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Anti-obesity Effects of Dark Tea Extracts by Down-regulation of C/EBPα and PPARγ

HYEONG-JOON LIM[1,2], TAEK JOO LIM[2], JIN HYUN LEE[2], JUN HEE LEE[2], MYEONG-OK KIM[2], JI YUN PARK[2], JONG-TAE KIM[3], MIN-JEONG KIM[3], SEONG-HEE JANG[3] and SEOK HWA CHOI[1]

[1]*College of Veterinary Medicine, Chungbuk National University, Cheongju, Republic of Korea;*
[2]*NDIC Co., Ltd., Hwaseong, Republic of Korea;*
[3]*Teazen Co., Inc., Haenam, Republic of Korea*

Abstract. *Background/Aim: Dark tea, made by fermentation of tea leaves using microorganisms, is well known for its anti-obesity effect; however, studies to identify this effect have not been sufficiently conducted. Herein, the anti-obesity effects of post-fermented dark tea were studied in high-fat diet mouse. Materials and Methods: Obesity was induced through a high-fat diet in C57BL/6 mice, and then dark tea extract powder (DTP) was orally administered daily for 12 weeks to evaluate the body and organ weights. Changes in the biochemical markers of obesity were evaluated to study the mechanism of the anti-obesity effects of DTP. Results: When DTP was administered to obesity mice, the weight and food intake reduced, blood aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C) decreased, whereas high-density lipoprotein cholesterol (HDL-C) increased. Histopathology showed that steatosis and inflammation scores were reduced within the liver and adipocyte sizes were reduced within epididymal adipocyte. In addition, a significant decrease in blood insulin and hepatic TG and a significant increase in blood adiponectin were also confirmed. The results of western blot and qPCR in week 12, showed a significant decrease in the mRNA and protein levels of C/EBPα, and the mRNA levels of PPARγ in the liver. Conclusion: Dark tea extracts are thought to have an anti-obesity effect by reducing the levels of the main transcription factors that promote adipocyte differentiation, such as C/EBPα, and PPARγ. Therefore, diet products using dark tea extracts could be developed.*

Correspondence to: Seok Hwa Choi, DVM, Ph.D., College of Veterinary Medicine, Chungbuk National University, 1 Chungdae-ro, Seowon-gu, Cheongju, Chungbuk 28644, Republic of Korea. Tel: +82 432613144, Fax: +82 432673150, e-mail: shchoi@cbnu.ac.kr

Key Words: Anti-obesity, anti-lipogenesis, dark tea (*Camella sinensis* L.), C/EBPα, PPARγ, *Eurotium cristatum*.

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Plant-derived natural foods are used as a potential material in various areas such as health, medicine, and beauty products in the modern world (1). In particular, westernized food culture causes lifestyle diseases such as obesity, diabetes, and cardiovascular disease, and increases unsaturated fatty acids in the body with high-fat consumption promoting differentiation of mastocytes that increases inflammation (2). Substances that can help reduce body fat to improve obesity are limited to conjugated linoleic acid (3), *Garcinia cambogia* (4), chitosan (5), and chito oligosaccharides (6). While various other substances have been developed for individual certification, no new substances have been shown to have a continual effect.

Tea is one of the most widely consumed drinks, only second to water, and has been spread in more than 160 countries and regions in the world (7). The functions of tea are closely related to its active chemical components, such as tea polyphenols, amino acids, and alkaloids. One of its essential functions is lipid-lowering and anti-hypolipidemic effects on the reduction of body fat and hepatic lipid, lean tissues increasement, and prevention of hepatic steatosis and fatty liver verified in numerous preceding investigations (8-11).

The tea culture in improving obesity using various kinds of tea has been developed. These kinds of tea including green tea extract (12), mate tea (13), and pu-erh tea (14) have been individually certified, and their related products are being actively developed as health supplements in domestic markets, but new substances have insufficient data to show its continuous effects. Dark tea is the product of *Camellia sinensis* L. leaves that have been processed. The classification of the tea depends on the degree of fermentation and the components of tea leaves change biochemically with the action of oxidizing and hydrolyzing enzymes of tea leaves during the processing steps (15). Tea

is divided into non-fermented tea, semi-fermented tea, and fully fermented tea depending on the tea leaves' degree of action of the enzymes. Dark tea, mainly produced in the Hunan Province of China, is a post-fermented tea that goes through the first process and then is deposited again (stacking of the first-processed tea leaves) or goes through pile fermentation (spraying the water on the first-processed tea leaves and stacking them, while increasing the temperature and humidity) to generate microorganisms during storage (16, 17). Compared with other teas, the manufacture technology of dark tea is unique for the "piling store", which is the post fermentation process.

The health benefits and chemistry of dark tea are increasing trends in the research field of teas. Deactivated leaves of *Camellia sinensis* are post-fermented under controlled conditions to make dark teas, the quality of which is dependent on the microorganisms like *Aspergillus, Penicillium* and *Eurotium* species in post-fermentation processes. Fungal flowering fermentation commonly called "flowering" is the most indispensable stage for good qualities of some dark teas. During this time, *Eurotium* spp., as the predominant bacteria, grow dynamically within the tea leaves at controlled temperature and moisture conditions (18, 19). Accordingly, various trials have been performed to explore the relationship between microbial activity and metabolic changes of tea (20, 21). PPARγ and C/EBPα are critical transcription factors highly expressed in adipocytes and regulate adipocyte differentiation and lipid metabolism (22, 23). Once the differentiation of adipocytes is induced by over-expression of the two genes, their expression is accelerated during the subsequent differentiation process. Therefore, studies on the down-regulation of PPARα and C/EBPα in adipose tissue are known to contribute significantly to the development of anti-obesity substances.

Although many studies on the anti-obesity effect of dark tea's fermented substances have been conducted (24), no specific mechanism-related studies have been conducted on the anti-obesity effect of dark tea. In this study, the effect of suppressing obesity was confirmed through various evaluation indicators by administering dark tea candidate substances to obesity-induced mice through high-fat diets, and the mechanism of the anti-obesity effect was analyzed using the target tissue.

Materials and Methods

Materials. Dark tea extract powder (DTP) was provided from TEAZEN Inc. (Haenam, Republic of Korea) and *Garcinia cambogia* extract was provided by Umalaxmi Organics Pvt. Ltd. (Vadodara, Rajasthan, India). The dark tea (*Camellia sinensis* L.) was extracted as follows; dried dark tea was extracted twice in hot water (w/v, 1:8) at 80˚C for 4 h. Filtered and vacuum-concentrated under reduced pressure to attain 35 Brix or more. Then, it was blended with dextrin, followed by spray-drying to obtain a DTP. DTP and dextrin were mixed in a ratio of 9:1 and dried to obtain dark tea powder.

Animals. C57BL/6 healthy mice aged 5 weeks and weighing 16-20 g were purchased from OrientBio (Seongnam, Republic of Korea). All animal care procedures followed the Laboratory Animal Care Procedures of NDIC (Hwaseong, Republic of Korea).

Experimental design. The mice were divided into six groups (n=6 in each group): G1, Sham-vehicle control; G2, High-Fat Diet (HFD) control; G3, *Garcinia cambogia* 200 mg/kg; G4, DTP 100 mg/kg; G5, DTP 200 mg/kg; G6, DTP 400 mg/kg. After group selection, Sham-vehicle (G1) was fed 2918C Teklad global diet (Harlan Teklad; Envigo, Indianapolis, IN, USA), and the High-Fat Diet-induced groups (G2-G6) were fed D12492 diet (60% energy from fat; Research diets, New Brunswick, NJ, USA). The test articles were orally administered once a day for 12 weeks. The body weight of mice was measured once a week, and food intake was measured three times a week. Their organ weights were measured at the sacrifice, and blood, liver, and epididymal adipose tissue samples were collected for further analysis. This study was performed upon the approval of NDIC's IACUC (The Institutional Animal Care and Use Committee) (Approval Number: P202020).

Biochemical analysis. The following were measured with a blood chemistry analyzer (Blood chemistry analyzer, AU480; Beckman Coulter, Brea, CA, USA). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), blood glucose, blood lipids [total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)] (25).

Measurement of blood insulin, leptin, adiponectin, and hepatic TG. The fasting blood insulin level was measured with Rat/Mouse Insulin ELISA Kit (#EZRMI-13K; Sigma-Aldrich, St Louis, MO, USA), and the fasting blood leptin level was measured with Mouse/Rat Leptin Quantikine ELISA Kit (#MOB00B, R&D System, Minneapolis, MN, USA). The fasting blood adiponectin was measured using Mouse Adiponectin/Acrp30 Quantikine ELISA Kit (#MRP300; R&D System) through an ELISA reader (#SpectraMax M2; Molecular Devices, San Jose, CA, USA).

In order to measure hepatic TG, liver tissue was homogenized with 300 μg/ml PBS (pH 7.4) and stainless-steel beads (Qiagen, Hilden, Germany) at 30 frequency/s for 1 min using a homogenizer (TissueLyser II; Qiagen). After the centrifugation of homogenates, the hepatic TG in the supernatant was measured using TG quantification kit (Cell Biolabs, San Diego, CA, USA) through an ELISA reader (26).

Histological examination. The epididymal fat and liver were fixed in 10% neutral-buffered formalin (BBC Biochemical, Mount Vernon, WA, USA), embedded in paraffin, and sections were stained with hematoxylin (BBC Biochemical) and eosin (BBC Biochemical) and observed under a light microscope (Nikon-Eclipse 50i, Tokyo, Japan). To analyze the accumulation of lipid droplets in the liver tissue and the degree of inflammation, the analysis was performed with the application of the criteria for non-alcoholic steatosis introduced in Table I. Also, the size of the adipocytes in the frozen section of epididymal tissue was measured using the Image J program (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis and reverse transcription and quantitative polymerase chain reaction (qPCR) analysis using liver tissue. After washing the liver tissue, the cells were dissolved in the Cell lysate

Table I. *Criteria for nonalcoholic fatty liver disease (NAFLD) activity score (NAS).*

Pathological appearance	Definition	Score
Steatosis		
Grade	<5%	0
	5%-33%	1
	33%-66%	2
	>66%	3
Inflammation		
Lobular inflammation	No foci	0
	<2 foci per 200× field	1
	2-4 foci per 200× field	2
	>4 foci per 200× field	3

master mix containing 1× cell lysate buffer (Sigma-Aldrich) 86 μl, 10× phosphatase inhibitor (Roche, Santa Clara, IN, USA) 10 μl, 25× protease inhibitor (Roche) 4 μl for 30 min on ice. Then, the supernatant was collected by centrifuging at 12,000 rpm at 4˚C for 10 min, and the protein was quantified using the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and western blot analysis was performed as follows. The tissue sample was reacted with the primary antibody CCAAT/enhancer-binding protein α (C/EBPα; Cell Signaling, Danvers, MA, USA), peroxisome proliferator-activated receptor γ (PPARγ; Santa Cruz, Dallas, TX, USA), fatty acid binding protein 4 (FABP4; R&D system), protein kinase B (AKT; Santa Cruz), phosphorylated protein kinase B (pAKT-Ser473; Cell Signaling), β-actin (Cell Signaling), then reacted with the horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling). The result was analyzed with luminol (ECL) solution (Cytiva, Marlborough, MA, USA) using Amersham imager 680 (GE Healthcare, Milwaukee, IL, USA). For qPCR analysis, the total RNA was separated from the tissue sample using trizol (TRI) reagent (Sigma-Aldrich), cDNA was synthesized using the Power cDNA synthesis kit (iNtRON, Seongnam, Republic of Korea), qPCR was performed with SYBR green and each primer, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. Using the Rotor-Gene Q Series Software 2.3.1. (Qiagen), the expression of the reference gene (*GAPDH*) compared to the target gene expression has been relatively quantitatively analyzed. The primer sequences and PCR conditions of the qPCR are listed in Table II.

Statistical analysis. The data are expressed as means±S.D. (standard deviation) and represent the average values of three experiments. Analysis of variance (ANOVA) was conducted, and Duncan's multiple range tests were used to determine the significance of differences between groups. A p-value <0.05 indicated statistical significance.

Results

Body weight and food intake in HFD-fed mice. The body weight (g) decreased significantly in the group administered *Garcinia cambogia* 200 mg/kg, from 2 weeks after administration to 6 weeks, whereas for the group administered DTP 100 mg/kg, the body weight decreased significantly after 2 weeks of administration to 8 weeks, and then at 10 weeks to completion of administration in comparison with the HFD-vehicle control group. The DTP 200 mg/kg administered group (21.40±0.37) and DTP 400 mg/kg administered group (21.36±0.37) showed a significant decrease in weights compared to the HFD-Vehicle control group (22.98±0.22) and this significant difference continued to the completion of administration (Figure 1A, p<0.001). The food intake (g) decreased significantly in the group administered DTP 200 mg/kg on the 49th day, 55th to 56th day, 78th day, 83rd to 84th day after administration, whereas the food intake (g) decreased significantly in the DTP 400 mg/kg administration group on the 49th day, 55th to 57th day, 63rd day, 78th day, 83rd to 84th day compared to the HFD-Vehicle control group. Regarding the weight of liver adipose tissue and epididymal adipose tissue (g), DTP 100 mg/kg administration group (0.8679±0.0209 and 2.0347±0.0820), DTP 200 mg/kg administration group (0.8287±0.0157 and 1.9185±0.0932), and DTP 400 mg/kg administration group (0.7811±0.0144 and 1.6086±0.0859) showed significant levels of absolute weight reduction compared to the HFD-Vehicle control (1.0300±0.0377 and 2.2732±0.0472).

Serum biochemistry in HFD-fed mice. The DTP 200 mg/kg administration group (56.50±2.47) had significantly decreased blood AST level compared to the HFD-Vehicle control group (72.33±3.67). In addition, the DTP 400 mg/kg administration group (22.33±0.33) showed a significant decrease in blood ALT levels compared to the HFD-Vehicle control group (45.33±5.36). Furthermore, the DTP 100 mg/kg administration group (63.50±2.69), DTP 200 mg/kg administration group (57.17±1.45), and DTP 400 mg/kg administration group (56.17±1.19) showed a significant decrease in blood TG levels compared to that of HFD-Vehicle control (86.33±4.59). All groups showed a significant increase in blood HDL-C measurement (mg/dl) compared with the HFD-Vehicle control (82.33±2.33), whereas regarding LDL-C, there was a significant decrease in the DTP 200 mg/kg administration group (11.17±0.31) and DTP 400 mg/kg administration group (10.67±0.33) in comparison with the HFD-Vehicle control group (13.17±0.79) (Figure 1B).

Histopathology in HFD-fed mice. As a result of the accumulation of lipid droplets in the liver tissue and the adipose inflammation level, liver tissues' steatosis scores of the DTP 100 mg/kg administration group (0.5±0.22), DTP 200 mg/kg administration group (0.3±0.21), and DTP 400 mg/kg administration group 0.0±0.00) significantly decreased compared to that of the HFD-Vehicle control group (1.5±0.22, p<0.01). The adipose inflammation within the liver tissue showed that the DTP 200 mg/kg administration group (0.0±0.00) and DTP 400 mg/kg administration group (0.0±0.00) had a significantly decreased inflammation score

Table II. *Primer sequence for RT-PCR amplification.*

Target	Forward	Reverse
GAPDH	AGC TTC GGC ACA TAT TTC ATC TG	CGT TCA CTC CCA TGA CAA ACA
C/EBPα	GCA GCC ACT TGA GTT CTC AGG	GAT GTA GGC GGA GAG GTC GAT
PPARγ	GAC ATC CAA GAC AAC CTG CT	TGT CAT CTT CTG GAG CAC CT
Adiponectin	GAA GCC GCT TAT GTG TAT CGC	GAA TGG GTA CAT TGG GAA CAG T
Leptin	CAC AGA GGT GGT GGC TTT GA	AGC ATT CAG GGC TAA CAT CCA
Fas	AGA GAT CCC GAG ACG CTT CT	GCT TGG TCC TTT GAA GTC GAA GA

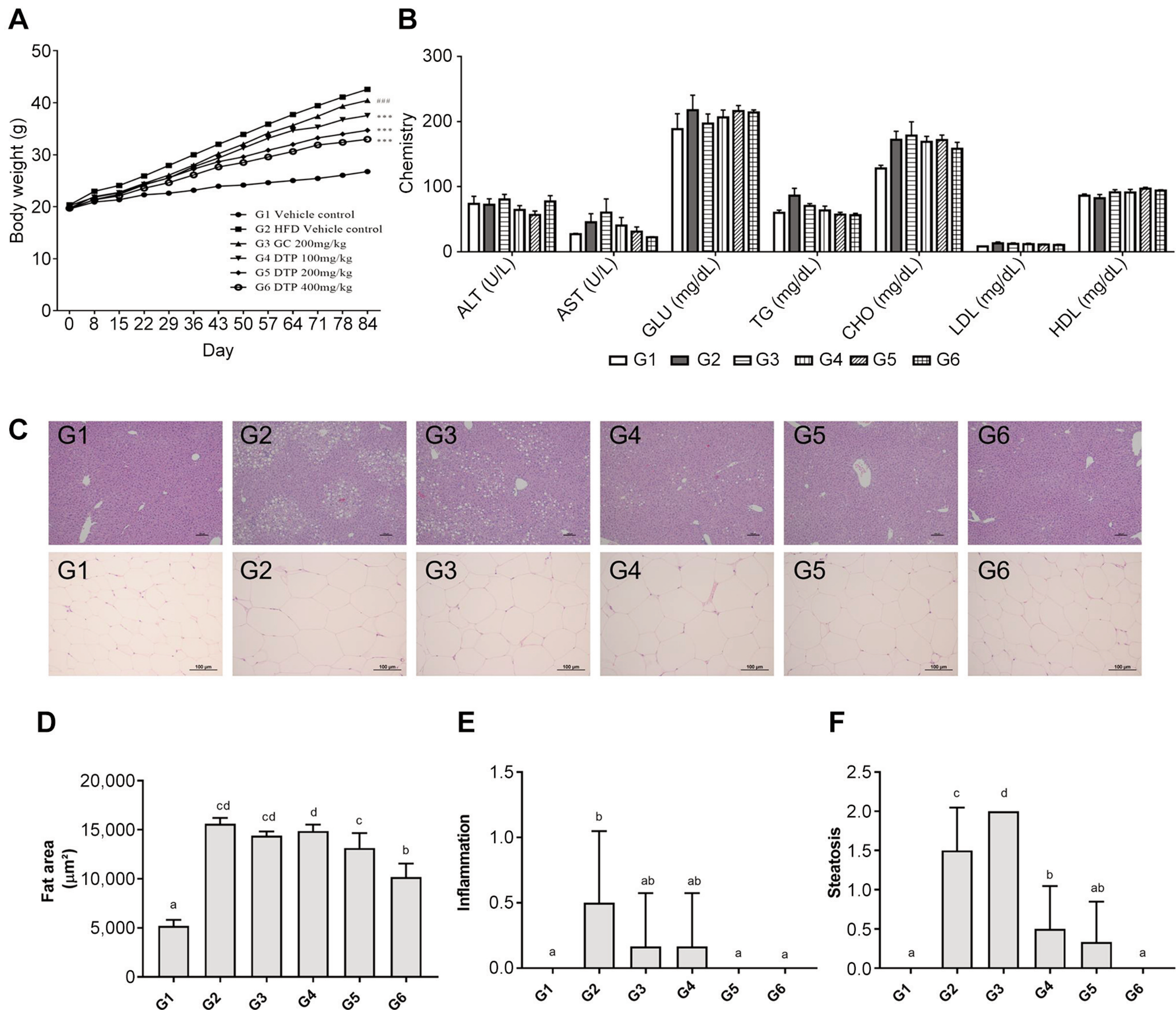

Figure 1. *Effects of dark tea extract powder (DTP) on body weight, the level of blood chemicals and cytokines, and the histopathological changes of the liver and epididymis. (A) Changes in body weight over 84-days period. (B) The levels of blood chemicals and cytokines. (C) Steatosis and inflammation score in the liver. (D) Average adipocyte size, (E) inflammation score, (F) Steatosis score. Data are expressed as mean±SD. Significant difference from G2 HFD vehicle control group was determined by One-way ANOVA. Significantly difference (p<0.05) was determined by Duncan's multiple range test. G1, Sham-vehicle; G2, High fat diet control, G3, Garcinia cambogia 200 mg/kg; G4, DTP 100 mg/kg; G5, DTP 200 mg/kg; G6, DTP 400 mg/kg. AST: Aspartate aminotransferase; ALT: alanine aminotransferase; GLU: glucose; TG: triglycerides; T-Chol: total cholesterol; LDLC: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol.*

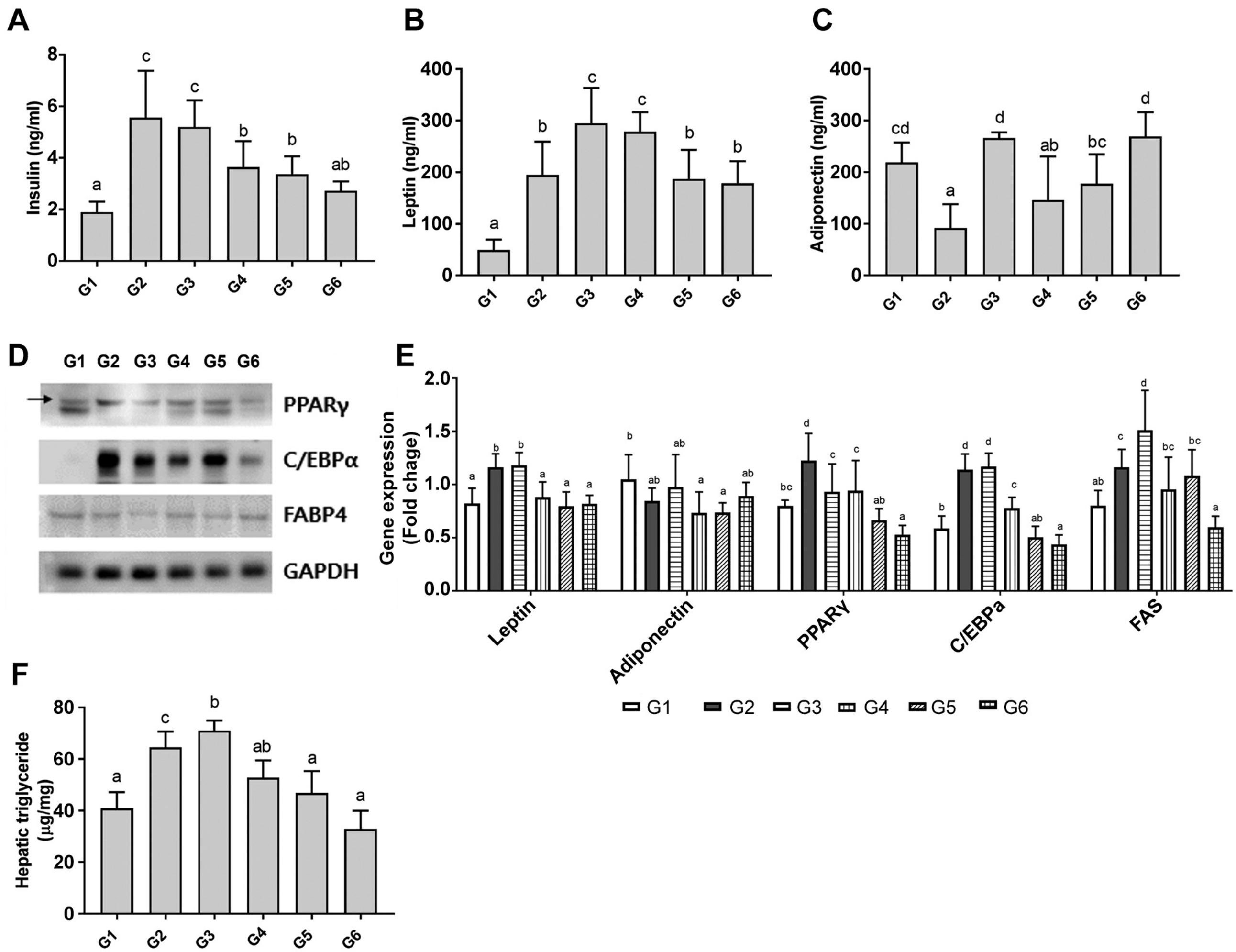

Figure 2. *Effects of dark tea extract powder (DTP) on obesity-related hormone levels in the serum, lipogenesis-related mRNA and protein levels in the liver, and hepatic TG levels. A) Fasting blood insulin level. (B) Fasting blood leptin level. (C) Fasting blood adiponectin level. (D) Determination of C/EBPα, PPARγ, and FABP4 protein expression (western blot). (E) qPCR mRNA expression in the liver. (F) Hepatic TG level. Data are expressed as mean±SD. Significant difference from G2 HFD vehicle control group was determined by One-way ANOVA. Significantly difference (p<0.05) was determined by Duncan's multiple range test. G1, Sham-vehicle; G2, High fat diet control, G3, Garcinia cambogia 200 mg/kg; G4, DTP 100 mg/kg; G5, DTP 200 mg/kg; G6, DTP 400 mg/kg.*

compared to the HFD-Vehicle control group (0.5±0.22) (Figure 1C, E, and F, p<0.05, p<0.01). The adipocyte size (um^2) within the epididymal adipose tissue was measured with the Image J program and its result confirmed a significant decrease only in the DTP 400 mg/kg administration group (10,196.52±557.53) compared to the HFD-Vehicle control group (15,607.26±243.89) (Figure 1D, p<0.05).

Inhibition of blood insulin, leptin, adiponectin, and hepatic TG. Regarding fasting blood insulin levels (ng/ml), a significant dose-dependent reduction was confirmed compared to the HFD-Vehicle control group (5.57±0.74) in the DTP 100 mg/kg administration group (3.64±0.41), DTP 200 mg/kg administration group (3.37±0.28), and DTP 400 mg/kg administration group (2.73±0.15) (Figure 2A, p<0.05). As shown in the next section, a significant reduction in leptin mRNA in the liver tissue was confirmed, but there was no significant difference in fasting blood leptin (Figure 2B and E, p<0.05). There was also a dose-dependent increase in fasting blood adiponectin levels in the test groups that were administered DTP. Among the test groups, DTP 200 mg/kg administration group (177.71±23.15), DTP 400 mg/kg administration group (269.21±19.23), and the comparator, *Garcinia cambogia* 200 mg/kg administration group (266.03±4.53) showed a significant decrease in fasting blood adiponectin levels compared to those of the HFD-Vehicle

control group (91.74±18.82) (Figure 2C, $p<0.05$). Furthermore, as a result of measuring TG levels in the liver tissue, a significant decrease in TG levels compared to those in the HFD-Vehicle control group was confirmed in all groups that were administered with DTP (Figure 2F, $p<0.05$).

Inhibition of adipogenesis gene expression in liver tissue. p-AKT, AKT, C/EBPα, PPARγ, and FABP4 proteins from the liver tissue were analyzed with western blotting. There was a significant decrease in *Garcinia cambogia* 200 mg/kg administration group (329.92±16.87), DTP 200 mg/kg administration group (299.11±32.04), and DTP 400 mg/kg administration group (283.69±45.91) in C/EBPα protein levels in the liver tissue compared to those in the HFD-Vehicle control group (502.37±107.54). However, for the rest of the other criteria (p-AKT, AKT, PPARγ, and FABP4), there were no significant differences in comparison with the HFD-Vehicle control group (Figure 2D, $p<0.05$). With qPCR analysis, it was confirmed that leptin mRNA in the liver tissue showed a significant decrease compared to that of the HFD-Vehicle control group (1.16±0.05) in the DTP 100 mg/kg administration group (0.88±0.06), DTP 200 mg/kg administration group (0.80±0.06), and DTP 400 mg/kg administration group (0.82±0.03). C/EBPα analysis also showed a similar trend, in that, the DTP 100 mg/kg administration group (0.78±0.04), DTP 200 mg/kg administration group (0.50±0.04), and DTP 400 mg/kg administration group (0.44±0.04) showed a significant decrease in C/EBPα mRNA levels compared to the HFD-Vehicle control (1.14±0.06, $p<0.05$). Regarding the qPCR results of PPARγ levels in the liver tissue, *Garcinia cambogia* 200 mg/kg administration group (0.93±0.11), DTP 100 mg/kg administration group (0.94±0.12), DTP 200 mg/kg administration group (0.67±0.04), and DTP 400 mg/kg administration group (0.53±0.04) confirmed a significant decrease in PPARγ mRNA compared to the HFD-Vehicle control (1.23±0.10). However, regarding FAS, only the DTP 400 mg/kg administration group (0.60±0.04) showed a significant decrease in FAS mRNA in comparison with the HFD-Vehicle control (1.17±0.07, $p<0.05$). There were no significant differences within all test groups in adiponectin in comparison with the HFD-vehicle control group (Figure 2E).

Discussion

This study was conducted to verify the suppression effect of adipocyte differentiation of dark tea and to determine its mechanism. Obesity in mice has been induced through an HFD. Then, DTP was orally administered daily for 12 weeks, and the evaluation criteria of obesity were compared to those of the comparator, *Garcinia cambogia*.

Through the result of the study, it was confirmed that DTP reduces weight and food intake in obesity-induced mice, and also reduces blood AST, ALT, TG, LDL-C levels while increasing HDL-C. Furthermore, the steatosis score and inflammation score of the liver tissue are decreased and it was confirmed that it decreases the size of adipocytes. As a result of blood hormone analysis, DTP was found to induce a significant decrease in blood insulin levels and a significant increase in blood adiponectin and it was also found to have a significant effect on reducing hepatic TG. Among natural extracts or nature-derived substances, the anti-obesity effect of polyphenol-containing substances has already been studied in many studies, and the common polyphenol-containing natural substances with the anti-obesity effect include green tea, pu-erh tea, tangerine peel tea (27-30), Reishi mushroom (31, 32), and Honeysuckle (33). The anti-obesity targets of these polyphenol-containing substances are known to be the factors involved in lipid homeostasis such as TG, TAG, HDL, and others (34).

The dark tea extract evaluated in this study also contains a large amount of polyphenol, and thus the anti-obesity effect of dark tea verified in the study can be expected to be the influence of the polyphenol contained in dark tea (35). It seems reasonable to conduct additional studies on the effect of polyphenols from dark tea on obesity and lipid metabolism.

C/EBPα and PPARγ identified in adipocytes are known as the major transcription factors to promote adipocyte differentiation (36). In particular, C/EBPα is identified in both white adipocytes and brown adipocytes, is over-expressed to induce adipocyte differentiation, and its expression is promoted during the latter half of the differentiation process (37).

PPARγ is selectively expressed in adipocytes, and it takes on an important role in controlling the metabolism of adipocytes and fatty acids (38). Particularly, it is highly related to adipocyte differentiation. It increases C/EBPδ and C/EBPβ during the beginning of differentiation and it continuously increases C/EBPα to promote differentiation (39). We confirmed that the mRNA of PPARγ was effectively reduced in the liver of mice administered with DTP in this study. At the same time, it was confirmed that not only the mRNA levels of C/EBPα were reduced in the same pattern as PPARγ, but also C/EBPα protein levels decreased at the same time. Therefore, the dark tea extract inhibited the production of adipocytes by inhibiting C/EBPα, and PPARγ transcription factors while reducing C/EBPα at the protein level (40, 41).

Taken together, when DTP was administered orally within the dose range of 200-400 mg/kg, it was confirmed that it decreases weight and food intake and also decreases the blood AST, ALT, TG, LDL-C while increasing HDL-C levels. In histopathology, the steatosis and inflammation scores decreased in the liver tissue and a reduction in the size of epididymal adipocytes was also confirmed. Also, a significant decrease in blood insulin and hepatic TG and a

significant increase in blood adiponectin levels were confirmed. Western blot and qPCR analyses were performed to verify the anti-obesity effect of DTP in the liver tissue and significant decreases in the mRNA levels of PPARγ and C/EBPα were confirmed. In conclusion, DTP can exhibit an equal to greater anti-obesity effect than *Garcinia cambogia* extract, which is registered as a herbal substance of health supplements; the same effect is caused by reducing the levels of C/EBPα and PPARγ mRNA involved in lipogenesis in adipocytes. However, it is necessary to confirm and verify the mechanism of the anti-obesity effect of DTP through cell-based studies in the future.

Conflicts of Interest

The Authors have no financial conflicts of interest to declare in relation to this study.

Authors' Contributions

This study was designated by Lim HJ and supervised by Choi SH. The test article was made by Kim JT, Kim MJ, and Jang SH. Investigation and data analysis were performed by Lim HJ, Lim TJ, Lee JH, Lee JH, and Kim MO. The manuscript was written by Lim HJ, Lee JH, Kim MO, and Park JY. All Authors approved the final version of the article.

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