(–)-Gossypol-enriched Cottonseed Oil Inhibits Proliferation and Adipogenesis of Human Breast Pre-adipocytes

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Abstract. *Background: Breast cancer is the most commonly diagnosed cancer in women. Obesity is an important risk factor for developing breast cancer and is one of few risk factors that women can modify to prevent cancer. (–)-Gossypol-enriched cottonseed oil [(–)-GPCSO] contains 65% (–)-gossypol and 35% (+)-gossypol. Previous studies have demonstrated that both (–)-gossypol and (–)-GPCSO have potent anticancer activity against multiple types of cancer, including breast cancer. In addition, (–)-GPCSO reduced body weight gain and food intake in young female rats. However, the role of (–)-GPCSO on adipogenesis in human breast pre-adipocytes remains unclear. Materials and Methods: Primary human breast pre-adipocytes were induced to differentiate in adipogenic medium in the presence of (–)-GPCSO. The proliferation of pre-adipocytes was determined with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium (MTS) assay. Lipid accumulation and glycerol 3-phosphate dehydrogenase (GPDH) activity were measured during adipocyte differentiation. mRNA expression of cyclin-D1, B-cell lymphoma-2 (BCL-2), Peroxisome Proliferator-Activated Receptor-γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα) and leptin was analyzed by real-time PCR. Results: (–)-GPCSO inhibited proliferation of pre-adipocytes and down-regulated the expression of cyclin-D1 and BCL-2. (–)-GPCSO also significantly decreased adipogenesis, as determined by inhibition of GPDH activity, triglyceride content (TG), and down-regulation of the expression of PPARγ, C/EBPα and leptin. Conclusion: These findings suggest that (–)-GPCSO has the potential as a food supplement to inhibit adipogenesis, and therefore, reduce obesity.*

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Breast cancer is the most common cancer among women in the US and is the second leading cause of cancer-related deaths in women worldwide. Obesity is associated with an increased risk for developing many types of cancer, including breast cancer, specifically in post-menopausal women. The risk of breast cancer doubles when post-menopausal women are obese (1-3). Obese women with breast cancer have lower survival rates, an increased risk of recurrence (4), and a poorer prognosis compared to normal-weight women with breast cancer (5).

Obesity is an epidemic in the US and is now a worldwide public health problem. The associated consequences of obesity – heart disease, cancer, and diabetes – are major causes of death (http://www.cdc.gov/cdctv/ObesityEpidemic). The relationship between obesity and breast cancer has received increased attention (2, 6).

Obesity is characterized by increased intracellular accumulation of lipids, which is significantly correlated with pre-adipocyte differentiation. Adipogenesis is the conversion of pre-adipocytes to adipocytes (fat cells) and is an indicator for the development of obesity. Pre-adipocytes and adipocytes are major cell types in the breast tumor microenvironment (2). Adipose tissue is the principal site of estrogen production in post-menopausal women. We previously demonstrated that exogenous leptin increased the expression of aromatase in normal human breast pre-adipocytes (7). This finding suggests that leptin may increase estrogen production in breast adipose tissues, which in turn may increase the proliferation of normal human breast epithelial cells. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion (8). Intra-abdominal tumors, such as ovarian cancer, have a

clear predilection for metastasis to the omentum, an organ primarily composed of adipocytes (9). Adipocytes provide fatty acids for rapid tumor growth, identifying lipid metabolism and transport as potential targets for the treatment of cancer where adipocytes are a major component of the microenvironment (9).

Plant-derived compounds have been extensively studied to prevent and treat cancer. In addition, phytochemicals have also been investigated to reduce both obesity and the risk of developing cancer due to obesity. Phytochemicals in fruits and vegetables reduce lipid accumulation and induce pre-adipocyte differentiation and adipocyte apoptosis. Therefore, these compounds have the potential to inhibit adipogenesis and induce apoptosis in adipose tissue (10-12) as well to prevent cancer (13, 14). Polyphenols have the potential to modulate obesity (15). Gossypol ($C_{30}H_{30}O_8$) (GP) is a polyphenolic compound found mainly in the cottonseed pigment glands of cottonseed plants. It constitutes of 20-40% of the gland weight and accounts for 0.4-1.7% of the whole kernel (16). Gossypol (GP) has diverse biological activities, including antifertility, antioxidative, antimicrobial, and antiviral activity (16). In addition, GP has antiproliferative activity against multiple types of cancer, including those of the breast, ovary, prostate and colon (17-20). GP also induces apoptosis of human lung cancer cells by up-regulation of TNF receptor superfamily, member-6 (FAS)/FAS Ligand (FAS/FASL) (21).

Cottonseed oil is extracted from the cottonseeds gland as a by-product of cotton production. Epidemiological data from independent states of the former Soviet Union found lower incidences of several types of cancer among those populations that used cottonseed oil containing GP for cooking than those that did not (22, 23). Naturally-occurring GP is a racemic mixture of (+)-GP and (–)-GP and, (–)-GP has greater *in vitro* anticancer activity than (+)-GP or racemic GP (17, 24, 25). (–)-GP-enriched cottonseed oil [(–)-GPCSO] from the USDA contains 65% (–)-GP and 35% (+)-GP (equivalent to 10.6 and 5.6 μM, respectively). (–)-GPCSO has a 120-fold greater anticancer activity against human breast cancer cells than (–)-GP when compared on an equimolar basis (26). Moreover, our laboratory demonstrated that (–)-GPCSO is a 110-fold more potent re-activator of the expression of the tumor suppressor gene protein tyrosine phosphatase receptor type-γ (PTPγ or PTPRG), in human breast cancer cells than (–)-GP when compared on an equimolar basis (unpublished data). These two important findings suggest that (–)-GPCSO contains additional compounds not present in pure (–)-GP that possess extremely potent anticancer activity.

In the present study, we determined if (–)-GPCSO can modulate the proliferation, differentiation, and adipogenesis of primary human breast pre-adipocytes isolated from human breast adipose tissues. Furthermore, we also investigated the expression of genes regulating apoptosis and adipogenesis in human breast pre-adipocytes in response to treatment with (–)-GPCSO in order to elucidate the mechanism behind the antiproliferative and antiadipogenic effects of (–)-GPCSO.

Materials and Methods

(–)-Gossypol-enriched cottonseed oil. (–)-GPCSO was kindly provided by the USDA Southern Regional Research Center, (New Orleans, USA). A 50% as a stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at 4˚C. Working stock solutions of (–)-GPCSO were freshly prepared before each experiment.

Isolation and culture of pre-adipocytes from human breast tissues. Adjacent normal breast tissue specimens from patients with human breast cancer were obtained through the US National Cancer Institute-sponsored Tissue Procurement Program at the Ohio State University Comprehensive Cancer Center Hospital (Columbus, OH, USA). Fresh tissue samples were placed in Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM/F12) (Atlanta Biologicals, Lawrenceville, GA, USA) without phenol red and stored at 4˚C before transfer to the laboratory. The tissues were sterilized in 70% ethanol for 30 sec, washed three times with fresh DMEM/F12, and then minced and digested in a humidified incubator (5% CO_2, 95% air, 37˚C) with 0.1% collagenase solution (Sigma, St Louis, MO, USA) for 18 h. The digested mixture was centrifuged at 200 ×*g* for 5 min at room temperature. Lipids were removed from the top of the centrifuge tube, and the lower layer of pre-adipocytes was carefully removed from the centrifuge tube. This fraction containing pre-adipocytes was centrifuged, and the cell pellet was washed three times and then re-suspended in 10 ml DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). Pre-adipocytes were identified under phase-contrast microscopy (Nikon Inc. Garden City, NY, USA), and photographs were recorded using a digital camera connected to a computer (Digital sight DS-Fi1; Nikon Corporation, Tokyo, Japan). The cell suspension was transferred to a 75 cm^2 culture flask and pre-adipocytes were propagated in a humidified incubator (5% CO_2, 95% air, 37˚C) The media were changed every 2 days. Cells at 85-90% confluence were harvested and subcultured in the DMEM/F12 medium with 5% FBS and subcultured.

Non-radioactive cell proliferation assay. A total volume of 100 μl medium containing 4,000 pre-adipocytes was seeded in each well of a 96-well plate in DMEM/F12 medium and incubated at 37˚C for 24 h. The culture medium was replaced with 100 μl DMEM/F12 supplemented with 0.2% BSA and incubated at 37˚C for another 24 h. The cells were treated with different doses of (–)-GPCSO for 48 h, whereas the control group was treated with 0.1% DMSO. Cell proliferation was determined by a non-radioactive 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium (Promega, Madison, WI, USA), MTS cell proliferation assay. Briefly, MTS was mixed with phenazine methosulfate (PMS) (Sigma) (20:1), and 20 μl of the mixture was added to each well and the cells then incubated for 1.5 h. The absorbance was measured at 490 nm using a kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA, USA) and analyzed with the Softmax Pro software (version 2.1.1).

Cell differentiation. Primary human breast pre-adipocytes were plated into 6-well culture plates and maintained in DMEM/F12 containing 10% FBS at 37˚C in 5% CO_2. Confluent cells were incubated in differentiation medium (DM) containing 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), 0.2 mM indomethacin, and 10 μg/ml insulin (Sigma) in DMEM with 10% FBS for two days. The culture medium was changed to post-DM containing 5 μg/ml insulin in DMEM with 10% FBS, and post-DM was replenished every two days until analysis was performed on day 8. (–)-GPCSO in DM was added at different concentrations in triplicate on day 2 of the 8-day differentiation assay. Triglyceride content and gene expression were measured at the end of differentiation.

Oil-Red-O staining. Differentiated adipocytes were stained using the Oil-Red-O method. In brief, differentiated apidocytes in culture wells were washed with cold PBS and fixed with 3.5% formaldehyde for 15 min. After two washes with propylene glycol, 1 ml of Oil-Red-O in isopropanol (5 mg Oil-Red-O/ml) was added to each well, and cells were incubated at room temp for 15 min and then washed with 85% propylene glycol. The plates were rinsed three times with distilled water. Differentiated adipocytes were examined by phase-contrast microscopy, and photographs were recorded as described above.

Glycerol-3-phosphate dehydrogenase (GPDH) activity and triglyceride accumulation. Adipocytes were harvested eight days after differentiation and were carefully washed twice with cold PBS. Adipocytes were lysed in 25 mM Tris, 1 mM EDTA, 1% (vol/vol) Triton X-100, pH 7.5, and GPDH activity in the cell lysates was measured as described by Wise and Green (27). Protein concentrations were determined using the Micro BCA™ protein assay reagent kit (Pierce Co., Rockford, IL, USA). Triglyceride accumulation was quantified with the AdipoRed™ Assay Reagent (Cambrex BioScience Walkersville, Inc., MD, USA).

RNA isolation and cDNA synthesis. After day 8 of differentiation, total RNA was extracted from cultured cells using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA). RNA concentrations were measured using a DU-70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The reverse transcription reaction for cDNA synthesis consisted of total RNA (1 μg), 200 U M-MLV Reverse Transcriptase (Invitrogen), 0.2 mM dNTP (1 μl mixture of 10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH), 1 μM random hexamers (Amersham, Piscataway, NJ, USA), 10 μl 5× First Strand buffer, 5 μl 0.1M Dithiothreitol and 40 U RNase Inhibitor (Invitrogen) in a total volume of 50 μl. The reaction was incubated at 37˚C for 50 min then followed by inactivation at 70˚C for 15 min in a gradient master cycle (Eppendorf, Westbury, NY, USA).

Quantitative real-time polymerase chain reaction (PCR). Real-time PCR was used to measure the expression of several genes critical for adipogenesis, including PPARγ, C/EBPα and leptin. Conditions were optimized for each primer pair and were performed in Stratagene Mx3005p (Agilent Technologies, Santa Clara, CA, USA). Briefly, newly-synthesized cDNA (2 μl) was used as a template for the reaction in a total volume (20 μl), which included 10 μl of 2× real-time master mix (Applied Biosystems, Warrington, UK), 3 μl ultra-pure water and 5 μl of primer mixer. The reactants were first incubated at 95˚C for 10 min, and then 40 cycles of amplification were carried out with each cycle consisting of denaturing at 95˚C for 30 seconds, annealing at 60˚C for 1 min and elongation at 72˚C for 1 min. Dissociation curves were constructed at the completion of each run to ensure that the PCR reactions produced the desired products as anticipated. The primer sequences for BCL-2 were 5'-CACACCTGGATCCAGGATAAC-3' (sense) and 5'-AGACAGCCAGGAGAAATCAAAC-3' (antisense); 5'-TTGGTTACAGTAGCGTAG-3' (sense) and 5'-TTATAGTAGC GTATCGTAGG-3' (antisense) were used for cyclin D1; 5'-CCTTTTGGTGACTTTATGGAGC-3' (sense) and 5'-GGCAAAC AGCTGTGA GGACT-3' (antisense) were used for for PPARγ; 5'-GGGTCTGAGACTCCCTATCCTT-3' (sense) and 5'-CTCATTGGT CCGCCAGGAT-3' (antisense) were used for C/EBPα; 5'-CCAAGATGACACCAAAACCC-3' (antisense and 5'-CCAGGCT GTCCAAGGTCTC-3' (antisense) were used for leptin; and 5'-TCCGATAACGAACGAGAC-3' (sense) and 5'-CTAAGGGCA TCACAGACC-3' (antisense) were used for 18s . The results of mRNA expression when compared to that of 18 sec in cells were analyzed using the ΔΔCt method (28).

Statistical analysis. The results were expressed as the mean±standard deviation (SD) of as least three replicates. Analysis was performed using SAS for Windows (SAS Institute Inc. Cary, NC, USA). Statistical differences were determined using the Student's *t*-test for independent groups. *p*-values of less than 0.05 were considered to be statistically significant.

Results

Primary human breast pre-adipoctyes for an in vitro model of adipogenesis. Pre-adipocytes were isolated from adjacent normal breast tissue specimens excised from patients with breast cancer by differential centrifugation and cultured in DMEM/F12 containing 10% FBS. Pre-adipocytes had a fibroblast-like appearance before adipogenic stimulation (Figure 1A). Following adipogenic stimulation, pre-adipocytes differentiated into mature adipocytes (Figure 1B) and accumulated lipid droplets (Figure 1C). The adipocytes were stained with Oil-Red-O on day 8 of adipogenesis (Figure 1D). Adipogenesis was confirmed by mRNA expression of known markers for adipogenesis including PPARγ, C/EBPα and leptin as determined by real-time PCR (Figure 6).

(–)-GPCSO inhibits the proliferation of pre-adipocytes. (–)-GPCSO reduced proliferation of pre-adipocytes after two days in a dose-dependent manner, as determined by an MTS assay (Figure 2). (–)-GPCSO at 500 and 1000 μg/ml reduced the number of pre-adipocytes by 41% and 60% ($p<0.05$), respectively, when compared to control cells.

(–)-GPCSO reduces intracellular lipid accumulation during adipocyte differentiation. During the differentiation of human breast pre-adipocytes into adipocytes, cells were treated with different concentrations of (–)-GPCSO for two days, and the number of intracellular oil droplets were determined by a triglyceride assay after day 8 of differentiation. Intracellular triglyceride accumulation was significantly reduced by treatment with (–)-GPCSO in a concentration-dependent manner (Figure 3) Lipid accumulation at the highest

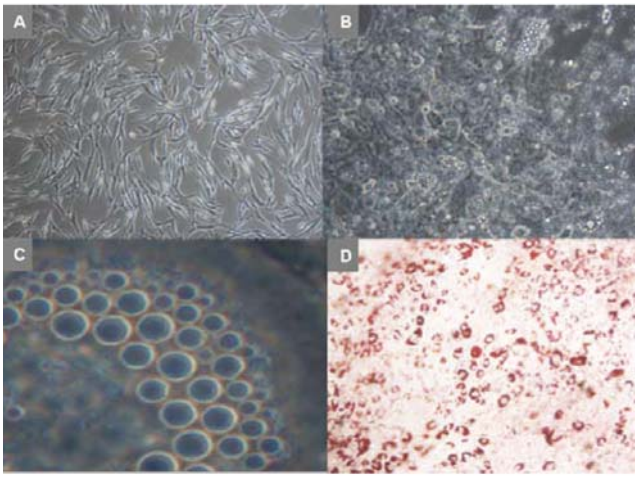

Figure 1. *Morphology of primary human breast pre-adipoctyes used for in vitro model of adipogenesis. A: Undifferentiated human breast pre-adipocytes. B: Differentiated human breast pre-adipocytes at day 8 after the initiation of differentiation. C: Lipid droplets of human breast differentiated adipocytes. D: Representative image of Oil Red O staining of differentiated human breast adipocytes.*

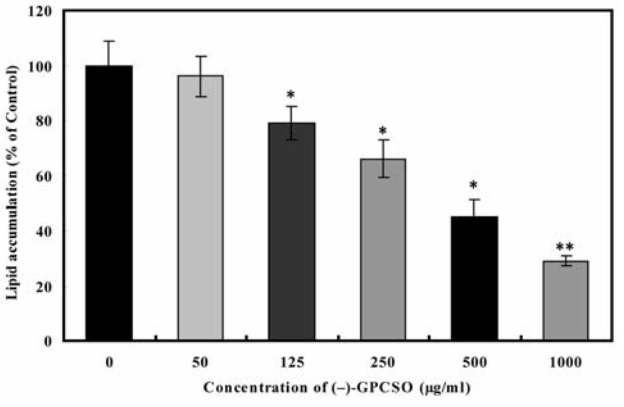

Figure 3. *Inhibition of intracellular triglyceride production in human breast adipocytes by (–)-Gossypol-enriched cottonseed oil, (–)-GPCSO. Pre-adipocytes were cultured in growth medium until confluence, and cells were then incubated in differentiation medium (DM) with or without (–)-GPCSO at day 2 of the 8-day differentiation , and then harvested. The values are expressed as mean±S.D. of three trials. Mean values are significantly different from that of the control (0) at *p<0.05, **p<0.01.*

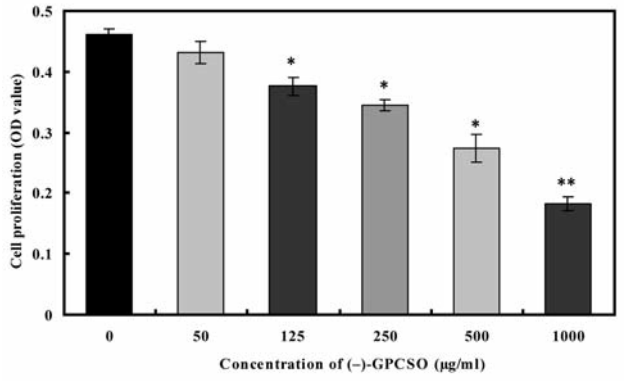

Figure 2. *Inhibition of proliferation of human breast pre-adipocytes by (–)-Gossypol-enriched cottonseed oil, (–)-GPCSO. Results are the means±SD (n=4). *p<0.05, **p<0.01, significantly different from that of the control (0).*

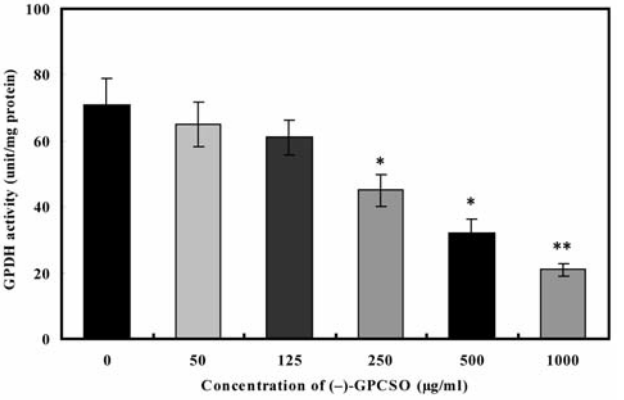

Figure 4. *Inhibition of glycerol 3-phosphate dehydrogenase (GPDH) activity in human breast adipocytes by (–)-Gossypol-enriched cottonseed oil, (–)-GPCSO. Pre-adipocytes were cultured in growth medium until confluence, and cells were then incubated in differentiation medium (DM) with or without (–)-GPCSO at the day 2 of the 8-day differentiation, and then harvested. The values are expressed as mean±S.D. of three trials. Mean values are significantly different from that of the control (0) at *p<0.05, **p<0.01.*

concentration (1,000 μg/ml) was reduced by 72% ($p<0.05$), when compared to the control cells.

(–)-GPCSO reduces GPDH activity in differentiated adipocytes. GPDH activity was measured on day 8 of the differentiation process. Administration of (–)-GPCSO two days after the initiation of differentiation reduced GPDH activity in a dose-dependent manner (Figure 4). (–)-GPCSO at 500 and 1,000 μg/ml reduced GPDH activity by 55% and 72% ($p<0.05$), respectively, when compared to control cells In contrast, GPDH activity was not detected in undifferentiated pre-adipocytes (data not shown).

(–)-GPCSO attenuates the expression of cyclin D1 and Bcl-2 genes in differentiated adipocytes. In order to determine whether (–)-GPCSO affects the expression of proliferative and apoptosis-related genes, the mRNA expression of *cyclin-D1* and *BCL-2* in matured adipocytes treated with (–)-GPCSO was investigated using real-time PCR. (–)-GPCSO at 500 and 1,000 μg/ml significantly ($p<0.05$) reduced *cyclin-D1* mRNA expression by 35% and 70%, respectively, when compared to control cells (Figure 5). (–)-GPCSO at

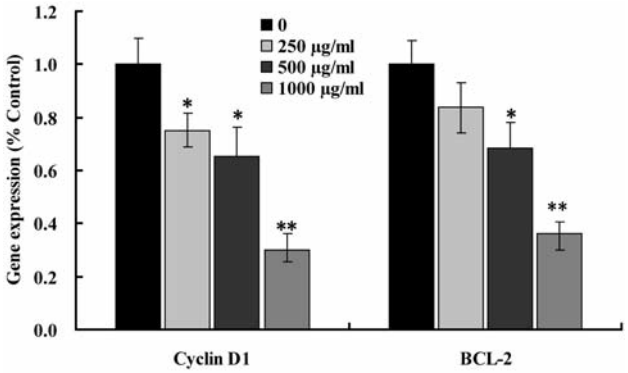

Figure 5. *Inhibition of mRNA expression of cyclin-D1 and B-cell lymphoma 2 (BCL-2) in human breast adipocytes by (–)-Gossypol-enriched cottonseed oil, (–)-GPCSO, as determined by real-time polymerase chain reaction (RT-PCR). The values are expressed as mean±S.D. of four trials. Mean values are significantly different from that of the control (0) at *p<0.05, **p<0.01.*

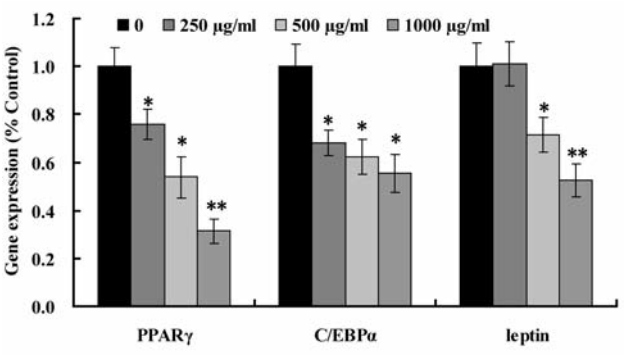

Figure 6. *Inhibition of the expression of Peroxisome Proliferator-Activated Receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα) and leptin in human breast adipocytes by (–)-Gossypol-enriched cottonseed oil, (–)-GPCSO, as determined by real-time polymerase chain reaction (RT-PCR). The values are expressed as mean±S.D. of four trials. Mean values are significantly different from that of control (0) at *p<0.05, **p<0.01.*

500 and 1,000 μg/ml also significantly ($p<0.05$) down-regulated *BCL-2* mRNA expression by 32% and 64%, respectively, when compared to control cells.

(–)-GPCSO attenuates the expression of adipogenic related genes PPARγ, C/EBPα and leptin in differentiated adipocytes. Adipocyte differentiation involves a series of programmed changes in gene expression. In order to determine whether (–)-GPCSO affects the expression of the key adipocyte differentiation markers PPARγ, C/EBPα and leptin, real time-PCR was performed on adipocytes to quantify mRNA expression of these genes. Cells were collected on day 8 of the differentiation process in the presence of different concentrations of (–)-GPCSO. (–)-GPCSO at 1,000 μg/ml reduced mRNA levels for *PPARγ*, *C/EBPα* and *leptin* by 69%, 55%, and 48% ($p<0.05$), respectively, when compared to control cells (Figure 6).

Discussion

Obesity has become a worldwide epidemic. The associated consequences of obesity - heart disease, cancer, and diabetes - are major causes of death (http://www.cdc.gov/cdctv/ ObesityEpidemic). Obesity costs the US about $150 billion or 10% of the national medical budget each year (29). The prevalence of obesity in the US more than doubled between 1976-1980 and 2003-2006 with more than 35% of US men and women and ~17% of youth classified as obese in 2009-2010 (30). Overall, adults aged 60 years and over are more likely to be obese than younger adults. Forty-two percent of women aged 60 years and over are obese when compared to 32% of women between at the ages of 20 to 39 years (30).

Obesity and being overweight are associated with an increased risk for developing many types of cancer, including those of the breast specifically in post-menopausal women, colon and rectum, endometrium, esophagus, kidney, and pancreas (30). Currently, being overweight and obesity are implicated in causing between 14 and 20% of all cancer-related deaths (30). Present trends in obesity predict that by 2030, obesity will be associated with ~500,000 additional cases of all types of cancer (31).

The risk of breast cancer doubles specifically when post-menopausal women are overweight or obese (32). While many studies have documented an increased risk of post-menopausal breast cancer incidence and mortality in women who are obese, the mechanisms for this risk remain poorly-understood.

Substantial progress has been made concerning our knowledge of bioactive components in plants to reduce obesity. For example, polyphenols and fatty acids (12, 33) have been investigated for their effects on obesity. Cottonseed oil, an edible oil derived from the seeds of cotton plants, contains compounds with anticancer and potentially antiobesity activities. Gossypol has potent antineoplastic effects against multiple types of cancer, including those of the testis, breast, cervix, skin (melanoma), colon, and others (16). Our laboratory demonstrated that (–)-GP is more potent than (+)-GP in inhibiting the growth of human breast cancer cells (17). Furthermore, we discovered that (–)-GP has potential antiobesity activity. Administration of (–)-GP to young female rats reduced both body weight gain and food intake when compared to control rats (34).

Wang and Jones (35) proposed a strategy to reduce the incidence of obesity that involves reducing pre-adipocyte

proliferation and differentiation. We showed that (–)-GP reduced the proliferation of human breast pre-adipocytes (7). Because (–)-GPCSO, which contains 65% (–)-GP and 35% (+)-GP, has a 120-fold greater anticancer activity against human breast cancer cells than (–)-GP when compared on an equimolar basis (26, 36), we hypothesized that (–)-GPCSO would reduce pre-adipocyte proliferation and differentiation.

In the present study, we investigated the effects of (–)-GPCSO on the cell proliferation and adipogenesis of human breast pre-adipocytes as part of an effort to reduce obesity. (–)-GPCSO reduced the proliferation of pre-adipocytes in a dose-dependent manner, as determined by the MTS assay (Figure 2). In order to elucidate the molecular mechanisms behind (–)-GPCSO inhibition of proliferation of human breast pre-adipocytes, the mRNA expression of *cyclin-D1* and BCL-2 in pre-adipoctyes in response to treatment with (–)-GPCSO was investigated using real-time PCR. *Cyclin-D1* gene is a critical mitogen-regulated cell-cycle control element whose transcriptional modulation plays a crucial role in breast cancer growth and progression (37). *BCL-2* is an antiapoptotic oncogene that blocks programmed cell death rather than promoting proliferation (38). Our study found that treatment with (–)-GPCSO markedly reduced cyclin-D1 and BCL-2 expression, suggesting that the decreased expression of both proto-oncogene *cyclin-D1* and antiapoptotic gene *BCL-2* may play a key role in antiproliferation of human breast pre-adipocytes.

We further investigated the effects of (–)-GPCSO on adipogenesis of human breast adipocyte. The accumulation of intracellular triglyceride and GPDH activity in human breast differentiated pre-adipocytes after treatment with (–)-GPCSO were determined to evaluated its anti-adipogenesis activity. Adipogenesis is cellular differentiation of pre-adipocytes into adipocytes, which is essential for fat formation. GPDH is involved in glycerol synthesis and catalyzes the formation of glycerol-3-phosphate from dihydroxyacetone phosphate. The activity of GPDH increases the differentiation of pre-adipocytes, and this enzyme is commonly used as an adipogenic marker (39). In this study, we found that the intracellular accumulation of triacylglycerols and the GPDH activity were significantly reduced in a dose-dependent manner in human breast pre-adipocytes undergoing adipocyte differentiation in the presence of (–)-GPCSO (Figure 3).

The adipogenesis process is regulated at the molecular level by transcription factors such as PPARγ, C/EBPα and CBP/p300. PPARγ is a member of the nuclear receptor superfamily of transcription factors and is predominantly expressed in adipose tissue. C/EBP (C/EBPα, β and δ) belongs to the basic leucine zipper family of transcription factors. These transcription factors appear to function as dominant activators of adipocyte differentiation (40). PPARγ plays an important role in the regulation of insulin sensitivity and glucose homeostasis (41). The present study discovered that (–)-GPCSO treatment down-regulated the mRNA expression of *PPARγ* and *C/EBPα* in human breast adipocytes (Figure 6), suggesting that (–)-GPCSO attenuated adipogenesis by affecting the transcriptional factor cascade upstream of PPARγ signaling. Leptin, which is the product of the OB gene, is a hormone secreted from adipocytes and reduces food intake and increases energy expenditure (42). Here, we also found that *leptin* mRNA expression was inhibited by (–)-GPCSO (Figure 6). Thus, the inhibitory effect of (–)-GPCSO on adipocyte differentiation, as indicated by a decrease in GPDH activity and triglyceride content, appears to be mediated through the down-regulation of expression of adipogenic transcription factors PPARγ and C/EBPα, and adipogenic-specific genes such as leptin.

In conclusion, we have demonstrated that (–)-GPCSO inhibited both the proliferation of human breast pre-adipocytes and their differentiation and adipogenesis of pre-adipocytes. These findings suggest that (–)-GPCSO has the potential to be used as a naturally derived inhibitor of adipogenesis. Further studies using animal experiments are required to confirm this adipogenesis-inhibitory effect *in vivo*.

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