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Reduced Leptin and Raised Glycerol Secretions in Mouse 3T3-L1 Adipocytes by Garlic-added *Kochujang*

Chang-Suk Kong, Ju-Hong Mun, Su-Ok Kim, Hye-Kyung Jung, In-Sook Ahn, Sook-Hee Rhee and Kun-Young Park†

Department of Food Science and Nutrition, Pusan National University, Busan 609-735, Korea

Abstract

In order to develop the improved *kochujang* with an anti-adipogenic effect, garlic-added *kochujang* was prepared and followed by fermentation at 30°C for 120 days. Anti-adipogenic effects of the garlic-added *kochujang* in cultured 3T3-L1 adipocytes were investigated by measuring leptin and glycerol levels as indicators of lipid accumulation-associated with lipolysis, respectively. Additional levels of garlic powder in the preparation of *kochujang* was determined from the result of a sensory evaluation test. Garlic-added *kochujang* showed the highest points when the added content was 3%. Fermentation of *kochujang* led to the decreased leptin secretion and increased glycerol release in the 3T3-L1 adipocytes. The addition of garlic to the *kochujang* also resulted in reduced leptin secretion and induced lipolysis. Since these results suggested that addition of garlic into *kochujang* can improve the anti-adipogenic effects of *kochujang*, it might be possible to develop garlic-added *kochujang* as an antiobesity-functional *kochujang*.

Key words: garlic, *kochujang*, leptin, glycerol, 3T3-L1 adipocytes, anti-adipogenic

INTRODUCTION

The obese population has been increasing worldwide. Obesity is a heavy accumulation of fat in the body's fat cells to such a serious degree that it greatly increases the risk of obesity-associated diseases. Among the risks are heart disease, hypertension, stroke, cancer, diabetes, and osteoarthritis (1,2). For these reasons, many studies have been conducted to find functional foods/agents including Korean traditional fermented foods for weight control.

The ob-protein leptin is secreted from adipose tissue and may be important in the development of obesity (3-5). Leptin concentration in the serum is directly related to the amount of body fat and the amount of energy stored in adipose tissue (6,7). Lipolysis in adipocytes is a cAMP-driven process. HSL (hormone-sensitive lipase) functions as the rate-limiting enzyme in lipolysis by mediating the hydrolysis of triacyglycerol to non-esterified fatty acids and glycerol. HSL is multiple phosporylated by protein kinase A (PKA) in the presence of cyclic AMP (cAMP). In addition, lipolysis includes other critical processes such as phosphorylation of perilipin and HSL translocation into the lipid droplets (8).

Kochujang, a fermented red pepper soybean paste, is the most famous traditional foods in Korea. Generally, traditional *kochujang* is prepared with glutinous rice, *meju* (fermented soybean blocks), red pepper powder and salt, and is fermented for several months or longer. The unique hot, sweet, salty and savory tastes, color, and flavors of *kochujang* are produced by the actions of microorganisms such as molds, bacteria, and yeasts during the fermentation process (9,10).

Garlic (*Allium sativum*) is a member of lily family that has been cultivated by humans as a food plant for over 10,000 years. Since ancient times, numerous medicinal properties of garlic have been discovered. Garlic and its associated sulfur compounds have been reported to suppress weight gain, TG and cholesterol contents (11). In addition it has been found that garlic and its associated sulfur compounds affect the normalization of plasma lipids, reduction of blood pressure and glucose, and inhibition of platelet aggregation (12-15). Sulfur compounds from garlic are known as alliin, allicin (diallyl thiosulfinate), diallyl sulfide (DAS), diallyl disulfide (DADS), etc. Alliinase catalyzes alliin from natural garlic into allicin, which breaks down rapidly to produce odorous oil-soluble sulfur compounds such as DAS, DADS, diallyl trisulfide (DATS) and ajoene (16).

In this study, garlic-added *kochujang* was made using garlic that contains active compounds, in order to develop an improved *kochujang* with an anti-adipogenic

†Corresponding author. E-mail: kunypark@pusan.ac.kr
Phone: +82-51-510-2839, Fax: +82-51-514-3138

effect. Anti-adipogenic effects of *kochujang* during fermentation and anti-adipogenic effects of the garlic-added *kochujang* on lipid accumulation in cultured 3T3-L1 adipocytes were investigated using leptin and glycerol levels as indicators of lipid accumulation associated with lipolysis.

MATERIALS AND METHODS

Ingredients and preparations of *kochujang*

Glutinous, malt flour, *meju* flour, and salt were purchased in a local market in Busan, Korea. Red pepper powder and garlic were purchased from Youngyang and Uiseong, Gyeongsangbuk-do, Korea, respectively. *Kochujang* was prepared by the standardized method (17) and fermented for 120 days at 30°C. Garlic was freeze-dried, powdered, and added in ratios of 0%, 3%, and 5% during the preparation of *kochujang*.

The prepared *kochujang* was freeze-dried, powdered, and extracted 3 times with 20-fold methanol. The methanol extract was concentrated using a vacuum rotary evaporator and followed by dissolution in dimethylsulfoxide (DMSO).

Sensory evaluation

The sensory qualities of *kochujang* were evaluated by twenty panelists. The panelists evaluated flavor, hot (spicy) taste, sweet taste, texture, appearance, and overall acceptability on a 9-point scale (18). Ratings were made according to a nine-point scale: 9, like extremely; 8, like very much; 7, like moderately; 6, like slightly; 5, neither like nor dislike 4, dislike slightly; 3, dislike moderately; 2, dislike very much; 1, dislike extremely.

Cell culture and adipocytes differentiation

3T3-L1 mouse cells were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Service Co. (USA). Methylisobutylxanthine (IBMX), dexamethasone (DEX), and insulin (INS) were purchased from Sigma Chemical Co. (USA). The 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% CO_2. At 1 day post-confluence (designated "day 0"), cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM), and insulin (5 μg/mL) in DMEM containing 10% FBS. On day 2 and day 4, the medium was replaced with DMEM containing 10% FBS and insulin (5 μg/mL) only. On day 6 and thereafter the medium consisted of only DMEM plus 10% FBS, which was subsequently replaced every 2 days.

The *kochujang* extract was treated into adipocytes at the concentration of 1 mg/mL at day 8 after inducing differentiation. After 24 hr, the medium was removed for analysis of leptin and glycerol.

Measurement of leptin and glycerol levels

Measurement of leptin levels 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) (19).

The glycerol level was determined using the enzymatic reagent, free glycerol reagent (Sigma, USA), directed by the protocol of GPO-TRINDER (Sigma, USA) (20).

Statistical analysis

Data were expressed as mean±standard error values ($n=3$). Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range tests. Each experiment was replicated at least 3 times.

RESULTS AND DISCUSSION

Effects of *kochujang* on leptin and glycerol secretions in 3T3-L1 adipocytes during fermentation

Changes in leptin secretion by traditional *kochujang* were investigated during fermentation for 120 days (Fig. 1). In a fed state, circulating leptin levels are correlated with the extent of obesity (21,22). To investigate the anti-adipogenic effect of traditional *kochujang* on the 3T3-L1 adipocytes during the fermentation, the leptin secretion

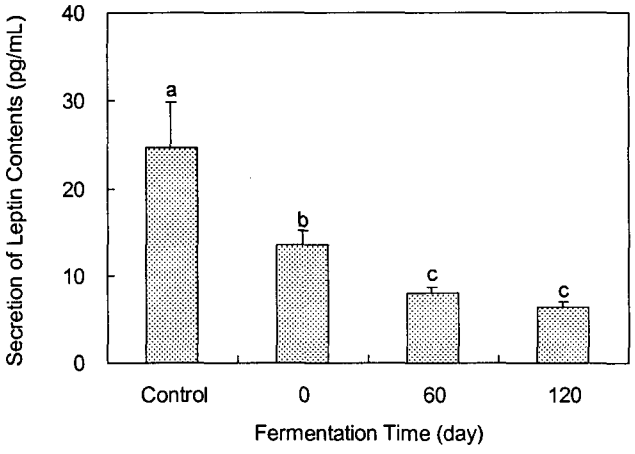

Fig. 1. Changes in leptin secretion of *kochujang* during fermentation. Adipocytes were treated for 24 hr at "day 8" after inducing differentiation with vehicle alone (control) or 1 mg/mL of *kochujang* fermented for 120 days. Data are expressed as mean±standard error values. Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.

was measured. Fermentation of traditional *kochujang* reduced leptin secretion in the medium. That is, leptin secretions of *kochujang* fermented for 0, 60, and 120 days were decreased by 15%, 68% and 74% compared to the control, respectively.

Lipolytic activities of traditional *kochujang* fermented for 120 days were investigated in order to examine the effect of the *kochujang* on lipid accumulation associated with lipolysis (Fig. 2). The lipolytic response of differentiated adipocytes was determined by measuring the amount of glycerol released in the medium. Fermentation of the *kochujang* considerably raised glycerol levels in the medium. The *kochujang* fermented for 0, 60, and 120 days induced increased glycerol levels in ratios of 7%, 27% and 72% compared to the control, respectively. These results reflect that fermentation of the *kochujang* induced the increased lipolytic activity of adipocytes.

From these results, the fermentation of *kochujang* resulted in reduced leptin secretion, which can result from decreased fat accumulation. The anti-adipogenic effect of *kochujang* may partly include the induction of lipolysis. The possible active components responsible for the anti-adipogenic effect might be capsaicin in red pepper powder, isoflavonoids produced from *meju*, and some products of *kochujang* fermentation, such as isomaltooligosaccharides and small peptides. *In vivo,* several studies have reported on antiobesity effects of the fermented traditional *kochujang* (23-26). The decrease in body weight, serum lipids, adipose tissues in rats, and body fat gain were also studied (23,24). Commercial *kochujang* also decreased the leptin secretion and adipocytes size in 3T3-L1 adipocytesby modulating adipogenesis and lipolysis (20). Therefore, the antiobesity effect of garlic-added *kochujang* can be examined in 3T3-L1 adipocytes.

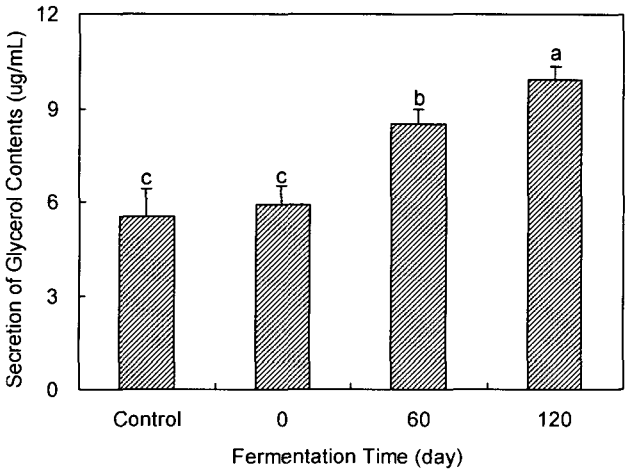

Fig. 2. Changes in glycerol secretion of *kochujang* during fermentation. Data are expressed as mean±standard error values. Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.

Effects of garlic-added *kochujang* on leptin and glycerol secretions

To investigate the effects of garlic addition to *kochujang* on lipid accumulation, leptin and glycerol secretions by treatment of garlic-added *kochujang* in 3T3-L1 adipocytes were examined. Since garlic includes active sulfur-compounds, the addition of garlic powder in the preparation of *kochujang* might induce a suppressive effect on lipid accumulation. Additional content of garlic powder in the preparation of *kochujang* was determined from the results of the sensory evaluation test. From the results of the sensory evaluation test, the addition of garlic powder induced an increased hot-taste of *kochujang*. Three percent garlic-added *kochujang* showed the highest

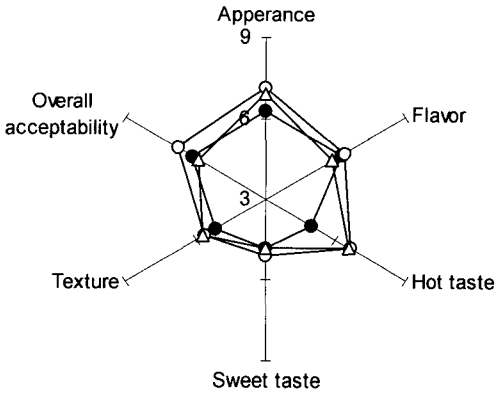

Fig. 3. QDA profile in garlic-added *kojuchang*.
—●—: Control *kochujang*.
—○—: 3% garlic-added *kochujang*.
—△—: 5% garlic-added *kochujang*.

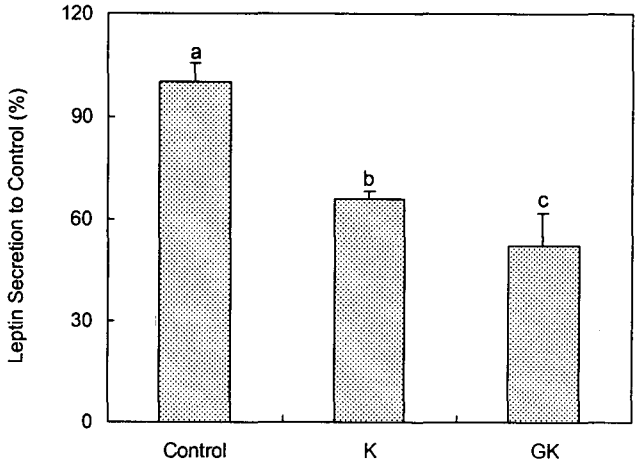

Fig. 4. Additional effects of garlic powder on leptin secretion of *kochujang* fermented for 120 days at 30°C.
Data are expressed as mean±standard error values (n=3). Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.
K: Control *kochujang*, GK: 3% garlic-added *kochujang*.

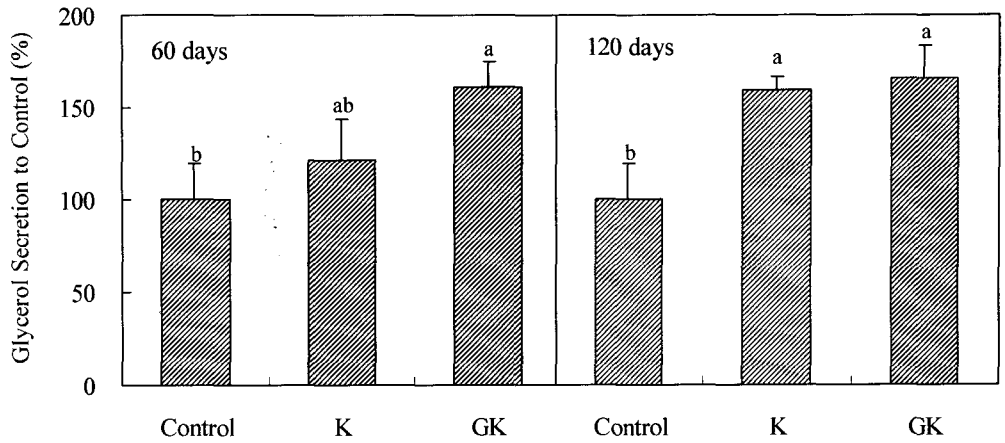

Fig. 5. Additional effects of garlic powder on glycerol secretion of *kochujang* fermented for 60 and 120 days at 30°C. Data are expressed as mean±standard error values (n=3). Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.

points compared to the control *kochujang* (0%) and 5% garlic-added *kochujang* (Fig. 3). Therefore, the garlic powder was added in preparation of *kochujang* in ratio of 3% concentration.

Leptin secretions caused by the garlic-added *kochujang* fermented for 120 days were examined (Fig. 4). Leptin secretions of the control *kochujang* and garlic-added *kochujang* were decreased by 34% and 48% to the control, respectively. The lipolytic activity of *kochujang* to the adipocytes was determined by the amount of glycerol released in the medium (Fig. 5). The addition of garlic powder into the *kochujang* resulted in reduced leptin secretion and the raised glycerol secretion in the medium. Glycerol secretions of the control *kochujang* and garlic-added *kochujang* fermented for 60 days increased by 21% and 61%, respectively. The control *kochujang* and garlic-added *kochujang* fermented for 120 days increased glycerol levels in ratios of 21% and 61%, respectively. Although the addition of garlic powder did not affect glycerol secretions when *kochujang* was fermented for 120 days, it increased glycerol secretion when *kochujang* was fermented for 60 days. The elevated glycerol level by garlic-added *kochujang* might be due to the induction of HSL mediated lipolysis. Improved anti-adipogenic effects of garlic-added *kochujang* might be due to a synergistic effect between garlic and the products of fermentation of *kochujang*. It has been reported that garlic and its associated sulfur compounds have various biological activities (27-33). Sulfur compounds from garlic are known as alliin, allicin (diallyl thiosulfinate), DAS, DADS, etc. (16). The effects of garlic powder on reducing total lipids, TG and cholesterol contents were studied (12-15). *In vivo*, garlic reduced weight gain, TG and cholesterol contents, and lipid values of adipose tissue (33). The addition of garlic during *kochujang* preparation also inhibited the growth of cancer cells in a concentration dependent manner and increased anticancer activities (34).

Since these results suggest that the addition of garlic into *kochujang* can improve the anti-adipogenic effects of *kochujang*, it is available to develop garlic-added *kochujang* as an antiobesity-functional *kochujang*.

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