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Epigallocatechin-3-Gallate Reduces Fat Accumulation in *Caenorhabditis elegans.*

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ABSTRACT: Epigallocatechin gallate (EGCG) is a polyphenol that is abundant in green tea. It has been reported that consumption of EGCG can contribute to weight loss, however, the underlying mechanism is not fully understood. To determine how EGCG reduces body fat, an organism model *Caenorhabditis elegans* was used, which is a useful animal model system in exploring crucial biological mechanisms that are readily applicable to humans. In this study, different strains were raised for two days on *Escherichia coli* OP 50 diet with or without 100 μM and 200 μM EGCG treatment. The current results showed that 100 μM and 200 μM EGCG significantly reduced the triglyceride content of wild type worms by 10% and 20% (P-value<0.01 and <0.001, respectively) compared to the control, respectively, without affecting its food intake and physiological behaviors. Additionally, EGCG could effectively reduce fat accumulation in *C. elegans* dependent on *atgl-1* (encoding a homolog of adipose triglyceride lipase), which suggests that EGCG controls the body fat by inhibiting adipogenesis.

Keywords: *Caenorhabditis elegans*, EGCG, fat accumulation, lipid metabolism

INTRODUCTION

Green tea is a popular beverage with a high content of flavonoids (a natural polyphenol) and is known to have many desirable health benefits, such as anti-carcinogenic, anti-inflammatory, and anti-oxidative effects (1). Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea, which is believed to be responsible for those bioactivities in green tea (2). EGCG has been reported to have fat reduction effects by inhibiting energy intake in diet-induced obese mice (3,4), inhibiting lipogenesis *in vitro* (5,6), inhibiting α-amylase activity *in vitro* (7), inhibiting lipid digestion and absorption in high fat-fed mice (8), stimulating energy expenditure *in vivo* (9), promoting fat oxidation both *in vivo* (10) and *in vitro* (11), and promoting lipolysis (12,13).

Caenorhabditis elegans has been used extensively in biological and medical studies due to their short life span of ~20 days, a reproductive cycle of 3 days, and a large brood size of about 300 eggs by self-fertilization (14). Moreover, it conserves 65% of genes related to human diseases (15), including those related to lipid metabolism, which makes it a great *in vivo* model for cellular and genetic studies. In *C. elegans*, EGCG has been previously shown to display antioxidant activities (16), reduce stress related responses (17), and extend longevity (18). However, there is no report of EGCG on the fat reduction effect in *C. elegans*. Therefore, this study was to examine whether EGCG could play a role in reducing fat content in *C. elegans*.

MATERIALS AND METHODS

Materials

The *C. elegans* strains and *Escherichia coli* OP50 used in this study were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA), including N2, bristal (wildtype); CE541, *sbp-1 (ep79) III*; RB754, *aak-2 (ok524) X*; RB1716, *nhr-49 (ok2165) I*; BX107, *fat-5 (tm420) V*; BX106, *fat-6 (tm331) IV*; BX153, *fat-7 (wa36) V*; RB1600, *tub-1 (ok1972) II*; GR1307, *daf-16 (mgdf50)*; OP50 and OP50-green fluorescent protein (GFP) *E. coli*. EGCG (purity >99%) and the Infinity™ Triglycerides Reagent were purchased from Fisher Scientific (Pittsburgh, PA, USA). The Coomassie Plus Protein Assay Reagent was obtained from Thermo Fisher Scientific (Middletown, VA, USA). 5-Fluoro-2'-deoxyuridine (FUdR) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Household bleach (The Clorox

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Company, Oakland, CA, USA) was used for bleaching the worms when synchronizing L1 worms.

Preparation of EGCG solution and worm culture

The EGCG was dissolved in sterilized water and filtered through a 0.22 μm-diameter membrane prior to use. Previously, it was reported that 200~400 μM EGCG was the optimal concentration for increasing lifespan in *C. elegans* (19), thus, we chose 100 μM and 200 μM EGCG as treatment groups to study the fat reducing effects in *C. elegans*.

M9 buffer, S-complete, and nematode growth media (NGM) agar were used in the *C. elegans* cultures (20). After synchronizing, all L1 worms were raised at 25°C in S-complete media supplemented with *E. coli* OP50 and treated with or without EGCG for 2 days.

Triglyceride quantification

After 2 days of EGCG treatment, *C. elegans* was collected and washed twice with water to remove *E. coli* and EGCG. *C. elegans* samples were dissolved in 0.05% Tween 20 solution. After sonication, the samples were used for the triglyceride (TG) and protein measurements. The TG assay was conducted with the Infinity™ Triglycerides Reagent and protein content was measured with the Coomassie Plus Protein Assay Reagent (21). TG content was then normalized with protein concentrations.

Measurement of growth rate, body size, movement, and food intake

After the 2-day treatment, nematodes were transferred to new agar plates to measure the growth rate. For each treatment group, ~50 worms were randomly selected and paralyzed using 10 mM NaN_3 (22,23). The numbers of worms at different stages were counted under an optical microscope (Olympus Corporation, Tokyo, Japan).

After the 2-day treatment, nematodes were transferred to new plates with fresh *E. coli* OP50 for the measurement of body size and movement (22). A 30-s video was recorded and used for the length, width, and moving speed of worms using the Wormlab tracking system (WormLab software version 3.1.0, MicroBrightField Inc., Williston, VT, USA).

To monitor food intake of the nematodes, age-synchronized N2 nematodes were cultivated on *E. coli* OP50-GFP bacterial lawns on NGM plates with or without EGCG (24). After treatment for 2 days, nematodes were washed twice with water, placed, and fixed onto slides, which were prepared with fresh 5% agar pads, and then visualized under a fluorescent microscope. The integrated density was quantified using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA) by determining the average pixel intensity. The pumping rate was also measured by counting the rate of pharyngeal muscle contractions from *C. elegans* under the optical microscope (25).

mRNA expression analysis

Total RNA was extracted from *C. elegans* using the TRIzol® reagent under RNase-free conditions (22,23). Total RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA, USA). Real-time polymerase chain reaction (PCR) was performed on a StepOne Plus real-time PCR system (Applied Biosystems). We used *cebp-2* (Ce02421574_g1), *hosl-1* (Ce02494529_m1), *atgl-1* (Ce02406733_g1), *mdt-15* (Ce02406575_g1), *pod-2* (Ce 02427721_g1), and *acs-2* (Ce02486193_g1) for TaqMan gene expression assays. Threshold values were analyzed using the comparative CT method. The RNA polymerase II large subunit *ama-1* gene (Ce02462726_m1) was used as an internal standard.

Statistical analysis

Data are expressed as means±standard errors (SE). Statistical analysis for all data was performed by the Statistical Analysis System (SAS version 9.4, SAS Institute, Cary, NC, USA). Data in Fig. 2B, 3A, 4, and 5 were analyzed by one-way ANOVA. Data in Fig. 1, 2A, and 3B~D were analyzed by two-way ANOVA (treatment and experiment). No interaction between treatment and experiment were found in all data. Tukey's multiple comparison test was used to determine treatment effects. Differences was defined at the $P<0.05$ level.

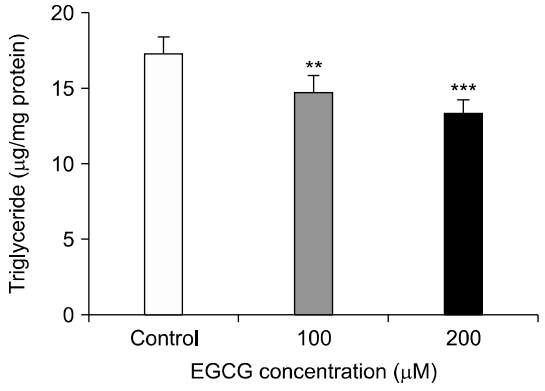

Fig. 1. Effects of epigallocatechin gallate (EGCG) on triglyceride accumulation in wild type *C. elegans*. EGCG treatment of *C. elegans* started from L1 stage for 2 days. Data are expressed as means±SE (n=11, collected from 3 independent experiments). Values with ** or *** show significant difference when compared with the control (*P*-value <0.01 or <0.001, respectively).

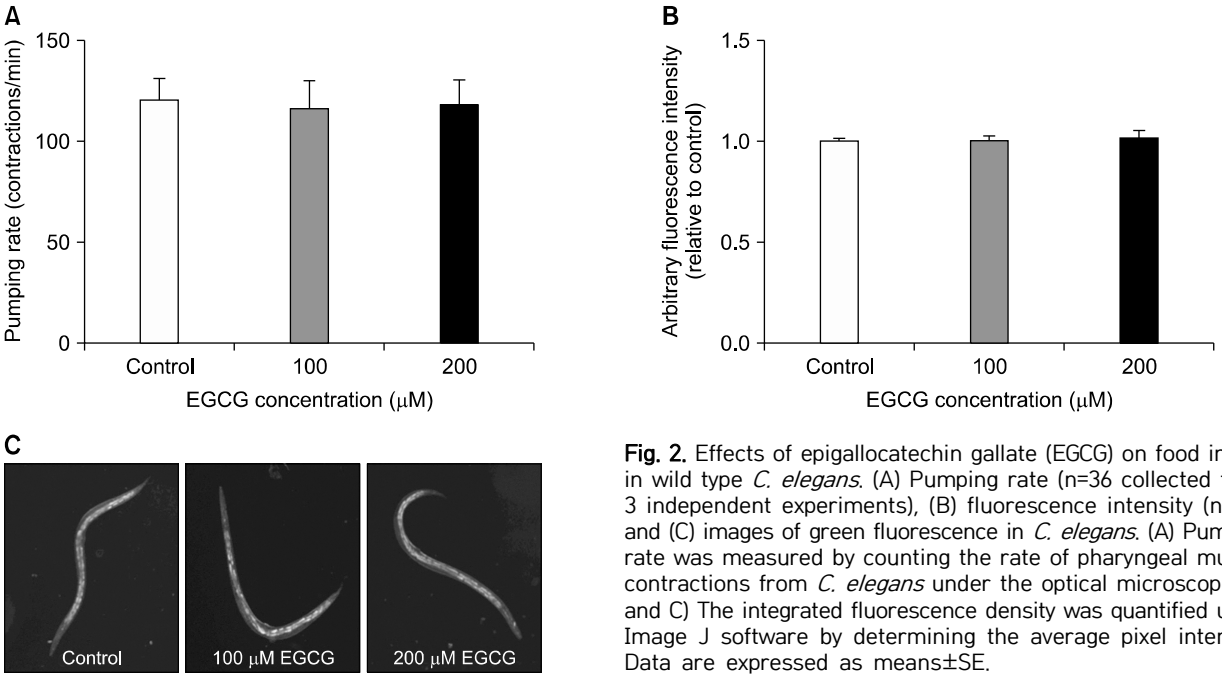

Fig. 2. Effects of epigallocatechin gallate (EGCG) on food intake in wild type *C. elegans*. (A) Pumping rate (n=36 collected from 3 independent experiments), (B) fluorescence intensity (n=12), and (C) images of green fluorescence in *C. elegans*. (A) Pumping rate was measured by counting the rate of pharyngeal muscle contractions from *C. elegans* under the optical microscope. (B and C) The integrated fluorescence density was quantified using Image J software by determining the average pixel intensity. Data are expressed as means±SE.

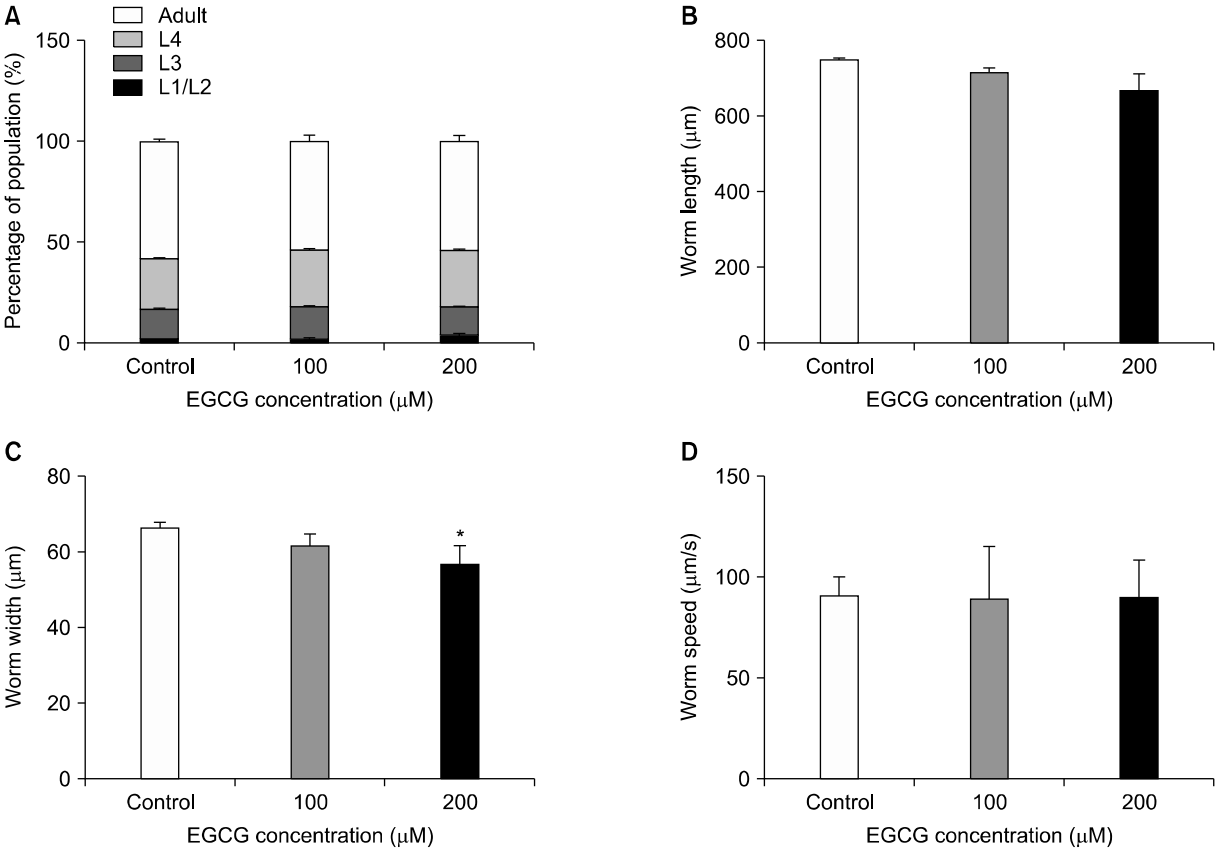

Fig. 3. Effects of epigallocatechin gallate (EGCG) on growth rate and physiological behavior in wild type *C. elegans*. (A) Growth rate: percentage of worms from L1 to adult between treatment and control groups after the 2-day treatment of EGCG. Numbers represented means±SE. (n=3 plates, each plate had ≈100 worms). (B) Worm length: average body length of N_2 worms after the 2-day treatment of EGCG. Numbers represent means±SE (n=143~155, collected from 3 independent experiments). (C) Worm width: average body width of N_2 worms after the 2-day treatment of EGCG. Numbers represent means±SE (n=143~155, collected from 3 independent experiments). (D) Worm speed: average locomotive activity of N_2 worms after the 2-day treatment of EGCG. Numbers represent means±SE (n=143~155, collected from 3 independent experiments). Value with * shows significant difference when compared with the control (*P*-value <0.05).

RESULTS

EGCG treatment decreased TG content without altering food intake

Treatment with EGCG significantly decreased the TG content in a dose-dependent manner, 10% (100 μM) and 20% (200 μM) decreases compared to the control (Fig. 1). Pumping rate is a mechanical movement, which represents food intake in *C. elegans* (25). Treatment of EGCG for 2 days had no effect on the pumping rate of wild type nematodes (Fig. 2A). We conducted another experiment using *E. coli* OP50-GFP to measure food intake. The analysis of fluorescent intensity (Fig. 2B) indicated that there was no significant difference between the treatment and control groups, which was consistent with the pumping rate. The representative images of *C. elegans* fed with *E. coli* OP50-GFP were shown in Fig. 2C. These results suggested that EGCG did not affect food intake in *C. elegans*.

EGCG had no effects on growth and development

Treatments of EGCG, at both 100 μM and 200 μM, showed no significant effect on growth rate (Fig. 3A), body length (Fig. 3B), and locomotive activities (Fig. 3D). While at 200 μM, the body width of the worms decreased compared with control (Fig. 3C), which might be due to reduced body fat observed in Fig. 1. Taken collectively, these data indicated that EGCG has no effect on the growth, body length, or locomotive activities, but has significant effect on body width at higher concentration in *C. elegans*.

EGCG potentiated lipolysis

Next, various mutant strains are known to be linked to lipid metabolism were tested, including *sbp-1* (encoding an ortholog of sterol response element binding protein), *nhr-49* (encoding nuclear hormone receptor and a functional ortholog of peroxisome proliferator-activated receptors), *aak-2* (encoding one of two homologs of the AMP-activated protein kinases), *tub-1* (encoding a homolog of TUBBY), *fat-5*, *6*, and *7* (endoding delta 9 desaturase homologs), and *daf-16* (encoding a homolog of the Forkhead box O transcription factor). Significant differences between the TG level of the control and EGCG treatment groups in these strains were observed, suggesting that effects of fat reduction by EGCG was independent to *sbp-1*, *nhr-49*, *aak-2*, *tub-1*, *fat-5*, *fat-6*, *fat-7*, and *daf-16* in *C. elegans* (Fig. 4).

We further determined expressions of genes involved in lipid metabolism in wild-type *C. elegans*, including *cebp-2* (encoding a homolog of the CCAAT/enhancer-binding protein), *acs-2* (encoding a homolog of acyl-CoA synthetase), *mdt-15* (a homolog of mediator complex subunit 15), *pod-2* (encoding a homolog of acetyl-CoA carboxylase α), *hosl-1* (encoding a homolog of hormone-sensitive lipase), and *atgl-1* (encoding a homolog of the adipose triglyceride lipase). EGCG significantly decreased the expression of *atgl-1* and *acs-2* at both 100 μM and 200 μM compared to the control, while other genes showed no difference (Fig. 5). These data indicated that the fat reduction effect of EGCG in *C. elegans* might be dependent on *acs-2* and *atgl-1*.

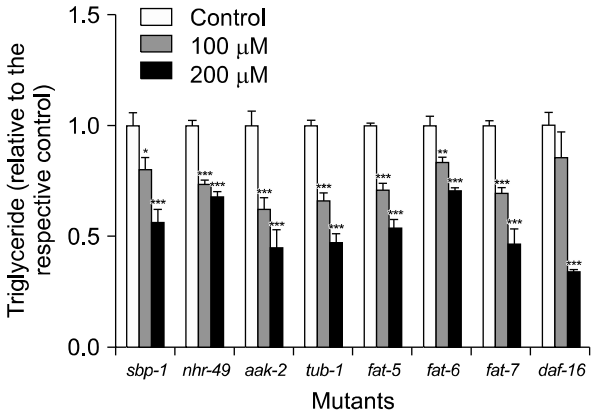

Fig. 4. Effects of epigallocatechin gallate (EGCG) on mutants involved in lipid metabolism. Numbers represent mean values± SE (n=3). Values with *, **, or *** show significant difference when compared with the respective control (*P*-value <0.05, <0.01, or <0.001, respectively).

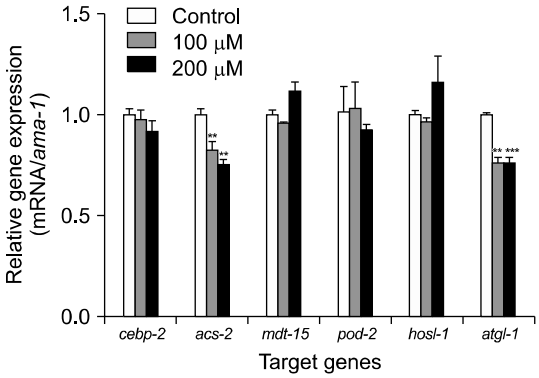

Fig. 5. Effects of epigallocatechin gallate (EGCG) on the expression of lipid metabolism-related genes in wild type *C. elegans*. Numbers represent mean values±SE (n=3). Values with ** or *** show significant difference when compared with the control (*P*-value <0.01 or <0.001, respectively).

DISCUSSION

In the present study, EGCG (100 μM and 200 μM) significantly reduced the fat accumulation in a dose-dependent manner in wild type worms without altering the food intake, growth rate, worm size, or locomotive activity. This is consistent with previous reports that EGCG treatments could reduce fat accumulation in high-fat fed rats (26) and that 100 μM EGCG decreased the lipid accumulation of 3T3-L1 preadipocytes (6). The current results fur-

ther indicated that EGCG does not influence energy intake or physical activity (as a part of energy expenditure), suggesting its metabolic involvement on fat reduction.

It was previously reported that green tea catechins could decrease lipogenesis by inhibiting the activity and/or expression of lipogenic enzymes, such as fatty acid synthase (FAS), sterol regulatory element-binding protein-1c, and stearoyl-CoA desaturase-1 in rodent animals (27). In addition, EGCG was reported to reduce fat storage by activation of AMP-activated protein kinase *in vitro* (28). However, the current results suggest that EGCG may not exert its fat reduction effects via *fat-5*, *fat-6*, *fat-7*, *sbp-1*, *nhr-49*, *daf-16*, or *aak-2* in *C. elegans*. Since we have not determine the role of EGCG in FAS, it is possible that EGCG may act via FAS along with additional lipogenic enzymes, such as fatty acid elongase, and 3-ketoacyl-CoA reductase (LET-767) (29). Alternatively, other catechins in green tea including epicatechin, epigallocatechin, and epicatechin-3-gallate (30), might contribute to decreasing fat content, not EGCG.

Tho and Wolfram (4) reported that dietary EGCG promoted fat oxidation in mice. Furthermore, one study in overweight/obese men showed that EGCG alone had the potential to promote fat oxidation and might thereby render an anti-obesity effect (10). In *C. elegans*, *acs-2* encodes an acyl-CoA synthetase, which catalyzes the conversion of a fatty acid to acyl-CoA for subsequent oxidation (31). However, the current result suggests that EGCG reduced the expression of *acs-2* in the wild type nematodes, which indicate that EGCG might inhibit fat oxidation although overall fat accumulation was still reduced in *C. elegans*. Similar discrepancies have been previously reported that low-fat phenotypes might lead to compensatory suppression of *acs-2* transcription to prevent further energy expenditure (32). Thus, we inferred that reduced expression of *acs-2* by EGCG has no significance on its effect on overall body fat in this model. Alternatively, it is possible that the other mechanisms, such as post-translational regulation of *acs-2* (33), is responsible for EGCG's fat reduction effect. This and other mechanisms may need to be further investigated.

There has been growing evidence that EGCG could enhance lipolytic activities in 3T3-L1 adipocytes (13). However, Söhle et al. (12) reported that EGCG had no contribution on the stimulation effect of white tea extract on lipolysis in human subcutaneous adipocytes. The controversial statements, therefore, suggested that EGCG had inconsistent effects on lipolysis. ATGL is an adipose triglyceride lipase, which is responsible for lipolysis (34). The current results showed that EGCG reduced the expression of *atgl-1* in wild type nematodes, suggesting that EGCG inhibit lipolysis in *C. elegans*. However, in addition to be used as a marker for lipolysis, ATGL is also known to be used as a marker for adipogenesis as it remains highly expressed in mature adipocytes (35). Thus, reduced *atgl-1* expression by EGCG might suggest inhibition of adipogenesis, rather than reduced lipolysis. This is consistent with the *in vitro* studies conducted by Hwang et al. (6) and Moon et al. (28). Overall the current results would not be enough to determine the potential mechanisms of fat reduction effects of EGCG, thus, additional research is needed to investigate the influence of *acs-2* and *atgl-1* at the post-translational level.

In summary, the current results conclude that effect of EGCG on fat reduction in *C. elegans* is dependent on *atgl-1* and *acs-2*. EGCG might inhibit adipogenesis to decrease the fat content in *C. elegans*, as shown by the decreased *atgl-1* gene expression level after EGCG treatment. Thus, consuming EGCG as a dietary supplement could potentially control the body fat content.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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