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Research paper

Anticancer(AC)-Functional Kimchi Exhibits Antiobesity Effects in Differentiated 3T3-L1 Adipocytes

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ABSTRACT

In vitro anti-obesity effects of anti-cancer (AC) functional kimchi in differentiated 3T3-L1 adipocytes were studied. We constructed three experimental groups: Control, standardized kimchi (SK), and AC functional kimchi (A-FK) that included active ingredients and *Lactobacillus plantarum*. Kimchi extracts did not show any cytotoxicity in pre-adipocytes in the concentration range of 1 – 5 mg/mL. A-FK significantly reduced fat droplet formation and absorbance in differentiated 3T3-L1 adipocytes, as shown by Oil red O staining, compared to Control and SK ($P < 0.05$). SK and A-FK reduced adipo-/lipogenesis related genes such as C/EBPα, SREBP-1, LPL, and LXRα compared to Control ($P < 0.05$). Especially, A-FK more greatly reduced SREBP-1 and LPL compared to SK ($P < 0.05$). A-FK up-regulated the β-oxidation related gene CPT-1c and down-regulated the pro-inflammatory cytokine IL-6 compared to Control ($P < 0.05$). Based on the results, A-FK exhibited anti-obesity effects by inhibiting fat droplet formation and adipo-/lipogenesis related genes by regulating the β-oxidation related gene CPT-1c and pro-inflammatory cytokine IL-6. In previous studies, A-FK kimchi already exhibited a strong anti-cancer effect. These results indicate that A-FK increased anti-obesity activity in this model system due to its functional ingredients and anti-cancer functionality.

Keywords Anti-obesity, kimchi, Adipogenesis, Lipogenesis, 3T3-L1 adipocyte

INTRODUCTION

Kimchi is a Korean traditional vegetable fermented food usually prepared by salting baechu cabbage and adding various ingredients. Kimchi has been reported to have inhibitory effects against obesity (Park et al., 2018), cancer (Kim et al., 2015), and inflammation (Lee et al., 2015). However, the variety of ingredients in kimchi can alter its health based functionalities. Addition of *Leuconotoc mesenteroides* or nano-sized *Lactoba - cillus plantarum* to kimchi has been shown to better suppress obesity and colon cancer compared to standardized kimchi (SK) (Cui et al., 2015, Kim et al., 2014). In addition, kimchi added with mustard leaf, Chinese pepper, and mistletoe extract was shown to better inhibit colon cancer and the pro-inflammatory cytokines TNF-α and IL-6 compared to SK (Kim et al., 2014). Therefore, kimchi has different health functionalities depending on its added ingredients. In this study, we used kimchi made according to an anti-cancer functional kimchi recipe (Kim et al., 2014), which has already been reported to have strong inhibitory effects against colon cancer (Kim et al., 2015, Lee et al., 2015, Kim et al., 2014). Therefore, we focused on the anti-obesity effects of this type of kimchi in differentiated 3T3-L1 adipocytes.

Obesity is related to many diseases such as osteoarthritis, diabetes, heart disease, and hypertension and is generally caused by an increase in the number of adipocytes (adipogenesis) and accumulation of fat (lipogenesis) (Yun, 2010). Adipo-/lipogenesis are regulated by various genes. CCAAT/enhancer-binding protein alpha (C/EBPα) is a major transcription factor of adipocyte proliferation processing genes (Wu et al., 1999). Sterol regulatory element-binding protein 1 (SREBP-1) is a transcription factor of decamer flanking the lipoprotein lipase (LPL) receptor gene and promotes sterol biosynthesis (Yokoyama et al., 1993, Magaña et al., 1996). LPL is involved in promoting cellular uptake of chylomicron remnants and free fatty acids (Mead et al., 2002). Liver X receptors (LXRs) are transcription factors and important regulators of fatty acids and glucose homeostasis (Schmuth et al., 2008). Therefore, it is possible to suppress obesity by controlling these adipo-/lipogenesis related genes. In addition, regulation of carnitine palmitoyltransferase I (CPT-1), which is a gene associated with β-oxidation, regulates obesity by promoting β-oxidation in mitochondria (Duplus et al., 2000). Moreover, when nutrients are overloaded in adipose tissue, nitric oxide synthase 2 (NOS2) and interleukin 6 (IL-6) are promoted in macrophages (M1) (Rocha et al., 2009). 3T3-L1 cells are used as an *in vitro* model of obesity, as these cells accumulate triglycerides during differentiation in growth medium (Cowherd et al., 1999).

In this study, we investigated whether or not AC functional kimchi also has an anti-obesity effect in 3T3-L1 adipocytes

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using Oil red O staining and analysis of the adipo-/lipogenesis related genes C/EBPα, SREBP-1, LPL, and LXRα, β-oxidation related gene CPT-1c, and inflammation-related gene IL-6 in differentiated 3T3-L1 adipocytes.

MATERIALS AND METHODS

Kimchi sample preparation

Kimchi samples were made by the Bongwoori Chan kimchi Co., (Namyangju, Gyunggi-do, Korea). Standardized kimchi (SK) was composed of the following ingredients: 100 g baechu cabbage, 8.0 g radish, 2.0 g green onion, 4.0 g kelp juice, 4.0 g glutinous rice paste, 4.0 g red pepper powder, 2.0 g crushed garlic, 0.4 g crushed ginger, 0.4 g anchovy powder, 3.4 g anchovy juice, 0.8 g salted shrimp, and 4.0 g solar salt (Song, 2018). AC-functional kimchi (A-FK) (Kim et al., 2014) was composed of the following ingredients: 100 g of baechu cabbage; 11.0 g radish, 2.0 g green onion, 2.5 g red pepper powder, 2.8 g crushed garlic, 0.6 g crushed ginger, 1.0 g sugar 7.5 g mustard leaf, 0.1 g Chinese pepper, 2.8 g pear, 5.0 g mushroom and sea tangle juice, 0.05 g mistletoe extract, 2.2 g bamboo salt, and 106 cfu/g Lactobacillus plantarum. Prepared kimchi samples were fermented in 4° C refrigerator. After 3 weeks, approached pH 4.3, all kimchi samples were freeze-dried (FD5512, Ilshin BioBase Co., Gyeonggi-do, Korea) at -80 ° C.

Dried kimchi samples were made into powder form, after which methanol equivalent to 20 times the amount of kimchi powder was added and stirred for 48 hours to obtain kimchi extracts. These extracts was concentrated using an evaporator (EYELA, Tokyo Rikakikai Co., Tokyo, Japan), after which dimethyl sulfoxide was added to make 250 mg/mL and used for *in vitro* experiments

Pre-adipocytes and differentiated adipocyte culture

3T3-L1 adipocytes were purchased from Korean Cell Line Bank (Seoul, Korea). Pre-adipocyte state 3T3-L1 adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Co., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich Co.) and 1% penicillin-streptomycin (PS, Welgene Inc., Gyeongbuk, Korea). Well-cultured pre-adipocytes were plated on a 6-well plate at a concentration of 2×10^5 cells/mL using a cell counter (Luna automated cell counter, Logos Biosystems, Gyeonggi-do, Korea) and incubated for 48 hours. After 48 hours, pre-adipocytes were converted into differentiated adipocytes by DMEM, 10% FBS, and 1% PS medium in a mixture of 0.5 mM isobutylmethylxanthine (Sigma-Aldrich Co.), 0.25 μM dexamet -hasone (Sigma-Aldrich Co.), and 1 μg/mL of insulin (Welgene Inc.) incubated for 48 hours. Then, cells were cultured for 6 days in DMEM, 10% FBS, 1% PS, and 1 μg/mL of insulin medium to prepare fully differentiated adipocytes (Zebisch et al., 2012) .

Cytotoxicity analysis of kimchi extracts in pre-adipocytes (MTT assay)

Pre-adipocyte cells were plated in 96-well plates to a concentration of 1.5×10^4 cells/mL using a cell counter (Luna automated cell counter, Logos Biosystems) and incubated for 24 hours. After incubation, the new medium was mixed with various concentrations of kimchi samples (1 - 5 mg/mL) and incubated for 24 hours, followed by addition of 500 μg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M TT) solution prepared with PBS and incubation for 4 hours. The formazan crystals were dissolved in DMSO incubated for 20 minutes and then measured at 550 nm using an Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA, USA) (Gerlier et al., 1986).

Lipid droplet analysis in differentiated adipocytes treated with kimchi samples (Oil red O staining)

Each of the kimchi extracts was mixed at a concentration of 5 mg/mL with medium containing well-differentiated adipocytes in a 6-well plate and cultured for 24 hours. The medium was then removed, washed with phosphate buffered saline (PBS), and fixed with 10% formalin. After fixation, cells were washed with distilled water, reacted with 60% isopropanol for 5 minutes, removed, and reacted with Oil Red O working solution (Sigma-Aldrich Co.) for 10 minutes. After washing with distilled water, samples were observed with a microscope (U-TVIX-2, OLYMPUS, Tokyo, Japan). After observation, 1 mL of 100% isopropanol was added to each well, and isopropanol mixed with Oil red O solution was measured at 490 nm with a Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer) (Park et al., 2018).

mRNA expression in differentiated adipocytes treated with kimchi samples (Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) assay)

Each of the kimchi extracts was mixed at a concentration of 5 mg/mL with medium containing well-differentiated adipocytes in a 6-well plate and cultured for 24 hours. After removing the medium, RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). Then, quantification was performed using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA), after which the quantified RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and then synthesized as cDNA. The prepared cDNA was amplified by incorporating each primer, SYBR green (Solis biodyne, Tartu, Estonia), and cDNA using the thermal cycler BioRad CFX-96 real-time system (BioRad, Hercules, CA, USA), and the expressed genes were analyzed. The gene primer sequences were 18s rRNA; forward 5' -TCGAGGCCCTGTAATTGGAA-3' and reverse 5'-CCCTCCAATGGATCCTCGTT-3', C/EBPα; forward 5' -TGCTGGAGTTGACCATGTAC-3' and reverse 5'-AAACCATCCTCTGGGTCTCC-3', SREBP-1; forward 5' -CGGAGACAGGGAGTTCTCAG-3' and reverse 5'-TGGGGGATATGCTCTACCAG-3', LPL; forward 5' -CAGCTGGGCCTAACTTTGAG-3' and reverse 5'-CCTCTCTGCAATCACACGAA-3', LXRα; forward 5' -GCAACTCAATGATGCCGAGT-3' and reverse 5'-CGTGGGAACATCAGTCGGTC-3', CPT-1c; forward 5' -TATCGCCACCTGCTGAACC-3' and reverse 5'-TTGAAGGTGACGAAGGTGGT-3', and IL-6; forward 5'-ATGAAGTTCCTCTCTGCAA-3' and reverse 5'-AGTGGTATCCTCTGTGAAG-3'(Livak and Schmittgen, 2001).

Statistical Analysis

In order to test the significance of the experimental results obtained from the control group and each sample, ANOVA (Duncan's multiple range test) was performed at a significance level of $P < 0.05$. Results were expressed as mean ± SD, and RT-qPCR data were expressed as mean ± standard error (SE). All statistical analyzes were performed using SPSS (v18.0, SPSS Inc., Chicago, IL, USA) statistical program.

RESULTS

Cytotoxicity of kimchi samples on 3T3-L1 pre-adipocytes

As shown in Fig. 1, SK (141.2±5.6%) and A-FK (134.6±12.0%) at concentrations of 1 mg/mL, SK (134.7±10.9%) and A-FK (119.5±8.9%) at 3 mg/mL, and SK (134.0±13.3%) and A-FK (103.5±15.1%) at 5 mg/mL showed no specific cytotoxicity in 3T3-L1 pre-adipocytes. Therefore, we decided to use a concentration of 5 mg/mL for the other experiments.

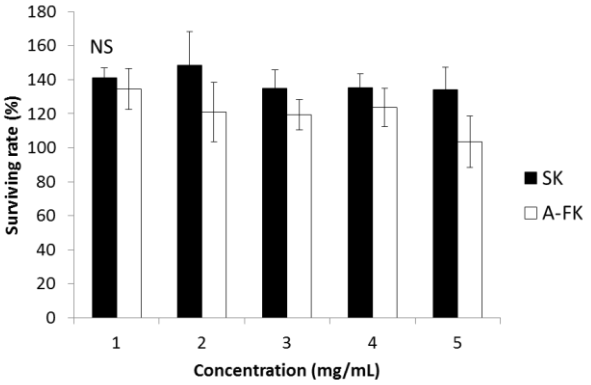

Fig. 1. Cytotoxicities of different kimchi samples in 3T3-L1 pre-adipocytes. The cells were induced with various concentrations of kimchi extract (1 -5 mg/mL) for 24 hours. SK: standardized kimchi, A-FK : anti-cancer functional kimchi. NS : not significa ntly different

Oil red O staining of differentiated 3T3-L1 adipocytes treated with kimchi samples

Oil red O staining was performed by visualizing the reddish stained area with Oil red O reagent, collecting the stained area using isopropanol, and then measuring the absorbance value to determine the difference (Cowherd et al., 1999). As shown in Fig. 2, SK and A-FK reduced the amounts of Oil red O stained fat compared to Control. In addition, when the OD value was 490 nm, SK and A-FK significantly reduced the OD value compared to Control (SK: 7%, A-FK: 15%) (P<0.05), and A-FK reduced the OD value compared to SK (P<0.05).

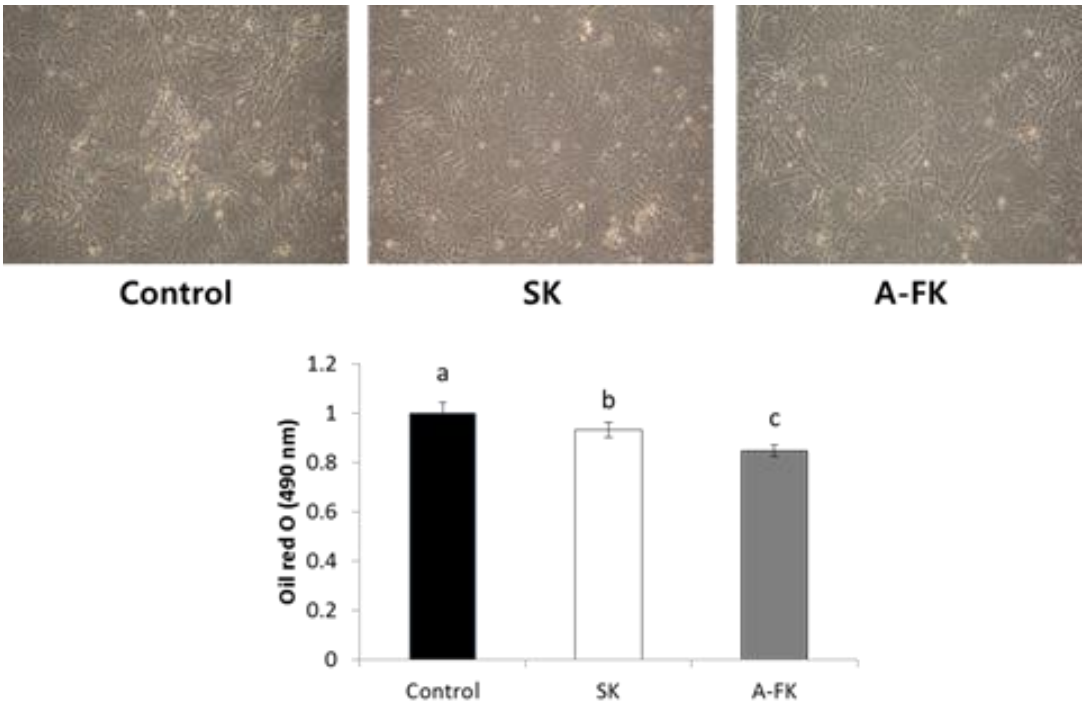

Fig. 2. Oil red O staining of differentiated 3T3-L1 adipocytes treated with kimchi samples. The cells were induced with 5 mg/mL of kimchi extract for 24 hours. Control fold ratio: 1 [a-c]Means the different letters on the bars are significantly different (P < 0.05) by Duncan's multiple range tests. SK: standardized kimchi, A-FK : anti-cancer functional kimchi

mRNA expression of adipo-/lipogenesis related genes C/EBPα, SREBP-1, LPL, and LXRα in differentiated 3T3-L1 adipocytes treated with kimchi samples

As shown in Fig. 3, mRNA expression of C/EBPα in adipocytes treated with SK (0.73±0.08) and A-FK (0.70±0.03) was significantly reduced compared to Control (P<0.05). mRNA expression of SREBP-1 in adipocytes treated with SK (0.63±0.05) and A-FK (0.37±0.02) was significantly decreased compared to Control (1.00±0.15) (P<0.05). mRNA expression of LPL in adipocytes treated with SK (0.66±0.09) and A-FK (0.48±0.02) was also significantly decreased compared to Control (1.00±0.05) (P<0.05). mRNA expression of LXRα in the samples treated with SK (0.84±0.03) and A-FK (0.68±0.06) was significantly decreased compared to Control (1.00±0.06) (P<0.05). In addition, A-FK significantly reduced SREBP-1 and LPL compared to SK (P<0.05). Therefore, all kimchi samples inhibited adipo-/lipogenesis via regulation of related gene expression, and A-FK showed better suppression of adipo-/lipogenesis than SK.

β-oxidation-related gene CPT-1c in differentiated 3T3-L1 adipocytes treated with kimchi samples

As shown in Fig. 4, A-FK (1.23±0.08) significantly up-regulated CPT-1c compared to Control (1.00±0.05) (P<0.05). Therefore, A-FK promoted β-oxidation in 3T3-L1 adipocyte mitochondria.

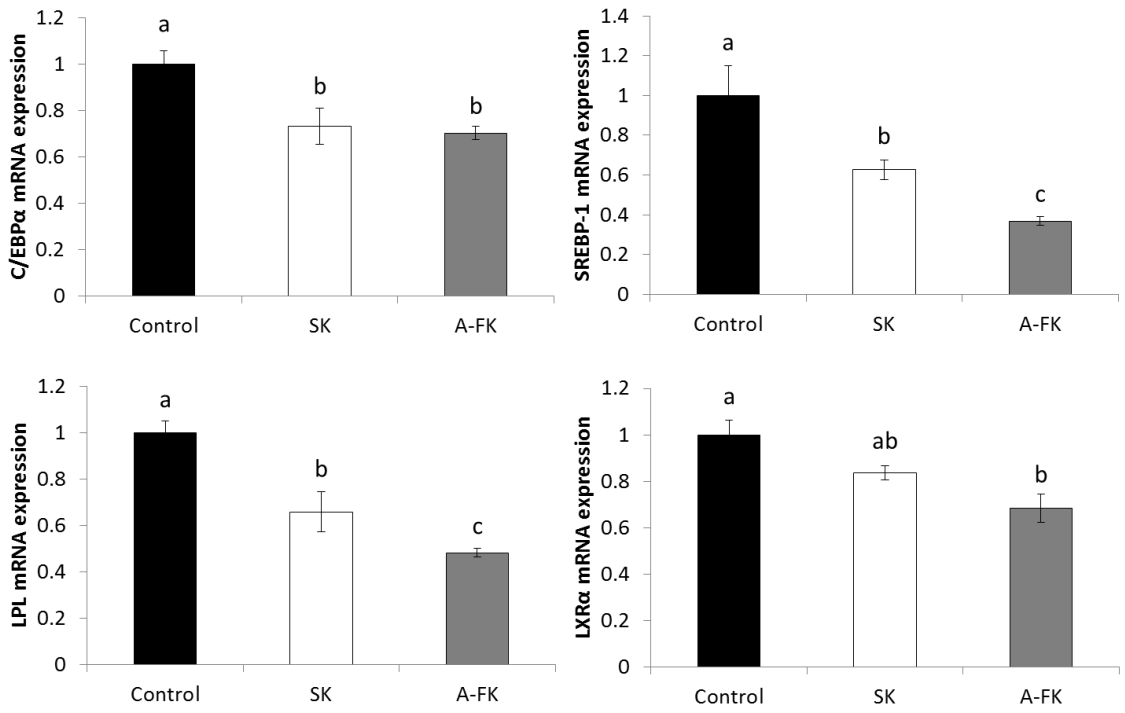

Fig. 3. Adipo-/lipogenesis related genes of SREBP-1, C/EBPα, LXR, and LPL in differentiated 3T3-L1 adipocytes treated with kimchi The cells were induced with 5 mg/mL of kimchi extract for 24 hours. 18s rRNA was used as a control (control fold ratio: 1). [a-c]Means the different letters on the bars are significantly different ($P < 0.05$) by Duncan's multiple range tests. SK: standardized kimchi, A-FK: anti-cancer functional kimchi.

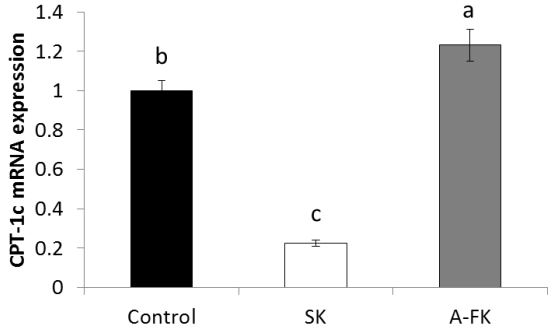

Fig. 4. β-oxidation related gene CPT-1c in differentiated 3T3-L1 adipocytes treated with kimchi. The cells were induced with 5 mg/mL of kimchi extract for 24 hours. 18s rRNA was used as a control (control fold ratio: 1). [a-c]Means the different letters on the bars are significantly different ($P < 0.05$) by Duncan's multiple range tests. SK: standardized recipe kimchi, A-FK: anti-cancer functional kimchi.

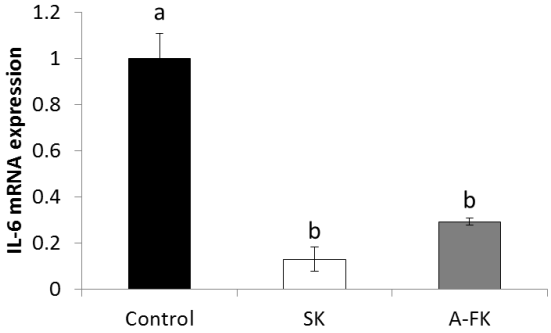

Fig. 5. Pro-inflammatory cytokine IL-6 in differentiated 3T3-L1 adipocytes treated with kimchi. The cells were induced with 5 mg/mL of kimchi extract for 24 hours. 18s rRNA was used as a control (control fold ratio: 1). [a,b]Means the different letters on the bars are significantly different ($P < 0.05$) by Duncan's multiple range tests. SK: standardized recipe kimchi, A-FK: anti-cancer functional kimchi.

mRNA expression of pro-inflammatory cytokine IL-6 in differentiated 3T3-L1 adipocytes treated with kimchi samples

As shown in Fig. 5, mRNA expression of IL-6 in the samples treated with SK (0.13±0.01) and A-FK (0.29±0.02) was significantly decreased compared to Control (1.00±0.11) ($P<0.05$). Therefore, SK and A-FK suppressed inflammation and reduced IL-6 levels.

DISCUSSION

Kimchi has already been studied in many *in vitro* and *in vivo* experiments, and experiments are generally performed using kimchi extract. Jeong et al. (2015) examined the toxicity of kimchi using RGM-1 normal gastric cells (0-7.5 mg/mL). We examined the toxicity of kimchi using 3T3-L1 pre-adipocytes (1-5 mg/mL). Therefore, kimchi extracts did not show toxicity in normal cells.

Kimchi has been reported to have anti-obesity function *in vitro* (Park et al., 2018, Lee et al., 2015), *in vivo* (Cui et al., 2015, Park et al., 2012), and in humans (Kim et al., 2011). Obesity is caused by the particular regulation of various pathways. Kimchi has been reported to generally inhibit obesity by regulating adipo-/lipogenesis. Kimchi was shown to reduce the expression of genes such as peroxisome proliferator-active receptor (PPARγ),

C/EBPα, and fatty acid synthase (FAS) in 3T3-L1 adipocytes (Lee et al., 2015). In addition, Cui et al. (2015) reported kimchi could inhibit the gene expression of PPARγ, C/EBPα, SREBP-1c, and FAS in high fat diet-induced obese mice, whereas Park et al. (2012) reported that kimchi also inhibited PPARγ, SREBP-1c, FAS, and LXRα in high fat diet induced obese mice. In this study, all kimchi samples suppressed C/EBPα, SREBP-1, LPL, and LXRα. Therefore, we suggest that kimchi controls obesity in differentiated 3T3-L1 adipocytes by controlling adipo-/lipogenesis related genes. The anti-obesity effect of kimchi is thought to be due to the various ingredients in kimchi. Park et al. (2014) reported that capsaicin, which is abundant in red pepper powder among the various ingredients of kimchi, is a major anti-obesity factor. In addition, radish, garlic, and ginger in kimchi also have been shown to have anti-obesity effects (Yoon, 2005). Therefore, these results are suggested to be due to the abundance of capsaicin and the several functional ingredients contained in kimchi.

A-FK showed better anti-obesity activity than SK in this study and regulated lipid profiles (Kim and Park, 2018). Thus, we speculate that A-FK has more effective anti-obesity phyto-chemicals than SK. A-FK alleviated inflammation and cancer better and showed elevated anti-oxidant activities than SK in various studies (Lee et al., 2015, Lee et al., 2015, Kim et al., 2014). In adipose tissue, excessive amounts of free fatty acids bind to toll-like receptor (Janeway and Medzhitov, 2002). Free fatty acids also induce the nuclear factor κB (NF-κB) pathway and expression of various inflammatory genes (Rocha et al., 2009). In this study, mRNA expression of IL-6 was similar to those of A-FK and SK. However, it was already reported that A-FK inhibited inflammation related genes such as tumor necrosis factor α (TNF-α) (50%), interferon gamma (IFN-γ) (40%), and inducible nitric oxide synthase (iNOS) (33%) better than SK in colitis associated colon cancer induced mice (Kim et al., 2014). In addition, A-FK (88%) showed better anti-oxidant effects than SK (73%) (Kim, 2013). Thus, the improved anti-inflammation and anti-oxidant effects of A-FK resulted in more powerful anti-obesity activities in differentiated 3T3-L1 adipocytes.

SK and A-FK were manufactured using different ingredients. Especially, A-FK includes Chinese pepper, mistletoe extract, and *Lactobacillus plantarum*. Chinese pepper (Irie et al., 1997), mistletoe extract (Jung et al., 2013), and *Lactobacillus plantarum* (Takemura et al., 2010) have been reported to inhibit lipid metabolism, fat size, and obesity. Chinese pepper includes integrifoliodiol, which inhibits α-glucosidase (Nguyen et al., 2016). Mistletoe includes triterpene, flavonoid, viscotoxin, and lectin (Ju et al., 2009). Triterpene (Moro and Basile, 2000) and flavonoid (Xu et al., 2014) also inhibit obesity. Due to the anti-obesity effects of these various materials and phytochemicals, A-FK may have better anti-obesity effects than SK. Moreover, A-FK enhanced the β-oxidation-related gene CPT-1c, which promote β-oxidation in mitochondria to regulate obesity (Duplus et al., 2000). In a recent study, various metabolites of kimchi were analyzed by liquid chromatography(LC)–mass spectro-metry(MS) and gas chromatography(GC)-MS, and the content of pyropheophobide a was increased by the difference of kimchi material(Song, 2018). Thus, these results necessitate additional research to identify major compounds.

In conclusion, A-FK suppressed obesity by regulating the adipo-/lipogenesis related genes C/EBPα, SREBP-1, LPL, and LXRα in differentiated 3T3-L1 adipocytes. Especially, A-FK showed better anti-obesity effects than SK due to regulation of SREBP-1, LXRα, and CPT-1c. A-FK has already been shown to have strong anti-cancer effects (Lee et al., 2015, Lee et al., 2015, Kim et al., 2014). However, it seems that changes in kimchi ingredients and fermentation method may have multi-functional effects on various chronic diseases such as obesity, cancer, metabolic syndrome, and diabetes by regulating inflammation, oxidative stress, and genes related to obesity and cancer.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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