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Original Article

Beakdugu-tang, Traditional Korean Digestant Medicine, Inhibits Hepatic Steatosis in Insulin Resistance Cell Model with HepG2 and THP-1

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Objectives: Beakdugu-tang (BDGT) consists of three medicinal herbs, and this prescription has long been used in treatment of various digestant problem in Korea. In this study, we designed to clarify mechanisms by which Korean traditional digestive medicine, BDGT, may exert anti-hepatic steatosis effects via improved insulin resistance cell model in human hepatocellular carcinoma (HepG2) and monocyte (THP-1).
Materials and methods: The preparation of BDGT and constituents were extracted with 70% ethanol. HepG2 and THP-1 were treated with different concentrations of BDGT and constituents in the presence and absence of stimulants such as free fatty acids (FFAs) and oxidized low-density lipoprotein (ox-LDL), respectively.
Results: The BDGT and its constituents inhibited the FFAs-stimulated lipid accumulation in HepG2 cells. Ethanol extracts of *Amomum cardamomum* (ACE) improved the ox-LDL induced insulin resistance in THP-1 cells. Also, treatment of monocytic cells with ACE increased anti-hepatic steatosis related gene levels including ABCA, ABCG and SR-B1.
Conclusion: The results suggest that the ethanol extract of BDGT and its constituents potently inhibit the FFAs- and ox-LDL induced liver steatosis via improved insulin resistance.

Key Words : Beakdugu-tang, hepatic steatosis, insulin resistance, digestant medicine

Introduction

Hepatic steatosis, considered the first step in the pathophysiologic continuum of non-alcoholic fatty liver disease (NAFLD), remains as a major global health problems[1]. NAFLD, is one of the types of fatty liver which occurs when fat is deposited in the liver, is also closely related with type 2 diabetes mellitus[2,3]. Type 2 diabetes mellitus is a metabolic disease characterized by insulin resistance (IR) and high blood glucose level[4,5]. Metformin, the first choice treatment for the type 2 diabetes, is known to suppress gluconeogenesis across various conditions of diabetes such as prediabetes and gestational diabetes[6-8]. However, metformin has relatively well known common side effects including diarrhea, nausea and abdominal pain. The mechanisms of these side effects of modern synthetic drugs used to

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treat hepatic steatosis remain unclear. Recently, traditional medicines and plant-derived drugs might be synergetic effects on the treatment of type 2 diabetes[9,10].

Beakdugu-tang, a traditional herbal prescription used for treatment of digestive disorders including coldness vomiting, consists of three medicinal herbs, *Ammomum cardamomum*, *Amomum xanthoides*, and *Pinellia ternate*. *In vitro* and *in vivo* studies have demonstrates that *Amomum cardamomum* protects against carbon tetrachloride-induced liver injury in our previously report[11]. Currently, circulating bile acids have been reported in patients related NAFLD[12]. For these reasons, traditional digestant have been used as an alternative to treat diverse human metabolic disorders and to maintain healthy conditions. This requires the discovery and development of products from traditional sources that may provide complementary and alternative interventions to recently approved medicinal herbs[13,14].

In this research, we prepared ethanol extracted Beakdugu-tang (BDGT) and constitutional herbs with the aim of improving its insulin resistance. Using a FFAs-induced human hepatocellular carcinoma and ox-LDL stimulated human monocytes with advanced molecular tools for lipid accumulation and gene expression. Our results indicate that BDGT and its constituents inhibit hepatic steatosis by decreasing intracellular TG in HepG2 and recover insulin sensitivity-related gene levels in THP-1.

Materials and methods

1. Cell Culture

HepG2, human hepatocellular carcinoma cell, was provided by Korean Cell Line Bank (KCLB, Seoul, Korea), and cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco). Human monocytic cell, THP-1 was also obtained from KCLB, and cultured in RPMI-1640 (Welgene, Korea) with 10% FBS and 1% antibiotics. Cells were incubated at 37°C in a humidified chamber contacting 5% CO_2. For all experiments, early passage cells were maintained and subcultured at 70~80% confluence and made quiescent by starvation for at least 24 h.

2. Preparation of 70% Ethanol Extract

BDGT and its three constituent herbs, *Ammomom cardamomum* (ACE), *Amomum xanthoides* (AXE), and *Pinellia ternate* (PTE) were purchased from Human Herb (Gyongsan, Korea). Ethanol extract of BDGT was prepared by mixing BDGT constituent herbs with 1:1:1 ratio, and respective materials also were extracted with 70% ethanol. All extracts were stirred at room temperature for three days, and then ethanol residue were evaporated using rotary evaporator at 50°C. Powder form samples were obtained by Labconco freezed-dry system (MO, USA). The yields of BDGT, ACE, AXE and PTE were 6.39%, 8.24%, 14.49% and 2.05%, respectively.

3. Cell Viability Assay

HepG2 cells seeded at a density of 1×10^5 cells/well in 96 well culture plates. The cells were then incubated with different concentrations of samples in DMEM for 24 h. THP-1, another cells for this experiment, were primed with 100 nM PMA for 24 h at a density of 5×10^5 cells/well in 96 well plates. The cell viability was determined using the EZ-Cytox cell viability assay Kit (Daeil Lab. Service, Seoul, Korea) as described by the manufacture. Briefly, 10 μl of the EZ-Cytox reagent was added to each culture well and incubated at 37°C in the CO_2 incubator for 2 h. After incubation, the viability of cells was measured at a wavelength of 450 nm using a microplate reader (VersaMax, Molecular Devices, USA).

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4. Determination of Intracellular Triglycerides (TG)

For experimental purpose, HepG2 cells were seeded at a density of 1×10^5 cells/well in 24 well plates. After 24 h incubation, the supernatant was discarded and replaced with fresh DMEM containing 1% bovine serum albumin (BSA). Hepatic steatosis conditions were induced by treating the cells in plates for another 24 h with a mixture of free fatty acids (FFAs, oleic acid : palmitic acid, 2:1, w/w). HepG2 cells were washed with ice-cold phosphate -buffered saline (PBS), scraped and transferred into tubes and then centrifuged at 3,000 rpm for 5 min. Intracellular triglycerides (TG) was measured using a Triglyceride assay kit (Asan pharmacology, Seoul, Korea) as described by the manufacture.

5. Lipid Accumulation Test (Oil Red O assay)

For this experiment, HepG2 cells were seeded at a density of 5×10^5 cells/well in 6 well plates. After the completion of desired treatment schedule, cells were washed with PBS and then fixed using 10% formalin solution for 5 min at room temperature. After fixation, cells were washed with 60% isopropanol and then stained with the working solution of Oil Red O in 60% isopropanol for 15 min. Result images were obtained by an inverted microscope (Olympus, CKX41, Japan), and the resultant solutions were measured at 520 nm using microplate reader.

6. Quantitative Real-time Polymerase Chain Reaction (qPCR)

For qPCR purpose, THP-1 cells were seeded at a density of 5×10^5 cells/ml in 100 mm culture plates. After the completion of desired treatment schedule, total RNA was isolated from THP-1 cells using TRIZOL reagent (Invitrogen, USA) according to manufacturer's instructions. For reverse transcription, a cDNA was synthesized from 1 μg of RNA using AccuPower RT PreMix kit (Bioneer, Korea) according to manufacturer's protocol. The cDNA was amplified by PCR with the following primers: ABCA, 5'-AACAGTTTGTGGCCCTTTTG-3' (sense) and 5'-AGTTCCAGGCTGGGGTACTT-3'(antisense); ABCG, 5'-GGTTCTTCGTCAGCTTCGAC-3' (sense) and 5'-GTTTCCTGGCATTCAGGTGT-3' (antisense); SR-B1, 5'-CTGTGGGTGAGATCAGGTGT-3' (sense) and 5'-GCCAGAAGTCAACCTTGCTC-3' (antosense); GAPDH, 5'-GGCCTCCAAGGAGTAAGACC-3' (sense) and 5'-AGGGGTCTACATGGCACCTG-3' (antisense). PCR amplification comprised 10 min at 95°C for 10 s, annealing at 52°C for 30 s, and extension at 72°C for 15 s. Results were analyzed using Light Cycler software (Roche Applied Science, Switzerland).

7. Determination of Glucose Uptake

In ox-LDL induced insulin resistance THP-1 cells model, glucose uptake was measured by 2-(N-(7-nirobenz-2-oxa1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG, Invitrogen) according to manufacturer's protocol. Cells were seeded on bottom clearly black well plate and then 50 μg/ml of ox-LDL exposure in each well plate. After the completion of desired treatment schedule, fluorescent images were obtained by a fluorescent microscope (Olympus, IX73), and the resultant solution were measured at excitation 485nm, emission 535nm using fluorescent microplate reader (Gemini EM, Molecular Devices).

8. Statistical Analyses

The results are expressed as mean ± standard deviation (SD), and all experimental data were analyzed by Tukey's Multiple Comparison Test using One-Way Anova on the Graph Pad prism 5.0 software (CA, USA). A $p\text{-value} < 0.05$ was considered statistically significant.

Results and discussion

In the present study, the inhibitory effect of BDGT and ACE on FFAs-induced hepatic steatosis or ox-LDL stimulated insulin resistance *in vitro* model, including HepG2 and THP-1 cell lines are investigated. BDGT is a traditional herbal prescription used for treatment of digestant, and its major medicinal herb is *Amomoum cardamomum* L., a member of Zingiberaceae, which are used as a spice in many countries and traditionally as a therapeutic for relief of dyspepsia, hiccupping, vomiting, and alcohol detoxification[15].

As the results, there was no significant toxicity shown by BDGT treatment to HepG2 cell for 24 h under 25 μg/ml concentrations. Among the ethanol extracted, ACE displayed the HepG2 cell viability for 24 h under 50 μg/ml concentrations by 84 % (Fig. 1A). Co-treatment of HepG2 with BDGT and its constituents resulted in markedly inhibited FFA-induced production of intracellular TG. Furthermore, exposure of FFAs-induced cells to ACE pretreatment (10 and 50 μg/ml) significantly decreased TG levels of lipid accumulation in a dose-dependent manner (1.05 and 0.98, respectively) (Fig. 1B). Several reports showed a decrease in the TG in the intracellular level of TG by the traditional medicines[16,17]. To investigate the effect of BDGT and ACE treatment on lipid accumulation, HepG2 cells exhibited typical morphological features of lipid staining in a dose dependent manner (Fig. 2A). In Both concentrations of ACE ameliorated hepatic lipid accumulation induced by FFAs. The ORO stain strongly appeared in lesser portion of HepG2 cells in ACE 50 μg/ml compared with FFAs exposure group (Fig. 2B). These results indicate that hyperlipidemia is closely related to the onset and development of hepatic steatosis.

Next, a qPCR assay was used to further characterize the insulin resistance-related genes expression observed.

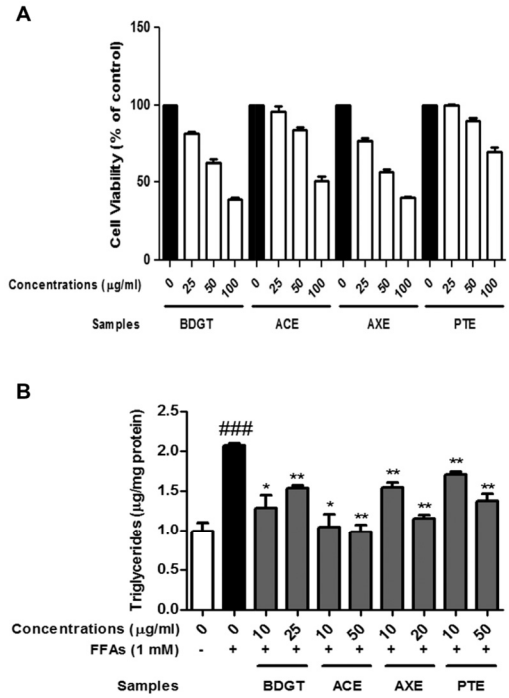

Fig. 1. The effect of Beakdugu-tang and the three constitutional herbs extract on cell viability (A) and FFAs-induced intracellular triglyceride levels (B) in HepG2 cells. Cell viability and intracellular triglycerides were determined using the cell viability and triglyceride detection kits, respectively. All data represent the means ± SD of three different experiments. Asterisk indicates statistically significant difference between FFAs-induced group (* 〈 0.05, ** 〈 0.01).

Pravastatin, used as positive control in this study, is a member of the drug class of statins, treated in combination with diet and exercise for lowering cholesterol[18,19]. ACE alone had no cytotoxicity on THP-1 cell viability at 10 μg/ml concentration by 103.7% (Fig. 3A). In agreement with this, we found that exposure of THP-1 cells to oxidized LDL at 50 μg/ml resulted in potently down-regulated intracellular gene levels including ABCA, ABCG and SR-B1 (1.15 fold, 0.34 fold and 0.47 fold to control, respectively) (Fig. 3B, C, and D). Besides, pravastatin (20 μM) treatment successfully increased insulin resistance-related genes. In previous studies,

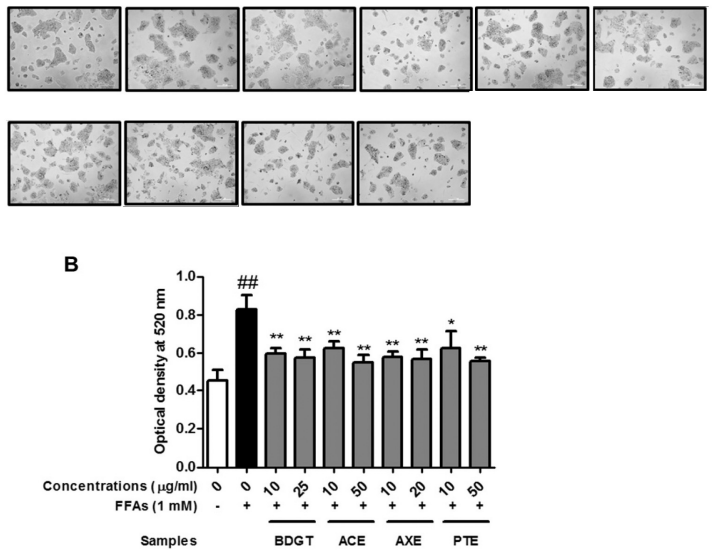

Fig. 2. The effect of Beakdugu-tang and the three constitutional herbs extract on FFAs-stimulated lipid accumulation. (A), Microscopic images and (B), resultant solutions were measured at 520 nm. All data represent the means ± SD of three different experiments. Asterisk indicates statistically significant difference between FFAs-induced group (* 〈 0.05, ** 〈 0.01).

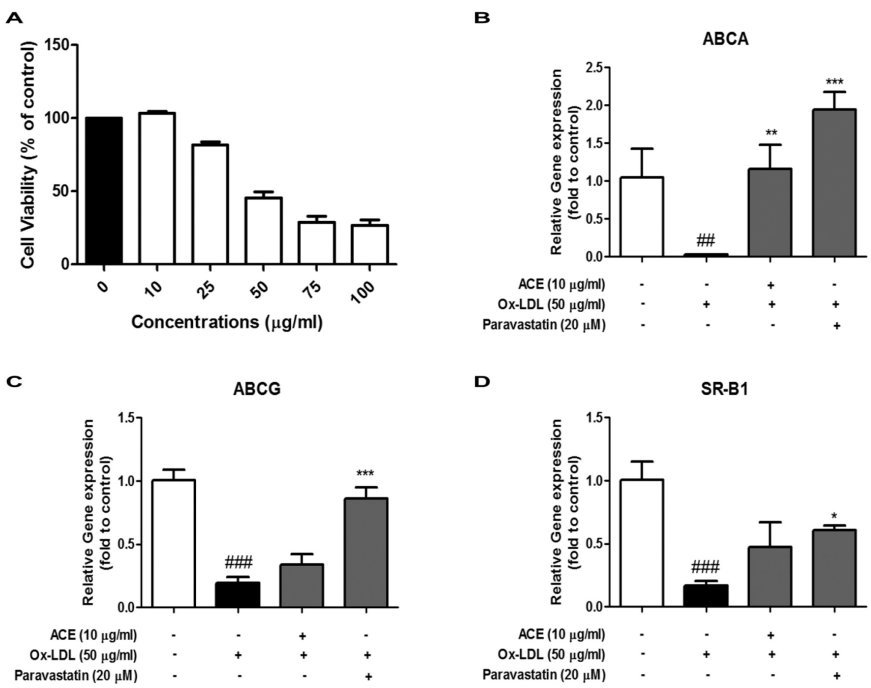

Fig. 3. The effects of *Amomum cardamomum* ethanol extract on THP-1 cell viability (A) and insulin resistance-related genes expression (B~D). All data represent the means ± SD of three different experiments. Asterisk indicates statistically significant difference between ox-LDL-stimulated group (* 〈 0.05, ** 〈 0.01, *** 〈 0.005).

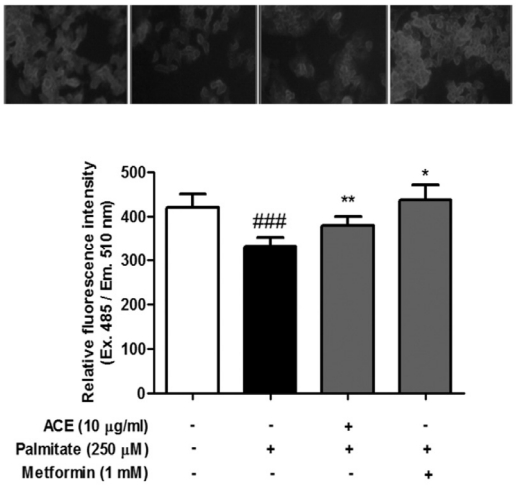

Fig. 4. The effects of *Amomum cardamomum* ethanol extract on ox-LDL-stimulated glucose uptake. (A), Fluorescence microscopic images and (B), resultant were measured at excitation 485 nm, emission 535 nm. All data represent the means ± SD of three different experiments. Asterisk indicates statistically significant difference between ox-LDL-stimulated group (* 〈 0.05, ** 〈 0.01).

insulin-resistance has been closely associated with numerous human diseases, such as type 2 diabetes, NAFLD, NASH and cardiovascular damage[20-22]. The present results show that exposure to oxidized LDL caused significant decrease in ABCA, ABCG, and SR-B1 mRNA levels due to insulin-resistance in THP-1 cells. However, pretreatment of ACE (10 μg/ml) markedly recovered insulin-resistance related-genes, which might be due to the phenolic compounds.

Finally, we demonstrate that ACE alleviated the palmitic acid-induced glucose uptake (Fig. 4A, B). The glucose uptake rate was significantly improved in free fatty acid-induced THP-1 cells at all measurement in response to the pre-treatment with both ACE (10 μg/ml) and metformin (1 mM).

Conclusion

The present study demonstrates for the first time the anti-hepatic steatosis effect of Korean traditional digestant improved insulin resistance. BDGT treatment alleviated lipid accumulation through down-regulated intracellular TG levels. Especially ACE, well known BDGT constituent, also significantly decreased intracellular TG on FFAs-induced hepatic steatosis *in vitro* model. ACE also recovered insulin resistant -related genes expression on ox-LDL stimulated THP-1 cells. In addition, ACE alleviated the palmitic acid-induced glucose uptake. These finding suggest that BDGT and ethanol extract of Amomum cardamoum could be therapeutic as a tradition herbal remedy in hepatic steatosis through improved insulin resistance.

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Competing Interests

The authors declare that they have no competing interests.

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