japan）, then isopropanol was added to supernatants （1:1, v/v）, after centrifugation at 12,000 x g for 10 min at 4℃, the samples were washed with 70% ethanol and the total RNA were solubilized in Diethylpyrocarbonate （DEPC） RNase-free water. RNA concentration was measured using a ND-1000 spectrophotometer （Nano Drop, Japan）.

Real-time PCR quantification of gene expression

SYBR green chemistry was used to perform quantitative determination of the mRNAs for peroxisome proliferator-activated receptor gamma （PPARγ）, adiponectin, tumor necrosis factor-alpha （TNF-α）, monocyte chemoattractant protein-1 （MCP-1）, interleukin-6 （IL-6）, and a housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase （GAPDH）, according to an optimized protocol [21, 22]. Five μg total RNA was employed for the synthesis of single-stranded cDNA using an oligo-dT primer and a SuperScript III cDNA synthesis kit （Invitrogen, USA）. The sense and antisense primers were designed using the Primer Express version 2.0.0 （Applied Biosystems） based on published cDNA sequences （Table 2）. Real-time PCR was performed using 1 μl cDNA in a 25 μl reaction volume with ABI PRISM 7000 （Applied Biosystems）. The double-stranded DNA-specific SYBR Green was incorporated into the PCR buffer provided in the QuantiTect SYBR PCR kit （TOYOBO, Japan） to allow for the quantitative detection of the PCR product. The temperature profile of the PCR reaction was 60℃ for 2 min, followed by 95℃ for 15 min, then 40 cycles of denaturation at 94℃ for 15 s, and annealing and extension at 60℃ for 1 min. GAPDH was used for normalization.

Statistical analysis

Date analyses were performed using Statview 5.0 （SAS Institute Inc., USA） software for Windows statistical program. All data were presented as mean ± standard error （SE）. The significances between group mean values were assessed using one-way ANOVA followed by Fisher's protected least significant difference. For all statistical analysis, a value of $P < 0.05$ was considered a statistically significant difference.

RESULTS

Effects of Kampos on 3T3-L1 preadipocytes differentiation into mature adipocytes by oil red O staining

We analyzed whether Kampo preparations could activate or inhibit 3T3-L1 preadipocytes differentiation into adipocytes and lipid accumulation by oil red O staining method. The preadipocytes differentiation into mature adipocytes is directly associated with oil red O stained cells due to accumulation of cytoplasmic lipid droplets. Microscopic views of adipocytes showed no significant difference in the lipid droplets accumulation, with the concentration at 100 μg/ml of each Kampo when compared to the control group （Fig. 1）. Although we performed image analysis of quantification, no significant difference was observed between the Kampo and control groups （data not shown）.

Effects of Kampos on mRNA expression of PPARγ and adiponectin in 3T3-L1 adipocytes

To explore whether Kampos suppress adipogenesis in 3T3-L1 cells at the molecular level, albeit no significant difference by oil red O staining, we further investigated the mRNA expression of PPARγ and adiponectin after 3T3-L1 cells differentiation for 14 days. As shown in Fig. 2, the mRNA expression levels of PPARγ and adiponectin were significantly decreased by BOT at 100 μg/ml （$P < 0.005$, $P < 0.005$）. In addition, SRI （100 μg/
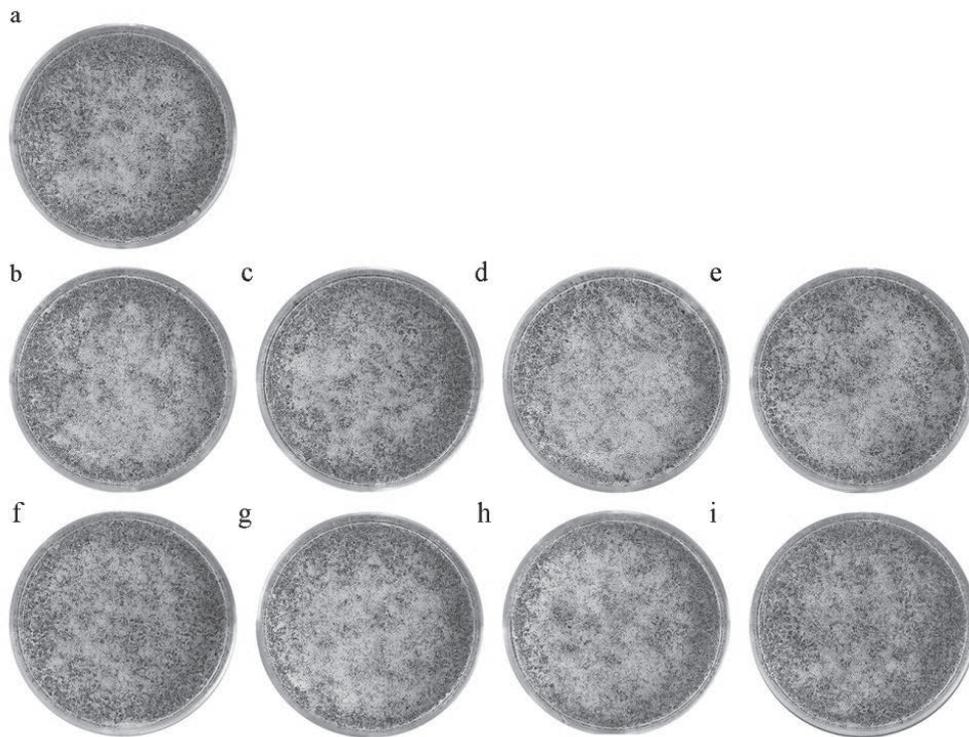
Fig. 1. The oil red O staining in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were subjected to a differentiation protocol in the presence of PBS control (a), 100 μg/ml test Kampos: Goshajinkigan (GJG) (b), Daisaikoto (DST) (c), Boiogito (BOT) (d), Bofutsushosan (BTS) (e), Hachimijiogan (HJG) (f), Seishinrenshiin (SRI) (g), Byakkokaninjinto (BNT) (h), Choijokito (CJT) (i).
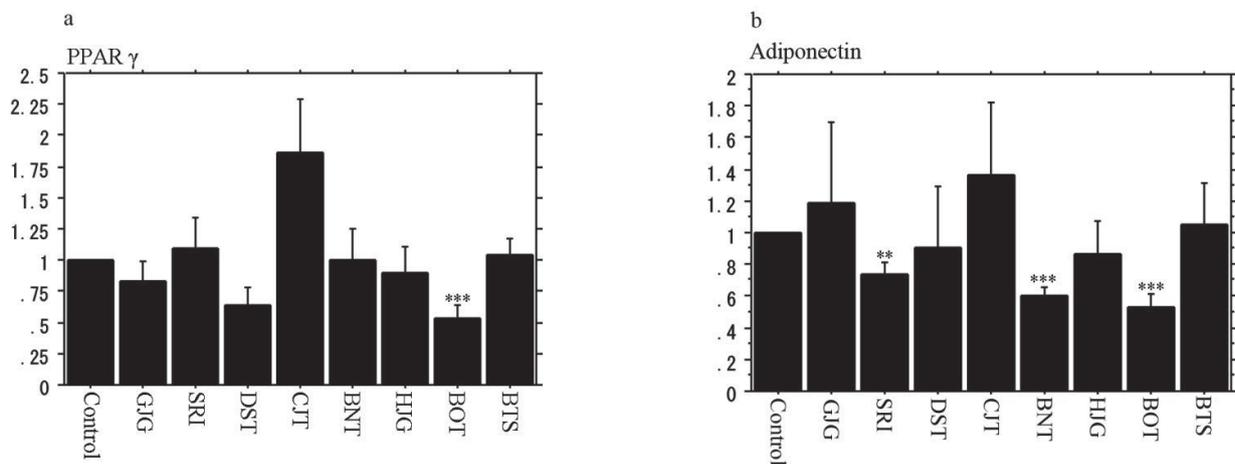

Fig. 2. Effects of Kampo medicines on mRNA expression of PPARγ and adiponectin in 3T3-L1 adipocytes. The 3T3-L1 preadipocytes were treated with Kampo samples (100 μg/ml) or PBS during induced differentiation for 14 days after they had become confluent. The relative mRNA expressions of PPARγ (a) and adiponectin (b) of the 3T3-L1 cells were determined against the control. The data show the mean ± SE (n = 4). ** $P < 0.05$, *** $P < 0.005$.

ml) and BNT (100 μg/ml) also significantly suppressed the mRNA expression levels of adiponectin ($P < 0.05$, $P < 0.005$), but no effect on the PPARγ

mRNA expression. The PPARγ mRNA level of the DST group was tended to be lower ($P = 0.0525$) when compared with the control group, whereas the

other Kampo groups showed no significant effects on PPARγ and adiponectin mRNA expressions.

Effects of Kampos on mRNA expression of proinflammatory factors in 3T3-L1 adipocytes

We further investigated the mRNA expression of TNF-α, MCP-1, and IL-6 which are secreted from adipocytes to explore the effects of anti-inflammation of Kampos in 3T3-L1 adipocytes [23]. As shown in Fig. 3, BOT, at 100 μg/ml, significantly decreased the mRNA levels of MCP-1 and TNF-α ($P < 0.05$, $P < 0.005$). DST, at 100 μg/ml, significantly decreased the mRNA level of TNF-α ($P < 0.005$), but no change in the other Kampo groups. There were no significant effects of the Kampos examined in the current study on IL-6 mRNA expression.

DISCUSSION

Obesity has become a major medical problem worldwide, frequently associated with the development of a series of metabolic abnormalities. During the development of obesity, adipocytes increase in size and number and their metabolic activity is dramatically altered [14]. Lipid accumulation-induced enlargement of the adipocyte size and an increase in the number of adipocytes are accompanied with abnormal adipocyte differentiation [24]. Accordingly, the inhibition of the differentiation of adipocytes may prevent the initiation and progression of obesity.

In the present study, we explored the direct inhibitory effects at the cellular level of 8 Kampos which are commonly-used for obesity on the 3T3-L1 preadipocyte differentiation into mature adipocytes first by oil red O staining. Lipid droplets produced in the adipocyte cytoplasm are selectively stained with oil red O. However, the result showed no significant difference between the test Kampo groups and control group. To explore the effect of the Kampo preparations on the differentiation at the molecular level, the mRNA levels of PPARγ and adiponectin were investigated using real-time PCR. The differentiation of 3T3-L1 preadipocytes into adipocytes is regarded to be induced by transcriptional activators. PPARγ is seen as a master regulator of adipogenesis and lipogenesis [25]. Adiponectin is one of multiple adipocytokines secreted by adipocytes and has been shown to promote preadipocyte differentiation and augment lipid accumulation in mature adipocytes [26]. In the present study, BOT significantly suppressed the PPARγ and adiponectin mRNA expression in 3T3-L1 adipocytes. BOT has a protective effect on the progression of obesity-related diseases such as T2DM and NAFLD [2]. Recent studies have demonstrated that BOT and its major components, ogi/astragalus and ginger, significantly alleviated the lipid metabolism and decreased the body weight gain and fat accumulation in experimental rats [5, 7, 27], and suppressed differentiation and lipid droplet accumulation in 3T3 adipocytes [28-30]. Yamakawa *et al.* [20] demonstrated
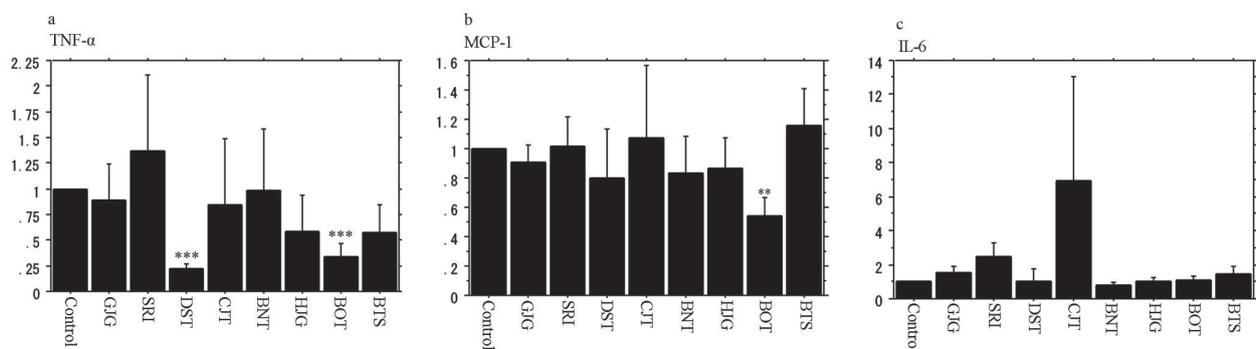

Fig. 3. Effects of Kampo medicines on mRNA expression of proinflammatory factors in 3T3-L1 adipocytes. The 3T3-L1 preadipocytes were treated with Kampo samples (100 μg/ml) or PBS during induced differentiation for 14 days after they had become confluent. The relative mRNA expressions of TNF-α (a), MCP-1 (b), and IL-6 (c) of the 3T3-L1 cells were determined against the control. The data show the mean ± SE (n = 4). ** $P < 0.05$, *** $P < 0.005$.

that intracellular lipid accumulation in cultured adipocytes significantly reduced over time by BOT in concentration-dependent manners through regulatory genes corresponding to obesity. Collectively, our observations proved that the anti-obesity effect of BOT is consistent with the differentiation-inhibitory effect on 3T3-L1 preadipocytes observed in the present study, and suggested that the mechanism involves the inhibition of PPARγ and adiponectin mRNA expression. The cause of the discrepancy between the findings by oil red O staining and those by PCR is unclear, but could be related with the relatively long differentiation induction period in the present study, which might saturate lipid droplet accumulation in the mature adipocytes for detection by oil red O staining. Since there are many factors, in addition to PPARγ and adiponectin, such as Dlk1, WNT family, BMPs, FGFs, and C/EBPs [31] that influence signaling pathways controlling complex differentiation process from preadipocytes to mature adipocytes, decrease in PPARγ expression alone may not lead directly to the trait change, or decrease in adiponection expression may not be large enough to produce difference in oil red O staining.

Whilst it is likely that there are multiple molecular mechanisms linking obesity to its complications, inflammation is a common feature that has been implicated in the pathophysiology of many obesity-associated disorders [32]. A state of low-grade chronic inflammation in obesity promotes the development of insulin resistance which is contributing to the development of T2DM and CVD [33]. The endocrine homeostasis is dramatically altered by the enlargement of adipose tissue mass in obese individuals. The adipocyte-derived proinflammatory cytokines including MCP-1, TNF-α, and IL-6 promote a proinflammatory environment and contribute to metabolic dysfunction and insulin resistance [16, 17]. Recent data have revealed that the plasma concentration of proinflammatory mediators such as TNF-α and IL-6 are increased in the insulin resistant states of obesity and T2DM both in experimental animals and in humans [34]. Weight loss is accompanied with the decline of plasma levels of TNF-α and MCP-1 [35, 36]. Among inflammatory molecules up-regulated in adipose tissue of obese animals and humans, MCP-1 has been viewed as one of the likely candidate adipokines initiating macrophage infiltration of the adipose tissue and inducing systemic insulin resistance. It was demonstrated that MCP-1 directly attenuated insulin signaling in myotube cells and insulin-stimulated glucose uptake in the skeletal muscle [37] and liver [38], suggesting that higher circulating MCP-1 may have a direct negative impact on glucose uptake in insulin target tissues. While the most effective means for ameliorating metabolic abnormalities associated with obesity is weight loss, treatment with anti-inflammatory medications may also be beneficial [39-41].

To examine whether Kampo preparations possess the direct inhibitory effect on inflammatory function of adipocytes, we investigated the effect of Kampos on the mRNA expression of proinflammatory cytokines, TNF-α, MCP-1, and IL-6 in 3T3-L1 adipocytes using real-time PCR. The present results demonstrated that BOT significantly inhibited the TNF-α and MCP-1 mRNA expression, suggesting that BOT has a potential anti-inflammatory effect on adipocytes. BOT with/without ginger has been shown to decrease mRNA expression of MCP-1 in the liver and adipose tissue in experimental rats [7, 27], coinciding with the effect of BOT on MCP-1 mRNA expression observed in the present study. These accumulating evidences support the anti-inflammatory activities of BOT in obesity as well as arthritis [2]. In the present study, DST tended to decrease PPARγ mRNA, and significantly decreased TNF-α mRNA expression. These findings are consistent with the reported effects of DST on suppressing body weight gain and visceral fat accumulation of experiment animals [9, 42]. Adiponectin mRNA was significantly decreased in the SRI and BNT groups in the present study. While the related studies are very few, SRI was reported to improve insulin resistance in streptozotocin-induced diabetic rats [43].

In summary, data of the current study demonstrate that BOT is capable of inhibiting the 3T3-L1 preadipocyte differentiation and expression of proinflammatory cytokines, decreasing the mRNA levels of PPARγ and adiponectin, as well as those of TNF-α and MCP-1 in the adipocytes. These results suggest the beneficial actions of BOT on obesity work at the cellular level in preadipocytes/adipocytes.

The other Kampo preparations in the present study showed only limited or no significant effect preadipocytes/adipocytes, whereas they have significant anti-obesity and lipid-lowering effects on experimental animals and obese individuals with diabetes [1, 2]. Since obesity is a complex pathological state in which different parts of body at different levels may have different pathological roles, further studies are required to ascertain the detailed mechanisms underlying the anti-obesity effects of the Kampo medicines.

Conflict of Interest: All authors have no conflicts of interest.

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