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RESEARCH NOTE

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Inhibition of Lipid Accumulation in 3T3-L1 Adipocytes by Extract of *Chokong, Rhynchosia nolubilis* Seeds Pickled in Vinegar

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Abstract The anti-obesity effect of *chokong*, *Rhynchosia nolubilis* seeds pickled in vinegars for 2 weeks at 4°C, was investigated. During the differentiation of 3T3-L1 adipocytes, the addition of ethanolic extracts of *chokongs* lowered the cellular triglyceride content by 8.1-9.0%, and glucose content by 12.2-27.6%, depending on the kinds of vinegar used. The activity of glycerol-3-phosphate dehydrogenase also decreased up to 56.0-59.3% by supplying those extracts. In addition, vinegars were superior to acetic acid, citric acid, and hydrochloric acid solutions, and distilled water in anti-obesity of the pickled seeds.

Key words: *Rhynchosia nolubilis* seed, vinegar, 3T3-L1 cell, triglyceride, glycerol-3-phosphate dehydrogenase

Introduction

In recent, obesity is rapidly increased due to the dietary and life style changes leading to the imbalance between energy intake and energy expenditure, and become a severe social problem in developing as well as developed countries (1,2). This metabolic disorder is characterized by the abnormal increase of fat mass resulted from hypertrophy and hyperplasia of adipocytes, in which triglycerides (TG) were accumulated (3,4). In relation to its close association with the development of diabetes, hypertension, hyperlipidemia, atherosclerosis, cancer, and osteoarthritis (5,6), the dietary resources to prevent or cure obesity had a growing concern, specifically, natural ones exhibiting low side-effects than synthetic ones (7,8).

Rhynchosia nolubilis seeds (RNS), called as *sumoktae* or *yakkong*, have been traditionally used in Korea for curing and/or preventing the various diseases such as neuralgia, kidney disease, senile dementia, and postmenopausal osteoporosis. These beneficial activities are thought to be associated with its higher content of antioxidant constituents (9,10) and isoflavones than other legumes especially (11,12). Nowadays, *chokong*, RNS pickled in vinegar, has of great interest as an anti-obesity foodstuff in Korea, but its effect wasn't evaluated yet even though the non-processed RNS itself was reported to be helpful for obesity and lipid metabolism (13,14).

In this study, therefore, the anti-obesity effect of *chokong* was tested by measuring the changes of TG accumulation, glycerol-3-phosphate dehydrogenase (GPDH) activity, and glucose content in 3T3-L1 adipocytes during their differentiation. The effectiveness of vinegars relative to other pickling solutions was evaluated at *chokong* preparation as well.

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Materials and Methods

Materials 3T3-L1 Preadipocyte cell line was obtained from Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified Eagle's media (DMEM), penicillin, streptomycin, and fungizone were purchased from Jell Biotechservices Inc. (Daegu, Korea). Fetal bovine serum (FBS) was the product of HyClone (Logan, UT, USA), and insulin, dexamethasone (DEX), and 1-methyl-3-isobutylxanthine (MIX) were those of Sigma-Aldrich Company (St. Louis, MO, USA). Foodstuffs including *Rhynchosia nolubilis* seeds (RNS), brown rice vinegar, and brewing vinegar were purchased from the local market in Daegu, Korea. All other reagents were of analytical grade.

Preparation of *chokong* and its extract *Chokong* was prepared by the pickling process, specifically, 20 g of RNS were soaked in 60 mL of vinegar (brown rice vinegar and brewing vinegar) for 2 weeks at 4°C. Other pickling solutions were also tested for the comparison; acetic, citric, and hydrochloric acid solutions with pH values similar to that of vinegar, and distilled water. After pickling, the seeds were washed with a sufficient amount of distilled water to remove the residual soaking solution followed by freeze-drying and grinding of the seeds. The resulting powder was extracted with 95% ethanol (1 : 10, w/v) with vortexing for 24 hr at room temperature, filtered (Whatman No. 1 filter paper), vacuum-evaporated at 40°C, and freeze-dried. For cell culture experiment, 1 g of freeze-dried extract was dissolved in 5 mL dimethyl sulfoxide (DMSO) and filtered through 0.2 μm membrane filter (polypropylene backed, MFS, Japan) to remove any contaminant.

Cell culture and differentiation 3T3-L1 Preadipocytes were cultured and differentiated as described elsewhere (15) with some modification. Briefly, the cells were cultured for 6 days in DMEM containing 5% FBS, 100 unit/mL of penicillin G sodium, 100 μg/mL of streptomycin sulfate, and 250 ng/mL of fungizone at 37°C under a

humidified 5% CO_2 atmosphere (NU-4750; NuAire, Polymouth, MN, USA). After reaching confluence, the differentiation to adipocyte was induced by incubating 1×10^5 cells/mL in DMEM containing 5% FBS, 0.25 μM DEX, 0.5 mM MIX, and 10 μg/mL of insulin. Four days later, the medium was then replaced with fresh DMEM containing 5% FBS, 10 μg/mL of insulin, and 20 μL of extracts dissolved in DMSO every 2 days. After 10 days of differentiation, the cells were used for the further analysis.

Oil red O staining Lipid accumulation was examined with Oil red O staining (16). The cultured cells were rinsed twice with phosphate-buffered saline (PBS) and fixed in 10%(v/v) formaldehyde for 1 hr. After the removal of formaldehyde, the cells were rinsed 3 times with deionized water and stained with a saturated solution of Oil red O in 60% isopropanol solution for 2 hr at room temperature. Microscopic image (Olympus, Tokyo, Japan) of the stained cells was obtained after removing the staining solution.

Measurement of triglyceride content The cellular TG content was determined spectrophotometrically using a TG determination kit (Cleantech TG-S; Asan Pharm Co., Ltd., Whasung, Korea) (17). Briefly, the cells were rinsed 3 times with PBS and scrapped off the plate with rubber policeman into individual 1.5 mL microcentrifuge tube. The cells were lysed in the lysis buffer [0.25 M sucrose, 1 mM sodium-ethylenediamine tetraacetic acid (EDTA), 5 mM Tris-base, and 1 mM dithiotreitol, pH 7.4] followed by sonication for 30 sec on 4°C. Twenty μL of the cellular lysate were mixed with 3 mL of the enzyme solution supplied, and incubated for 10 min at 37°C. The absorbance at 550 nm was measured within 60 min. The protein concentration was determined by using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity The GPDH activity was measured as described elsewhere (18) with some modification (19). After the centrifugation of the obtained cellular lysates for 5 min at 12,500×g, 100 μL of the supernatant was mixed with 0.4 mL of the reaction buffer containing 125 mM triethanolamine-EDTA premix, 0.22 mM NADH, 1.0 mM dihydroxyacetone phosphate and 0.125 mM β-mercapto-ethanol. The absorbance reduction at 340 nm for 5 min was measured to estimate the rate of NADH oxidation during the GPDH-catalyzed reduction of dihydroxyacetone phosphate. One unit of enzyme activity corresponded to the oxidation of 1 nmol NADH/min and the results were expressed as units per mg protein.

Measurement of glucose content The glucose content was analyzed using a commercial kit (Glucose, Asan Pharm Co., Ltd.) (20) according to the manufacturer's instructions. The supernatant of 20 μL was mixed with 3 mL of the enzyme solution supplied, and incubated for 5 min at 37°C, and the absorbance at 500 nm was monitored within 40 min.

Statistical analysis All experiments were determined in triplicate and represented as mean±standard deviations. The significance was verified by performing Duncan's multiple range tests using the SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) software package.

Results and Discussions

In order to estimate the anti-obesity effect of *chokong*, we used 3T3-L1 cells established by Green and Kehinde (21), which is well known model system for *in vitro* adipocyte

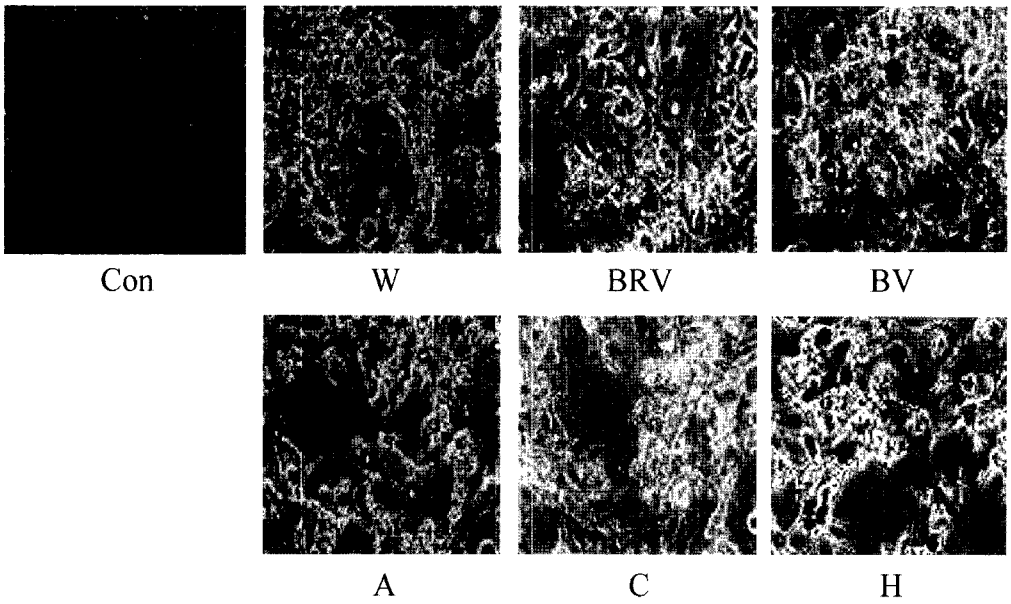

Fig. 1. Lipid accumulation during differentiation of 3T3-L1 peadipocyte under the treatments of the pickled seeds. Lipid droplet were stained with Oil Red O. Abbreviations W, BRV, BV, A, C, and H designated the treatment of extracts of RNS pickled in water, brown rice vinegar, brewing vinegar, acetic acid solution, citric acid solution, and hydrochloric acid solution, respectively. Con indicated no extract treatment.

research and widely used for the obesity-related researches (4,22).

When the lipids accumulation was observed by Oil red O staining after 20 μL of each extract was applied to culture medium during differentiation, both *chokongs* pickled in brown rice vinegar and brewing vinegar showed the considerable inhibitions on lipid deposit compared to the non-treated control as shown in Fig. 1. The seeds soaked in acetic and citric acid solution seemed to inhibit it a little, but those soaked in distilled water and hydrochloric acid solutions didn't. No toxicity was observed for cells treated with concentration of extracts up to 20 μL (data not shown).

For the quantitative comparison of the lipid accumulation, we analyzed TG contents in the cells (Fig. 2a). Similarly with the morphological results, their significant reductions were induced by *chokongs* pickled in brown rice vinegar and brewing vinegar up to 9.0 and 8.1%, respectively, while no reductions were done by the seeds soaked in water and hydrochloric acid solution. Those soaked in acetic and citric acid solutions seemed to lower TG contents a little but not significantly. Morphology and TG content of the cells indicated vinegars' effectiveness than other solutions for the preparation of *chokong* to prevent lipid accumulation.

As the important marker for the lipid accumulation in 3T3-L1 cells, it was compared the activity of cytosolic enzyme GPDH which performs a central role in TG synthesis pathway by catalyzing the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (18,23). *Chokongs* pickled in brown rice vinegar and brewing vinegar suppressed GPDH activity most effectively in the similar way as in TG content, that is, up to 59.3 and 56.0%, respectively. This enzyme was inhibited also by the seeds soaked in other solutions from 44.2% (acetic acid solution) to 25.4% (hydrochloric acid solution).

The analysis of cellular content of glucose, major energy source in adipocytes (21), showed that *chokong* pickled in brown rice vinegar mostly reduced it. Other pickled seeds lowered also its content significantly except for that pickled in hydrochloric acid solution.

Although proteins was well known to be in charge of the anti-obesity of soybean among various ingredients such as proteins, peptides isoflavones, soyasaponins, and lipids etc. (24), they would merely contribute to the anti-obesity property of *chokong* because we used 95% ethanol extracts for the experiment although the raw RNS contains proteins over 38% (25). Liu *et al.* (26) has reported the ethanol extracts of soybean mainly composed of lipids, carbohydrate, ash, and flavonoids such as isoflavone etc.

Considering their higher content in RNS than other soybean species (9-12), isoflavones were presumed to attribute to the anti-obesity effect of *chokong*. Specially, genistein is well known to inhibit cellular tyrosine kinase (27), CCAAT/enhancer-binding protein β activity (28), lipoprotein lipase expression (29), and GLU4-mediated glucose uptake (30,31) including still unknown mechanisms. In our previous report (32), the pickling process raised the content of aglycones, genistein, and daidzein (to about 300 to 780 μg/g) which was not detectable in raw seeds. Therefore, these aglycones of *chokong* could contribute to the inhibition of the cellular lipogenesis accompanying with the decreases of glucose uptake and GPDH activity during the differentiation of 3T3-L1 adipocytes. The increase of aglycones might be the result from the action of endogenous β-glucosidase in *chokong* for which vinegars contribute to the destruction of intact seed structure and the proper pHs (4.01-4.15). In the mean while, acid hydrolysis

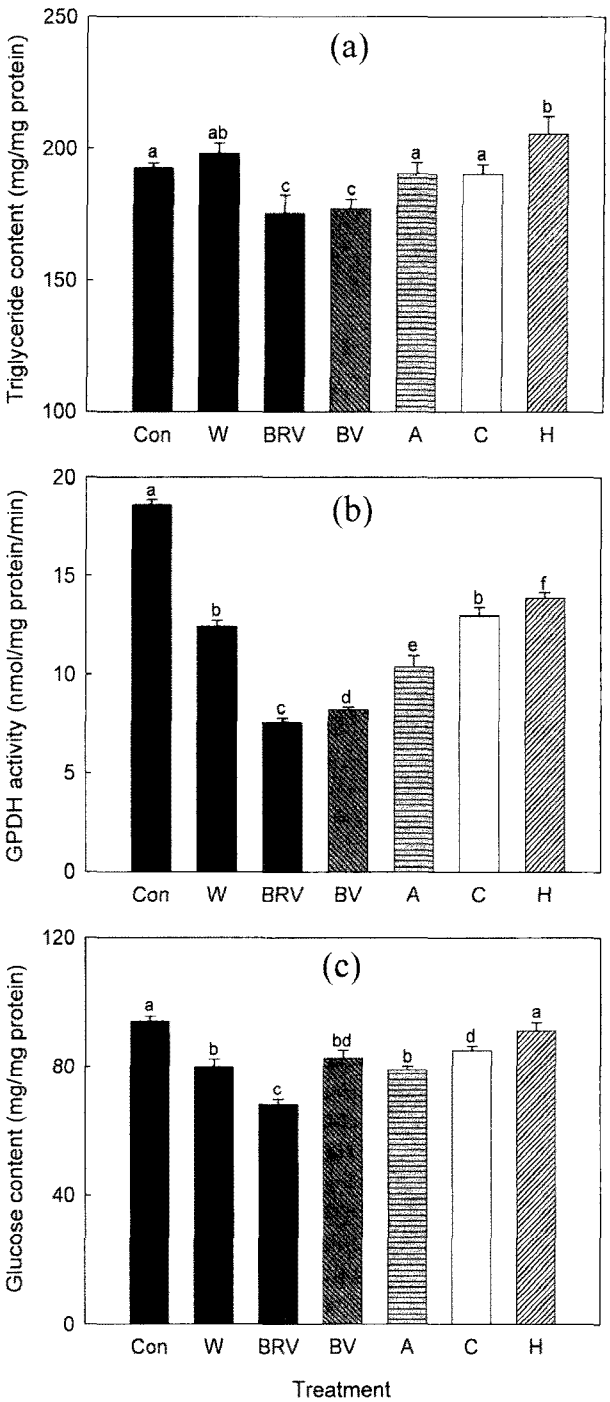

Fig. 2. The effect of RNS pickled in various solutions on lipogenesis in 3T3-L1 cells. TG content (a), GPDH activity (b), and glucose content (c) were evaluated as the indices of the cellular lipogenesis. Abbreviations W, BRV, BV, A, C, and H designated the treatment of extracts of RNS pickled in water, brown rice vinegar, brewing vinegar, acetic acid solution, citric acid solution, and hydrochloric acid solution, respectively. Con indicated no extract treatment. Means with different letters are significantly different at $p<0.05$.

would not primarily serve for the increase of aglycones based on the fact that the heating to prevent enzyme activity resulted in the similar amount of aglycones in the seeds pickled both in vinegars and in water (32).

Their increase, however, could not be only or major cause for the anti-obesity of *chokong* because the contents (about 180 and 219 μg/g) were not significantly higher in *chokong* than the other pickled seeds (about 169 to 430 μg/g). In particular, these aglycones seemed not to have a role on the decrease of GPDH activity as reported by Iwashita *et al.* (33) who revealed this enzyme was not inhibited by genistein and rather enhanced by daidzein. Instead of those, other flavonoids with strong antioxidant activity such as quercetin, kaempferol, and isorhamnetin were revealed to suppress GPDH activity highly in 3T3-L1 cells. Another study (34) also reported that antioxidant phenolic acids including chlorogenic acid, gallic acid, coumaric acids inhibited the proliferation of 3T3-L1 cells. Therefore, some antioxidants high in RNS specially (10,11) was assumed to be important to inhibit GPDH activity, in turn, to decrease TG content, where the superior activity of *chokong* was thought to be originated from the vinegars' effectiveness for the utilization of those antioxidants. As we have previously revealed (32), in fact, vinegar was better than other solutions for eluting the antioxidant matters because of its more hypertonic property than other solutions used, which causes greater osmotic disruption of intact seed structure. Additionally, it could be postulated that the pickling in vinegar induced the higher conversion of various glucosides flavonoids into their aglycones exhibiting stronger anti-obesity effect (33,35). In other words, vinegar might support the better action of endogeneous β-glucosidase causing their conversion by presenting the more disruption of seed structure, and sustaining the optimum pH range for this enzyme during pickling process (32), even though studies on this enzymatic conversion are limited to isoflavones till now (36).

As another possible anti-obesity ingredient of *chokong*, linoleic acid, most plentiful lipid in soybean, could be considered from its inhibition property on the lipogenesis in 3T3-L1 cells (37) and the nature of extract, but it is hard to explain its exact role at present.

In summary, the treatment of *chokongs* reduced TG content in part through the decreases of GPDH activity and glucose content in 3T3-L1 adipocytes. Moreover, vinegars were superior to other acidic solutions (acetic, citric, and hydrochloric acid) of similar pH to vinegars, and water at the TG reduction. However, further work is needed to elucidate the principal components and their exact mechanisms related to the anti-obesity activity of *chokong*.

Acknowledgments

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