

Reduced Leptin Secretion by Fucoidan-Added *Kochujang* and Anti-adipogenic Effect of Fucoidan in Mouse 3T3-L1 Adipocytes

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Abstract

In order to improve the antiobesity effect of *Kochujang*, 1% of sea tangle powder, alginic acid extract, and fucoidan extract were added to *Kochujang*. Sea tangle powder-added *Kochujang* decreased leptin secretion by only 12% compared to *Kochujang*, whereas alginic acid or fucoidan-added *Kochujang* significantly decreased leptin secretion by more than 60% in 3T3-L1 adipocytes. Fucoidan, one of the active components of sea tangle, decreased leptin secretion by 56%, 60%, and 60% compared to the control in the concentrations of 1 μ M, 2.5 μ M, and 5 μ M, respectively. To see the effect of fucoidan on TG formation during adipocyte differentiation, 3T3-L1 cells were treated with 1 μ M and 5 μ M concentrations of fucoidan during adipocyte differentiation (from "day 0" to "day 6"). Oil red O staining showed fucoidan decreased the amount of TG droplets and 5 μ M fucoidan potently inhibited TG formation. To see the effect of fucoidan on lipolysis, differentiated 3T3-L1 adipocytes were treated with fucoidan. The secretion of glycerol, which is used to measure lipolytic activity, was increased by 21%, 37%, and 53% compared to the control in the concentrations of 1 μ M, 2.5 μ M, and 5 μ M, respectively. Oil red O staining showed fucoidan decreased TG amount at 1 μ M and 5 μ M concentrations. These results suggest that fucoidan decreases leptin secretion and TG accumulation by inhibition of adipocyte differentiation and induction of lipolysis. Since fucoidan is reported to have various biological activities in addition to an anti-adipogenic effect, it seems valuable to develop fucoidan-added *Kochujang* as a multi-functional *Kochujang*.

Key words: *Kochujang*, fucoidan, leptin, adipocyte differentiation, lipolysis

INTRODUCTION

The obese population has been increasing worldwide and obesity has become a major socioeconomic problem. Obesity raises more concerns by the accumulation of the knowledge that obesity is related directly or indirectly to diseases such as diabetes, hypertension, cancer, and osteoarthritis (1). For these reasons, many studies have been conducted to find functional foods/agents for weight control. Korean traditional fermented foods are also belong in these.

Kochujang is one of the most famous Korean traditional fermented foods. The antiobesity effect of *Kochujang* has been reported in several studies. In rats, it decreases lipid levels in adipose tissue and serum (2). It increases energy expenditure that results in reduction of body fat gain (3). In 3T3-L1 adipocytes, when treated with *Kochujang*, decreased the fat accumulation by modulating adipogenesis and lipolysis (4).

To increase the antiobesity effect of *Kochujang*, we can consider adding functional foods/agents such as green tea, garlic, and HCA, which are known to have effects on weight control. Sea tangle (*Laminaria religiosa*) contains a lot of dietary fiber (32~75% of sea tangle). Fifty-one to eighty-five percent of the dietary fiber in sea tangle is soluble dietary fiber containing laminaran, alginic acid, fucoidan, etc. The soluble dietary fiber of sea tangle decreases food digestibility and nutrient absorption. It reduces blood TG and glucose and body weight (5). Alginic acid is the viscous and indigestible fiber that reduces cholesterol level and has been proposed as dietary fiber to reduce body weight (6). Alginic acid also helps to excrete harmful metal ions such as copper, cadmium, zinc, or lead from the body since it has a high affinity to divalent cations (7). In 3T3-L1 cells, alginic acid restricts adipocyte differentiation and the creation of triglycerides (8). Fucoidan (fucan sulfate) is a fucose-containing sulfated polysac-

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charide. Since fucoidan has a low viscosity and high water-solubility, it can be used as a material for water-soluble dietary fiber. The structure and function of fucoidan are similar to heparin in blood. It exhibits various biological activities such as anticoagulant (9), antiviral (10), and antitumor (11). Fucoidan decreases lipid and total cholesterol in serum and liver in hyperlipidemia-induced rats (12). However, there has been little investigation into fucoidan's effect on weight control.

The aim of this study is to see whether fucoidan, when added to *Kochujang*, improve antiobesity effect of *Kochujang*. To do this, we prepared sea tangle, alginic acid, and fucoidan-added *Kochujang* and tested their effect on leptin secretion by them. We also investigated the anti-adipogenic effect of fucoidan, and whether fucoidan affects adipocyte differentiation or lipolysis.

MATERIALS AND METHODS

Preparation of *Kochujang*

Kochujang used in this study were provided by H Co. (Chungnam, Korea). Sea tangle powder, alginic acid extract, and fucoidan extract were provided by MSC Co. (Kyoungnam, Korea). The commercial preparation of this *Kochujang* included several steps: steamed wheat flour was inoculated with *Aspergillus oryzae* at 35°C for 3 days and followed by mixing with the steamed wheat grains and salt; the mixture was matured in the presence of yeast at 30°C for a week and followed by homogenization for 30~40 days; and then mixed with red pepper powder (*Kochujang*). In the final step of preparation of materials for experiment, 1% of sea tangle powder, alginic acid extract, and fucoidan extract were added to *Kochujang*. The prepared *Kochujang* was freeze-dried, powdered, and extracted 3 times with 20-fold methanol. The methanol extracts (*Kochujang* extract, KE; sea tangle-added *Kochujang* extract, SKE; alginic acid-added *Kochujang* extract, AKE; fucoidan-added *Kochujang* extract, FKE) were concentrated using a vacuum rotary evaporator and then dissolved in 20% dimethylsulfoxide (DMSO).

Cell culture and adipocyte differentiation

Mouse 3T3-L1 preadipocytes were grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. At 1 day postconfluence (designated "day 0"), cell differentiation was induced with a mixture (DM) of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 µM), and insulin (5 µg/mL) in DMEM containing 10% FBS. After 48 hours ("day 2"), the medium was removed and replaced by DMEM

(post-DM) containing 10% FBS supplemented with insulin (5 µg/mL) alone. The medium was changed every 2 days. Samples were added into culture medium of adipocytes on "day 8". After the 24-hour treatment of *Kochujang* or fucoidan (Sigma, USA), the medium was removed for analysis of glycerol and leptin secretion.

Cytotoxicity of fucoidan (Sigma, USA) ranging from 1 µM to 50 µM was evaluated by MTT assay after the incubation with fucoidan for 3 days.

Measurement of leptin and glycerol

Measurement of leptin was performed with a sandwich enzyme-linked immunosorbent assay (ELISA). Anti-mouse leptin, recombinant mouse leptin, and biotinylated anti-mouse leptin antibody were purchased from R&D Systems (MN, USA) and followed the protocol.

The glycerol level was determined using the enzymatic reagent, free glycerol reagent (Sigma, USA), directed by the protocol of GPO-TRINDER (Sigma, USA).

Oil red O staining

The accumulation of triglyceride was measured by Oil red O staining using a previously published method (13). To see the effect of fucoidan on triglyceride formation during adipocyte differentiation, we cultured cells in the differentiation or postdifferentiation medium containing fucoidan (Sigma, USA) from "day 0" to "day 6". To see the effect of fucoidan on lipolysis, the differentiated adipocytes on "day 8" were cultured in the medium containing fucoidan for 3 days. After the culture, adipocytes were fixed with 10% fresh Formalin (Sigma, USA). They were rinsed in phosphate buffered saline (Biowhittaker, USA) and incubated in filtered Oil red O (Sigma, USA) stock solution at 4°C for at least 1 hour. After the staining solution was removed, the dye retained in the cells was eluted into isopropanol and OD₅₄₀ was determined.

Statistical analysis

The results were expressed as means ± SE. The data were analyzed by the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS Institute, 1999~2001). The significance differences between groups was determined by carrying out Student's *t*-test.

RESULTS AND DISCUSSION

Effects of *Kochujang* extracts containing sea tangle, alginic acid, and fucoidan on leptin secretion

Our previous studies showed that *Kochujang* (which is used in this experiment too) decreased leptin secretion and adipocyte size in 3T3-L1 cells by modulating adipogenesis and lipolysis (6). It also decreased the body

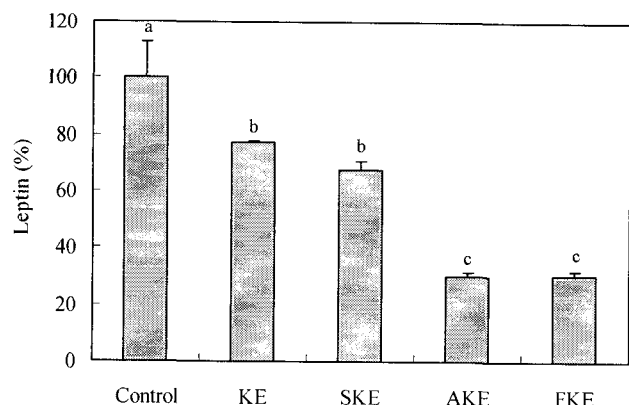


Fig. 1. Effects of sea tangle, alginic acid, and fucoidan-added *Kochujang* on leptin secretion. 3T3-L1 cells were differentiated by the method described in Materials and Methods. Adipocytes were treated for 24 hours starting at “day 8” after inducing differentiation with vehicle alone (Control), or 1 mg/mL of KE (*Kochujang* extract), TKE (*Kochujang* extract containing sea tangle), AKE (*Kochujang* extract containing alginic acid), FKE (*Kochujang* extract containing fucoidan). The leptin secretion was quantified by enzyme-linked immunosorbent assay. Data are expressed as mean \pm standard error values ($n=3$). Means with different letters are significantly different ($p<0.05$) by Duncan’s multiple range tests.

weight, serum lipid, and adipose tissues in rats (14). In order to improve the anti-obesity effect of *Kochujang*, we added 1% of sea tangle, alginic acid, and fucoidan to *Kochujang*. Sea tangle-added *Kochujang* extract (SKE) decreased leptin secretion by 12%, which showed no significant difference compared to *Kochujang* extract (KE). *Kochujang* extracts containing alginic acid (AKE) and fucoidan (FKE) decreased leptin level by 62% and 66%, respectively (Fig. 1). Leptin is a 16-KD protein, which is mainly secreted by adipocytes. It regulates appetite and energy metabolism in human and rodent (15,16). In fed state, leptin is secreted by adipocytes in proportion to their TG stores, and circulating leptin levels are correlated with the extent of obesity (17,18). There is a study on the anti-adipogenic effect of alginic acid in 3T3-L1 adipocytes (8), but not for fucoidan. Therefore, we studied the anti-adipogenic effect of fucoidan by investigating leptin secretion, TG formation and lipolysis in 3T3-L1 adipocytes.

Effect of fucoidan on leptin secretion

We performed MTT assay to exclude the possibility of cytotoxicity by fucoidan that could cause us to misinterpret cell death. As shown in Table 1, noticeable cell death was not observed up to the concentration of 10 μ M. To investigate the anti-adipogenic effect of fucoidan on 3T3-L1 adipocytes, leptin secretion was measured. Leptin secretion in the medium was decreased by 56%, 60%, and 60% compared to the control group when adipocytes were treated with fucoidan at the

Table 1. Cytotoxicity of fucoidan on 3T3-L1 adipocytes

Fucoidan (μ M)	Relative cell survival (%)
Control	100.00 \pm 3.98 ¹⁾
1	106.35 \pm 3.58
10	88.50 \pm 1.75
20	85.62 \pm 2.94 **
50	66.20 \pm 3.40 **

¹⁾Values are mean \pm SE ($n=3$).

The significance differences between groups was determined by carrying out Student’s *t*-test. ** $p<0.01$ versus control.

concentrations of 1 μ M, 2.5 μ M, and 5 μ M, respectively (Fig. 2). There were no significant differences in leptin secretion among the three groups treated with fucoidan.

Effect of fucoidan on triglyceride formation during adipocyte differentiation

To determine whether fucoidan affected TG formation during differentiation of 3T3-L1 adipocytes, we cultured cells in the differentiation or postdifferentiation medium containing fucoidan for 6 days (from “day 0” to “day 6”). Since there was no significant difference in leptin secretion among the groups treated with three different concentrations (Fig. 2), we treated only lower (1 μ M) and higher (5 μ M) concentrations of fucoidan in the medium. After the culture, we stained adipocytes with Oil red O and eluted dye which contained TG. The Oil red O stain is commonly used to identify exogenous or endogenous lipid deposits. The inhibitory effect on TG formation could be observed as the changes in the morphology and the number of lipid droplets (Fig. 3a). Fucoidan treatments resulted in a decrease in cytoplasmic deposition of TG droplets and 5 μ M fucoidan potentially inhibited TG formation. The OD values of

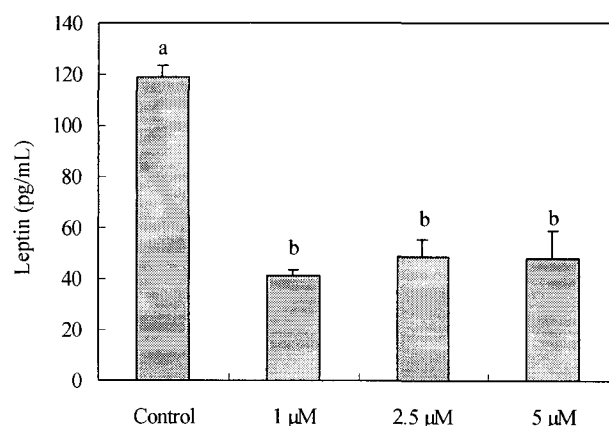


Fig. 2. Effect of fucoidan on leptin secretion. Adipocytes were treated for 24 hours at “day 8” after inducing differentiation with vehicle alone (Control), or 1 μ M, 2.5 μ M, or 5 μ M fucoidan. Data are expressed as mean \pm standard error values ($n=3$). Means with different letters are significantly different ($p<0.05$) by Duncan’s multiple range tests.

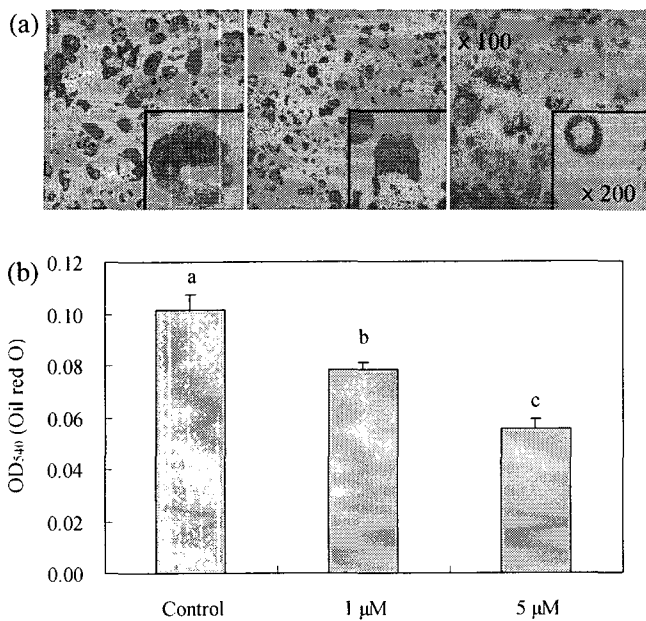


Fig. 3. Effect of fucoidan on triglyceride formation during adipocyte differentiation. Cells were cultured in the differentiation or postdifferentiation medium containing 1 μM or 5 μM fucoidan (Sigma, USA) from “day 0” to “day 6”. (a) Morphology of adipocytes influenced by fucoidan. Magnification, ×100 and ×200 for each group. (b) OD values of eluted dye. Data are expressed as mean ± standard error values (n=4). Means with different letters are significantly different ($p < 0.05$) by Duncan’s multiple range tests.

eluted dye (TG) significantly decreased in both concentrations (Fig. 3b), which means that fucoidan inhibits TG formation during the adipocytes differentiation. Adipogenesis is induced through the action of several enzymes, including FAS, ACC, acyl-CoA synthetase (ACS), glycerol-3-phosphate acyltransferase. The expressions of these genes are regulated by transcription factors such as PPAR- γ , C/EBPs and SREBP-1c. Glucose and fatty acid uptake are other important factors for adipogenesis (19). There has been no study into how fucoidan works on adipocyte differentiation, resulting in a decrease of TG formation. It will be interesting to study the action mechanism of fucoidan in 3T3-L1 adipocytes.

Effect of fucoidan on lipolysis

In order to examine whether fucoidan induces lipolysis, we measured glycerol secretion from 3T3-L1 adipocytes by treatment of 1 μM, 2.5 μM, and 5 μM fucoidan. Glycerol secretion increased 21%, 37%, 53%, respectively (Fig. 4a). We also cultured fully differentiated adipocytes in the culture medium containing fucoidan for 3 days (from “day 8” to “day 11”) and stained adipocytes with Oil red O. The OD values of eluted dye significantly decreased in both concentrations (Fig. 4b). The increased glycerol secretion and the reduced TG accumulation imply that the lipolysis of adipocytes

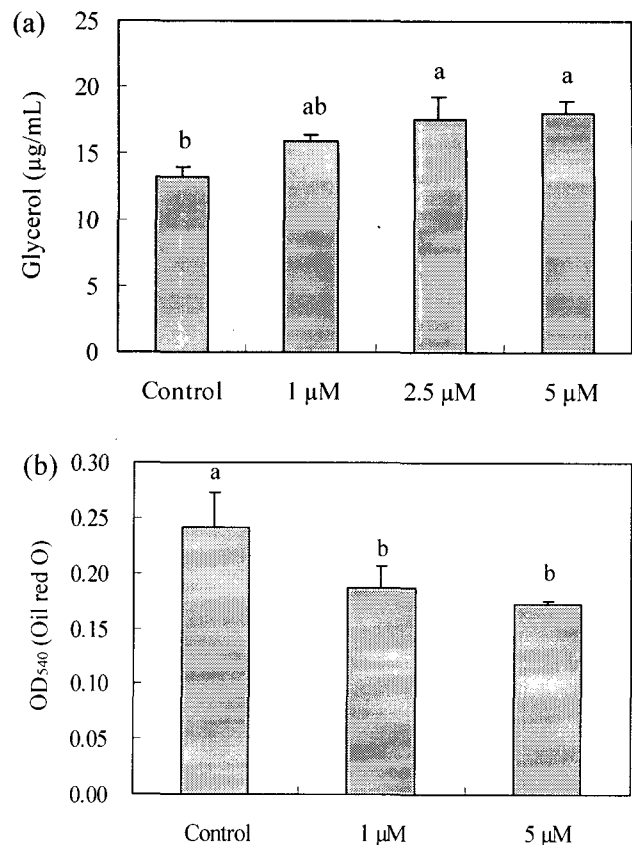


Fig. 4. Effect of fucoidan on lipolysis. (a) Glycerol secretion. Cells were cultured in the medium contained with 1 μM, 2.5 μM, or 5 μM fucoidan for 24 hrs. Data are expressed as mean ± standard error values (n=4). (b) OD values of eluted dye. Cells were treated with 1 μM or 5 μM fucoidan at “day 8” for 3 days and stained with Oil red O. Data are expressed as mean ± standard error values (n=3). Means with different letters are significantly different ($p < 0.05$) by Duncan’s multiple range tests.

was induced by fucoidan. Hormone-sensitive lipase (HSL) is an important mediator of lipolysis of TG to non-esterified fatty acids and glycerol. Lipolytic hormones such as catecholamines or beta adrenergic ligands stimulate hydrolysis TG through protein kinase A (PKA)-mediated phosphorylation of HSL in the presence of cyclic AMP (cAMP) (20). In addition, lipolysis includes other critical processes such as phosphorylation of perilipin and HSL translocation into the lipid droplet (21). Fucoidan seems to work as a beta adrenergic ligand or an activator of factors associated with TG hydrolysis.

The experiments of fucoidan in this study show that fucoidan decreases leptin secretion and TG accumulation by inhibition of adipocyte differentiation and induction of lipolysis. As leptin is secreted in proportion to TG stores, and circulating leptin levels are correlated with the extent of obesity (17,18), it is not surprising that fucoidan-treated cells secreted less leptin (Fig. 2) because they reduced fat accumulation. In Fig. 3b we can see that

the treatment of 5 μ M fucoidan reduced OD value of Oil red O (TG) more effectively than 1 μ M fucoidan. The other results show no significant differences between the two groups treated with 1 μ M and 5 μ M fucoidan (Figs. 2 and 4). To observe the dose-dependent effect of fucoidan, we need to test in lower concentrations of fucoidan.

Some reports revealed that *Kochujang* containing 2~6% of sea tangle showed a low acceptability or no change in sensory properties in the sensory evaluation (22,23). Alginic acid is highly viscous and may not be suitable to be added to *Kochujang*. However, fucoidan is highly water-soluble and not too viscous, and thus might be used as a soluble dietary material. A further study for sensory evaluation of alginic acid or fucoidan-added *Kochujang* is suggested.

In conclusion, *Kochujang* with 1% alginic acid or fucoidan significantly decreased leptin secretion compared to *Kochujang* or sea tangle-added *Kochujang*. Fucoidan, one of the active components of sea tangle, significantly decreased leptin secretion. It inhibited TG formation during adipocyte differentiation as well as induced lipolysis. These results show that fucoidan is effective in decreasing fat accumulation, which suggests that adding fucoidan to *Kochujang* can improve the anti-adipogenic effect of *Kochujang*. Since fucoidan is reported to have various biological activities in addition to an anti-adipogenic effect, it will be valuable to develop fucoidan-added *Kochujang* as a multi-functional *Kochujang*.

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