ABSTRACT Today obesity is an epidemic, and its prevalence has increased significantly over the last few decades. To avoid excessive accumulation of fat, optimum energy intake along with regular exercise is mandatory. Polyphenols present in green tea, grape seeds, orange, and grapefruit combat adipogenesis at the molecular level and also induce lipolysis. However, very little is known regarding the role of blueberry polyphenols on adipocyte differentiation. Hence we tested the dose-dependent effects of blueberry polyphenols on mouse 3T3-F442A preadipocyte differentiation and lipolysis. 3T3-F442A preadipocytes were incubated with three doses of blueberry polyphenols (150, 200, and 250 μg/mL [BB-150, BB-200, and BB-250, respectively]), and intracellular lipid content, cell proliferation, and lipolysis were assayed. Blueberry polyphenols suppressed adipocyte differentiation determined by Oil Red-O staining and AdipoRed assay. Intracellular lipid content in control (11,385.51 ± 1,169.6 relative fluorescence units) was significantly higher ($P < .05$) than with the three doses of blueberry polyphenols (8336.86 ± 503.57, 4235.67 ± 323.17, and 3027.97 ± 346.61, respectively). This corresponds to a reduction of 27%, 63%, and 74%, respectively. Cell proliferation was observed to be significantly higher in the control (0.74 ± 0.03 optical density units) than with BB-150 (0.51 ± 0.031), BB-200 (0.49 ± 0.023), and BB-250 (0.45 ± 0.012). However, when tested for lipolysis, there was no significant difference observed among the groups. We conclude that blueberry polyphenols may play an effective role in inhibiting adipogenesis and cell proliferation.

KEY WORDS: • adipocyte differentiation • cell proliferation • lipolysis • obesity • polyphenols

INTRODUCTION

OBESITY IS BECOMING AN EPIDEMIC in both children and adults. Its prevalence has increased significantly over the last few decades, and it is the major risk factor for cardiovascular diseases and type 2 diabetes mellitus. Surplus energy from a well-fed state is stored as triglycerides in the adipocytes. Combating unnecessary adipogenesis at the molecular level can be beneficial to prevent diseases at a very early stage. Therefore, in vitro studies to identify compounds that will interfere and inhibit adipogenesis are warranted.

Fruits, vegetables, and legumes are not only rich in fiber but also abundant in antioxidants like polyphenols. Some polyphenols improve endothelial function and reduce total cholesterol and triglycerides and thereby are effective in the treatment of cardiovascular diseases. They also demonstrate anti-inflammatory effects. Anthocyanins, compounds present in polyphenols, have antioxidant effects and suppress development of obesity in mice fed a high fat diet. Polyphenols stimulate adipokine secretion and attenuate gene expression of adipocyte-specific genes like peroxisome proliferator-activated receptor γ and CCAAT/enhancer binding proteins (z and b) in 3T3 L1 preadipocytes isolated from rats. Polyphenols present in green tea, grape seeds, orange, and grapefruit have been shown to inhibit adipogenesis and initiate lipolysis.

Blueberry polyphenols have shown promising results in the treatment of cognitive impairment, ischemic heart disease, oxidative stress, and neurological degeneration. Blueberries have also been reported to attenuate diet-induced atherosclerosis in mice. Moreover, purified blueberry anthocyanins and blueberry juice are effective in preventing obesity in C57BL mice. However, to our knowledge, there has been no investigation of the effect of blueberry polyphenols on adipogenesis. Therefore, in this study we examined the effect of blueberry polyphenol extract on adipocyte differentiation and lipolysis.

MATERIALS AND METHODS

Blueberry polyphenol extraction

Polyphenol extraction was performed by modification of a previously described procedure by Kim et al. Freeze-dried blueberry powder was provided by the US Highbush Blueberry Council (Folsom, CA, USA) and stored at −20°C in the dark. The blueberry powder consisted of a blend of Tifblue and Rubel blueberries in a 1:7 ratio. In brief, blueberry polyphenols were extracted with 80% ethanol in
subdued light. After sonication of the extract for 20 min at room temperature under nitrogen, the extract was filtered through a Buchner funnel. The filtrate was then concentrated by rotary evaporation and lyophilized. The lyophilized polyphenol extract was stored in an amber-colored bottle at −20°C.

### Polyphenol assay

Total polyphenol content of blueberry extract was determined using gallic acid as a standard and expressed as gallic acid equivalents using a previously described procedure.22

### Cell culture

3T3-F442A cells were obtained from Dr. Howard Green (Harvard Medical School) and cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum. After 24 h, that is, on Day 0, differentiation was initiated with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 167 nM insulin in the presence and absence of different concentrations (150, 200, and 250 μg/mL of medium) of blueberry polyphenol (BB-150, BB-200, BB-250, respectively). After 48 h (Day 2), the cells were switched to Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum with or without blueberry polyphenol. Thereafter, the culture medium was replaced every 48 h with fresh medium of identical constitution. Analyses were performed on Day 8.

### Oil Red-O staining

Cells were plated in a six-well plate at a density of 4 × 10⁴ cells per well. They were allowed to differentiate in the presence and absence of blueberry polyphenols. On Day 8, cells were fixed with 10% formalin and then stained with Oil Red-O by modification of a previously described procedure.23

### AdipoRed assay

Cells were plated in six-well plates at a density of 4 × 10⁴ cells per well and allowed to differentiate in the presence and absence of blueberry polyphenols. On Day 8 of differentiation, cells were treated with AdipoRed (Lonza, Houston, TX, USA), and total lipid content was determined as per the manufacturer’s instructions.

### Cell titer

The effect of blueberry extract on cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI, USA) as per the manufacturer’s instructions. In brief, 4 × 10⁴ cells were plated per well of a six-well plate. Cells were allowed to differentiate in the presence and absence of blueberry extract as mentioned in the above procedure. Cell viability was determined on Day 8. The cells were washed with Hanks’ buffered salt solution and incubated with MTS reagent for 1–4 h at 37°C in a CO₂ (5%) incubator. Absorbance was then measured at 490 nm in a plate reader.

### Fatty acid assay

Cells were plated in six-well plates at a density of 4 × 10⁴ cells per well and allowed to undergo differentiation without blueberry. On Day 8, varying doses of blueberry polyphenols were added to the wells, and the cells were placed at 37°C in a 10% humidified CO₂ incubator for 3 days. On Day 11, free fatty acid content of the media was determined using the free fatty acid assay kit from BioVision (San Francisco, CA, USA). Values were corrected for the absorbance obtained for cell-free incubations.

### Statistics

One-way analysis of variance along with Tukey’s post hoc test was performed to assess the significance of differences among the groups. Data are mean ± SE values. Results were considered significant when P < .05.

### RESULTS

When the cells were examined on Day 8 of differentiation, blueberry polyphenols caused a dose-dependent suppression of intracellular lipid accumulation within the 3T3-F442A cells (Fig. 1A).

In order to highlight actual lipid accumulation, cells were fixed with 10% formalin and then stained with Oil Red-O. The result showed the dose-dependent decrease in triacylglycerol content with increasing concentrations of blueberry polyphenols (Fig. 1B). When the BB-250 group was observed under a high-resolution microscope, the cells had fine red-colored lipid droplets surrounding the inner lining of the cell membrane.

To quantify the qualitative results obtained with Oil Red-O staining, we performed the AdipoRed assay. AdipoRed is used to quantify the intracellular lipid droplets formed during adipogenesis. The results confirmed that control cells had the highest amount of lipid concentration measured in relative fluorescence units (Fig. 2). All blueberry polyphenol concentrations had significantly reduced cellular lipid content compared with the control (P < .05). The reduction was 26.8%, 62.8%, and 73.4% for blueberry doses of 150, 200, and 250 μg/mL, respectively. When the three blueberry doses were compared among themselves, BB-150 had significantly higher intracellular lipid content than BB-200 and BB-250 (P < .05); however, there was no significant difference between BB-200 and BB-250 (P > .05).

Cell viability was analyzed by MTS assay to determine the dose-dependent effect of blueberry polyphenols on cell proliferation (Fig. 3). Compared with all the blueberry groups, cell proliferation was observed to be significantly higher in the control (P < .05). However, there was no significant difference among the three doses of blueberry polyphenols. Thus, blueberry polyphenol seems to inhibit cell proliferation.

The free fatty acid assay was performed to determine the effect of blueberry polyphenols on adipocyte lipolysis. There were no significant differences among the three doses of polyphenols (Fig. 4).
DISCUSSION

Our study demonstrated that polyphenols extracted from blueberry inhibit 3T3-F442A preadipocyte differentiation. Cells treated with blueberry polyphenols showed a dose-dependent reduction in intracellular lipid accumulation as demonstrated by Oil Red-O staining and AdipoRed assay (Figs. 1 and 2). Oil Red-O stains only the fat droplets and gives a qualitative estimate of triacylglycerol accumulation in the cell. When cells start accumulating triacylglycerol, they become spherical in shape. Although we did not directly measure cell size, visual comparison of cell sizes and fat content between the control cells and blueberry polyphenol-treated cells indicated that both the cell size and cellular lipid content decreased with increasing concentrations of blueberry polyphenols. Previous studies have reported that polyphenols from green tea, as well other polyphenolic compounds, such as resveratrol, genistein, and quercetin, also inhibited adipogenesis in 3T3-L1 cells.15,24 Green tea polyphenols belong primarily to the catechin family, but blueberry polyphenols are a mixture of different polyphenolic compounds, including flavonoids, anthocyanins, proanthocyanidins, and hydrocinnamic acids.25–28 Thus, polyphenols as a whole appear to inhibit adipogenesis regardless of the nature of the individual compound.

Preadipocyte differentiation involves the activation of several adipocyte genes, including peroxisome proliferator-activated receptor γ, CCAAT/enhancer binding protein α, and differentiation-dependent factor 1/sterol regulatory element binding protein isoform. Collectively, they regulate the sequence of adipocyte differentiation. Polyphenolic compounds such as epigallocatechin gallate from green tea,15 resveratrol from red grapes,24 and curcumin from turmeric29 have been shown to suppress one or more of these genes and thus inhibit differentiation of 3T3-L1 cells. Although in this study we did not investigate the mechanism of blueberry polyphenol-induced adipocyte differentiation, it is likely that blueberry polyphenols also inhibit adipocyte differentiation by one of the above mechanisms.

Blueberry polyphenols decreased cell viability as determined by the MTS assay (Fig. 3). In this procedure, 3T3-F442 preadipocytes were differentiated in the presence and absence of varying doses of blueberry polyphenols, and cell proliferation was determined on Day 8 of differentiation. Although cell proliferation was reduced significantly in the blueberry-treated cells compared with control cells, cell proliferation did not differ across the three doses of blueberry polyphenols. We also observed inhibition of cell proliferation in cultures incubated with a polyphenol concentration as low as 75 µg/mL (data not shown). Recent studies have shown that epigallocatechin gallate from green tea15 and a combination of genistein, quercetin, and resveratrol also

![FIG. 1. Dose-dependent inhibition of intercellular lipid accumulation: (A, I–IV) differentiated cells on Day 8 (magnification, ×20); (B, V–VIII) corresponding Oil Red-O–stained cells (magnification, ×40). BB-150, BB-200, and BB-250 denote blueberry polyphenol concentrations of 150, 200, and 250 µg/mL of medium, respectively (n = 18). These are representative photographs from three independent experiments, each performed in duplicate.](http://www.liebertpub.com/mlt/download/figs/mlt/mlt-450-0450-fig1.png)

![FIG. 2. Quantification of dose-dependent inhibition of intracellular lipid accumulation. Cells were differentiated with or without blueberry polyphenol. The AdipoRed assay was performed on Day 8 (n = 12). Histograms with different superscript asterisks are significantly different from each other (P < .05).](http://www.liebertpub.com/mlt/download/figs/mlt/mlt-450-0450-fig2.png)
inhibited proliferation of 3T3-L1 preadipocytes in culture.\textsuperscript{24} Inhibition of preadipocyte proliferation by blueberry polyphenols could reduce the number of differentiated adipocytes. Thus, it appears that polyphenols reduce adipocyte lipid accumulation not only by inhibiting intracellular lipid content but also by reducing adipocyte number.

The net intracellular lipid content in differentiated adipocytes represents a balance between total triacylglycerol synthesis and lipolysis. Increased triacylglycerol synthesis accompanied by reduced $\beta$-oxidation will result in substantial intracellular lipid accumulation. Therefore, in our study in order to determine whether blueberry polyphenols affect adipocyte lipolysis, we performed lipolysis assay in mature adipocytes exposed to different polyphenol doses. The result indicates that blueberry polyphenols have no effect on adipocyte lipolysis (Fig. 4). However, it is possible that the assay was not sensitive enough to detect very small changes in lipolysis. The sensitivity limit of the current assay was 2\textmu M free fatty acid. Use of a more sensitive method might have produced different results.

The blueberry polyphenol doses used in our study were selected based on preliminary results of cell viability and adipogenesis. Concentrations lower than 150\textmu g/mL had very little inhibitory effect on adipogenesis. Similarly, polyphenol doses higher than 250\textmu g/mL appeared to cause excessive cell loss. Our test dose of 250\textmu g/mL blueberry polyphenols is equivalent to approximately 8 g of freeze-dried blueberry powder or about one-half cup of fresh blueberry. Although this dose of blueberry consumption is easily achievable in humans, we cannot extrapolate the results of cell culture studies to humans.

Blueberry polyphenols have been shown to improve insulin sensitivity in 3T3-L1 adipocytes\textsuperscript{28} and obese, insulin-resistant men and women.\textsuperscript{30} Furthermore, consumption of whole blueberries decreased cardiovascular risk factors in people with metabolic syndrome.\textsuperscript{31} Our study demonstrates for the first time that blueberry polyphenols also inhibit adipocyte differentiation in 3T3-F442 preadipocytes. Further studies are needed to elucidate the physiological significance of these findings.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist for any of the authors.

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