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The Juhwangjam (JH) 3rd day of fifth instar silkworm exhibits antioxidative properties and prevents high-fat diet-induced hypercholesterolemia

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Abstract

The silkworm is a food material that can simultaneously ingest phytochemicals from mulberry leaves, proteins with essential amino acids, and fatty acids. They are known to have hypoglycemic properties; however, further functional investigation is needed. In this study, four varieties of 3rd day of fifth instar silkworm with different cocoon colors, namely Baegokjam (BG), Goldensilk (GS), Yeonnokjam (YN), and Juhwangjam (JH), were compared in terms of antioxidant properties and cholesterol-lowering effect. JH, which had the highest polyphenol content (+38% vs. GS, $p<0.05$) showed high antioxidant efficacy. Treatment with JH also resulted in the lowest cholesterol biosynthesis enzyme activity (28% vs. control, $p<0.05$). In the animal study, high-fat diet (HFD)-fed mice that were orally administered JH extract for 12 weeks showed lower body weight gain (-10.4% vs. HFD, $p<0.05$) and serum total cholesterol levels (-12.7% vs. HFD, $p<0.05$). Comparing the varieties, JH had the highest effect. In future studies, analysis of the active ingredients according to their variety should be done.

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Introduction

Sericulture produces various bioactive products including 3rd day of fifth instar larva, mature silkworms, pupae, and silk peptides. Among the silkworm products, larvae are considered as a functional ingredient in Korea because of their hypoglycemic effect. The bioactive compound in silkworm larvae, 1-deoxynojirimycin (1-DNJ), has a structure similar to that of glucose alone. This structural similarity acts as an inhibitor of α-glucosidase, which is a polysaccharide-degrading enzyme. Therefore, it delays the absorption of glucose after a meal and helps maintain normal blood glucose levels. 1-DNJ in silkworms is derived from mulberry leaves, with which silkworm are fed and accumulate at a high density. Similarly, the polyphenol components of mulberry leaves have also been detected in silkworms. The silkworm is a food source that can simultaneously consume the bioactive components of mulberry leaves as well as insect-derived proteins and lipids.

Silkworms exhibit various bioactivities depending on their growth stage. Mature silkworms including hongjam have an effect on Parkinson's disease, improves memory in mild cognitive deficiency animal model (Nguyen *et al*., 2016), protective effect on liver and gastrointestinal tract (Park *et al*.,

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2022). In addition, differences in nutritional content have been observed depending on the variety (Ji *et al*., 2016). Silk affects bone regeneration and antibacterial activity, and differences also exist depending on the variety (Lee *et al*., 2022; Ghalei and Handa, 2022). Silkworms produce different cocoon colors depending on their variety. This is likely due to differences in pigment absorption, metabolism, and accumulation processes in the silk gland. Further studies on the pigment components of the cocoons are required.

In a previous study by Kim *et al*. (2021), the Baegokjam (BG) variety was reported to show hypocholesterolemic effects through various mechanisms, including inhibition of cholesterol oxidation, biosynthesis, and accumulation. In this study, we compared four types of silkworm varieties with different cocoon colors. The selected silkworm varieties were BG, Golden silk (GS), Yeonnokjam (YN), and Juhwangjam (JH), which are white, yellow, yellow-green, and orange in color, respectively (Kang *et al*., 2011; Kim *et al*., 2020; Kweon *et al*., 2012; Lee *et al*., 1984). The polyphenol content, antioxidant activity, and inhibitory effects on HMG-CoA reductase (HMGCR) were compared. In addition, the lipid-lowering effect of the JH silkworm variety in a high-fat diet (HFD) mouse model was examined.

Materials and methods

Sample preparation and extraction

Silkworms with four different cocoon colors were selected for the study: BG, GS, YN, and JH. These silkworm larvae (3rd day of fifth instar) were obtained from the National Institute of Agricultural Sciences (Wanju, Korea). Silkworm larvae were lyophilized and used for extraction. Extraction was performed at 25 ℃ using 50% ethanol as the solvent for 2hr which showed a high extraction rate of phenolic components in a previous study (Kim *et al*., 2021).

Determination of total polyphenol content

The total phenolic content of the silkworm larvae was measured using the Folin-Ciocalteu colorimetric assay. First, 10 μL of larvae extract was added to 200 μL of a 2% Na_2CO_3 solution and stand for 3 min at 25 ℃. Subsequently, 50% Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) was added (10 μL) and mixed by pipetting then stand for another 3 min at 25 ℃. The total phenolic content was measured by absorbance of 750 nm. The absorbance was converted to mg gallic acid equivalents (GAE)/g of sample. Gallic acid was the reference material (Lee *et al*., 2018).

Free radical-scavenging activity

Antioxidant activity was measured using the radical reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The DPPH was dissolved in ethanol as a 0.2 mM solution. To prepare the ABTS solution, a mixture of 7 mM ABTS (Sigma-Aldrich) and 2.5 mM potassium persulfate (Sigma-Aldrich) was allowed to react for 24 h without light. The larvae extracts (10 μL) were transferred to a 96-well plate. DPPH solution (200 μL) or ABTS solution (200 μL) was added to each well and stand for 10 min at 25 ℃. The color changes were measured by spectrophotometer (Multiskan GO; Thermo Fisher, Waltham, MA, USA) at 520 nm for DPPH scavenging assay and 734 nm for ABTS scavenging assay. Trolox was the standard compounds and the levels of each radical scavenging activity were calculated as mg Trolox equivalents (TE)/g of samples (Lee *et al*., 2019).

Determination of HMGCR activity

The enzyme assay was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, silkworm extracts and a positive control (pravastatin) were aliquot (1 μL) into 96-well plate. The assay buffer, nicotinamide adenine dinucleotide phosphate substrate solution, and HMGCR was mixed prior to assay and added to each test well. The HMGCR enzyme activity was measured at 340 nm (Multiskan GO) every 30 s for 10 min (Kim *et al*., 2021).

Animal care and plasma analysis

Male C57BL/6 mice (7 weeks old) were purchased from Doo Yeol Biotech (Seoul, Korea) and maintained at 23±1℃ and 56% relative humidity. They were fed a 60% HFD with or without silkworm extract (JH variety, 50% EtoH). The test samples were orally administered food every day for 12 weeks. To analyze plasma lipid levels, mice were sacrificed and their blood samples were collected through the abdominal vein. Plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels were measured using a blood analyzer (XL-200; Erba). The animal experiments were preceded according to Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health of

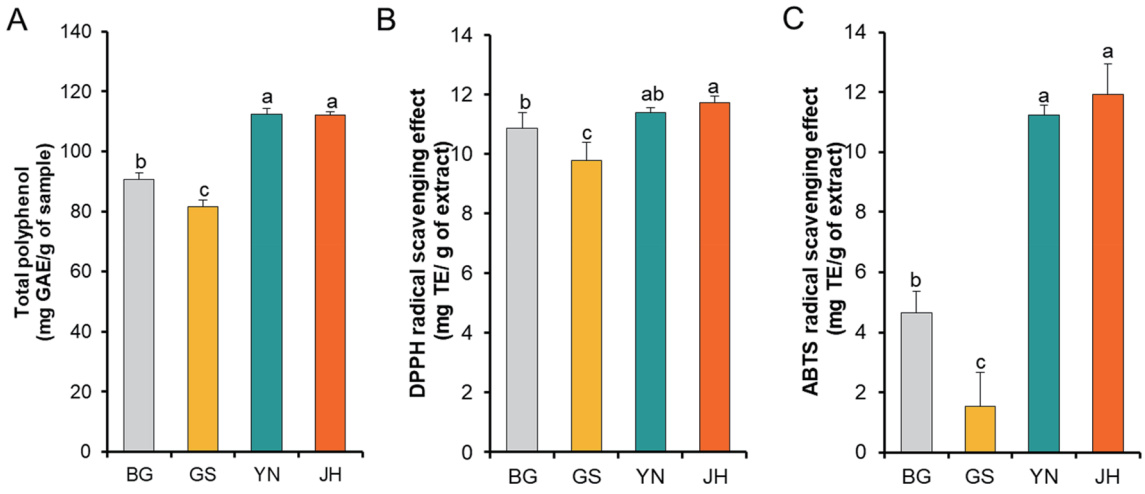

Fig. 1. Antioxidant activity and polyphenol content of silkworm extracts. (A) Total polyphenol content, (B) DPPH radical scavenging effect, and (C) ABTS radical scavenging effect were compared among the four types of silkworm varieties. BG, Baegokjam; GS, Goldensilk; YN, Yeonnokjam; JH, Juhwangjam; GAE, gallic acid equivalent; TE, trolox equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). The different letters indicate significant difference ($p<0.05$) according to Duncan's analysis of variance (ANOVA). The data represents mean±SD.

Korea. And the experiment was approved by the Institutional Animal Care and Use Committee of KIOM (approval number KIOM-D-22-010).

Statistical analysis

The results are presented as the mean ± standard deviation. Analysis of variance was performed using the SAS Enterprise Guide 7.1 program (SAS Institute Inc., Cary, NC, USA) with Duncan's multiple range tests or Student's t-test. Statistical significance was set at p-value < 0.05.

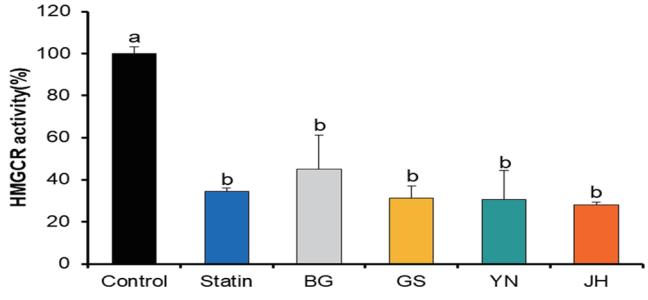

Fig. 2. Relative HMGCR activity after treatment with silkworm extract. Comparison of the HMGCR inhibitory effects of the four types of. A statin (pravastatin) was used as a positive control for HMGCR inhibition. Different letters indicate significant differences ($p<0.05$), according to Duncan's ANOVA. The data represents mean±SD.

Results and Discussion

Effect of silkworm extracts on free radical scavenging

The amount of polyphenol, which is a representative antioxidant group that has radical removal ability, was compared among the silkworm varieties. JH showed the highest antioxidant efficacy, while YN showed a similar effect. The total polyphenol content and DPPH- and ABTS-radical scavenging activities were 38%, 21%, and 672% higher in JH than in GS, respectively ($p<0.05$). Polyphenols are secondary metabolites of plants; however, they are detected in silkworms due to intake of mulberry leaves. Flavonoids commonly found in silkworms are rutin, myricetin, trans-resveratrol, luteolin, and kaempferol, which are similar to the components of mulberry leaves (Wannee and Luchai, 2020). Polyphenol content was found to be correlated with antioxidant activity. Polyphenols prevent oxidative damage via radical stabilization (Perron and Brumaghim, 2009).

Effect of silkworm extracts on HMGCR inhibition

Cholesterol synthesis in the body occurs through reactions involving more than 20 enzymes. Among these enzymes, HMGCR is a rate-limiting enzyme that converts HMG-CoA

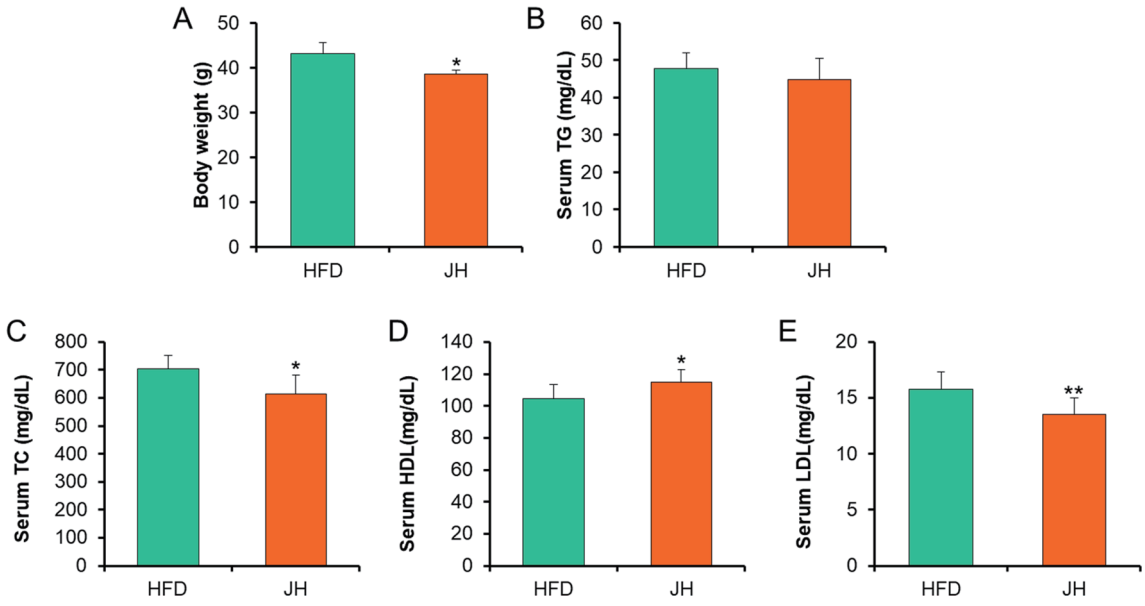

Fig. 3. Animal study using HFD-fed mice treated with JH extract. The mice were orally administered JH extract for 12 weeks during HFD feeding. Serum samples were collected, and the levels of biomarkers for metabolic disorders were measured. (A) Body weight, (B) serum TG, (C) serum TC, (D) serum HDL, and (E) serum LDL levels. HFD, high-fat diet; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; and LDL, low-density lipoprotein. *p<0.05 and ** p <0.01 vs. the HFD group.

to mevalonic acid. HMGCR inhibitors effectively inhibit this process, thereby reducing cholesterol synthesis and preventing cardiovascular diseases by lowering serum cholesterol levels (Sharpe and Brown, 2013). In this study, the inhibition of HMGCR activity by four silkworm varieties and a statin (pravastatin), cholesterol-lowering medicine, was compared. Among the silkworm varieties, JH resulted in the lowest HMGCR activity (28%). All silkworm treatment groups showed significantly reduced HMGCR levels ($p<0.05$); however, no differences were observed between varieties.

Effect of JH administration on serum cholesterol level in HFD-fed mice

In an in vitro experiment that examined the antioxidant activity and HMGCR inhibitory ability of the silkworm varieties, JH was found to be the best variety. Therefore, animal experiments were conducted using JH. Mice were fed the silkworm extract along with an HFD for 12 weeks. This confirms the efficacy of preventing obesity when silkworms are fed a high-calorie diet. Silkworm consumption for 12 weeks suppressed body weight gain by 10.4% compared with that in the HFD group. In the JH-fed group, serum TG, TC, and LDL levels decreased by 6.3%, 12.7%, and 14.6%, respectively, while HDL increased by 9.9%. In addition, cholesterol-related markers were significantly altered compared with the HFD group ($p<0.05$). Polyphenol content and cholesterol metabolism were found to be strongly correlated. In previous studies, polyphenol-fed groups induced bile acid excretion and lowered total and LDL levels. Chambers *et al*. (2019) reported that the results were due to reverse cholesterol transport with increased CYP7A1 gene expression, reduction of bile acid transporters in the small intestine, and changes in the intestinal microbiota. To identify the active substance of JH, an accurate component analysis according to the silkworm variety is needed.

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