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Cyanidine-3-O-Galactoside Enriched *Aronia melanocarpa* Extract Inhibits Adipogenesis and Lipogenesis via Down-Regulation of Adipogenic Transcription Factors and Their Target Genes in 3T3-L1 Cells

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Abstract

Aronia melamocarpa (AM) is a rich source of anthocyanins, which are known to help prevent obesity. The cyanidine-3-O-galactoside enriched AM extract (AM-Ex) containing more cyanidine-3-O-galactoside than conventional AM extract was recently developed. The objective of this study was to examine the effect of AM-Ex on adipogenesis and its action mechanisms in vitro using 3T3-L1 adipocytes. To examine the anti-obesity effect of AM-Ex, 3T3-L1 cells were induced adipocyte differentiation and incubated with various concentration of AM-Ex. Lipid accumulation, cellular triglyceride content, mRNA expression of transcription factors and adipogenic genes were analyzed. Treatment with 100 - 400 μg/mL of AM-Ex resulted in a dose-dependent decrease in adipocyte differentiation and triglyceride accumulation. mRNA expression of adipogenic transcription factors, such as peroxisome proliferator-activated receptor gamma, CCAAT/enhancer binding protein *α*, sterol regulatory element-binding protein 1 were decreased. The level of gene expression of adipogenesis and lipogenesis-related genes, such as adipocyte protein 2, lipoprotein lipase, acetyl-CoA carboxylase, ATP-citrate lyase and fatty acid synthase were decreased. These results suggest that AM-Ex alleviated risk factors related to obesity by modulating multiple pathways associated with adipogenesis.

Keywords

Obesity, Adipocyte, Adipogenesis, Lipogenesis, Transcription Factor, Adipocyte Protein 2

1. Introduction

The prevalence of obesity has increased dramatically worldwide and has become a major global health problem. Obesity is related to increased mortality and morbidity in a number of chronic diseases, such as metabolic syndrome and vascular disease [1] [2]. Therefore, it is important to control obesity to prevent various chronic diseases and improve health.

Various drugs have been developed and applied to treat obesity through regulating appetite and controlling fat absorption and oxidation. However, these anti-obesity drugs have been reported to cause negative side effects and rebound weight gain when the medication was ceased [3] [4]. Thus, the need exists for functional foods or drugs with high efficacy and no negative side effects. Recently explored substances which may potentially cure and prevent obesity include numerous foods and plant-derived bioactive compounds, such as catechin [5] [6] and hydroxycitric acid [7] [8].

Aronia melamocarpa (AM), known as aronia or chokeberry, belongs to the Rosaceae family, and originated in North America. Its red-purple fruits are frequently made into juice or jams. Also, it has traditionally been used in Russia and some Eastern European countries to treat chronic diseases [9]. Currently, AM has attracted a lot of research attention because of its high phytochemical content, compounds known to be beneficial to health. AM contains various phenolic compounds, including anthocyanins, phenolic acids, and flavonoids [10]. *In vitro* and *in vivo* studies suggested that the phenolic compounds contained in AM possess a wide range of beneficial health functions, including antioxidant [11] [12], anti-hyperlipidemic [13] [14], anti-diabetic [15] [16], hepatoprotective [17], cardioprotective [18] [19] [20], and gastroprotective [21] activities. Recent animal studies demonstrated that AM reduced diet-induced obesity [22] [23]. Kim *et al*. [24] reported that the content of cyanidin-3-O-galactoside (C-3-Gal), a major anthocycnin in AM, is present in different amounts in AM extracts depending on the extraction method, and that the greater the C-3-Gal content in the extract, the greater the anti-obesity effect. However, information regarding the anti-obesity effect of AM is limited, and the molecular mechanism underlying these effects has yet to be elucidated.

Adipogenesis is constituted by a set of processes, which include preadipocyte proliferation, differentiation, and fatty acid synthesis, and is regulated by various molecular factors. Adipogenesis is related to both the occurrence and development of obesity [25]. To understand whether and how AM exhibits anti-obesity effect, we investigated the anti-adipogenic effect of AM.

Recently, we developed a new kind of C-3-Gal enriched AM extract (AM-Ex), containing more C-3-Gals than conventional AM extract. In the present study, we investigated the effect of AM-Ex on adipogenesis and its action mechanisms *in vitro* using 3T3-L1 adipocytes in order to elucidte the anti-obesity of AM-Ex.

2. Materials and Methods

2.1. Materials

The materials used in this study were purchased from the indicated suppliers: Dulbecco's Modified Eagle's Medium (DMEM) and other miscellaneous cell culture reagents from Welgene (Daegu, Korea); fetal bovine serum (FBS) and bovine calf serum (BCS) Gibco-Thermo Fisher Scientific Inc. (Waltham, MA, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-isobutyl-1-methylxanthine, dexamethasone, insulin, and Oil red O from Sigma-Aldrich Co. (St. Louis, MO, USA); and cyanidine-3-*O*-galactoside (C-3-Gal) from Polyphenols AS (Sandnes, Norway). Unless noted otherwise, all other materials were purchased from Sigma-Aldrich Co.

2.2. Preparation of Cyanidine-3-O-Galactoside Enriched Aronia Melanocarpa Nero Extract (AM-Ex)

The freeze-dried powder of *Aronia melanocarpa* Nero fruits harvested from Goseong (Korea) was purchased from Goseong Happy Aronia Farm (Goseong, Korea). The dried fruit powder was extracted with 70% ethanol by adding 100 g of the dried powder to 2 L of 70% ethanol using a high pressure homogenizer (Micronox, Seongnam, Korea) at room temperature. The intermediate extract was additionally extracted under reduced pressure using a rotary evaporator at 40 Mpa pressure at 30˚C for 2 h. The extract was concentrated with a rotary evaporator, and lyophilized for 72 h in a lyophilizer (IlshinBioBase, Dongducheon, Korea). The resulting powder was used as *Aronia melanocarpa* Nero extract (AM-Ex) and stored at −20˚C until further use. The control 70% ethanol extract (C-AM-Ex) was extracted for 24 h at 80˚C from 100 g of the dried fruit powder placed into 2 L of 70% ethanol. This C-AM-Ex was concentrated with a rotary evaporator, and lyophilized for 72 h in a lyophilizer (IlshinBioBase).

2.3. High-Performance Liquid Chromatography (HPLC) Analysis

Both AM-Ex and C-AM-Ex were analyzed using HPLC (Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) with Jupiter 5 μ C18 columns (150 × 4.6 mm, 3000 A, Phenomenex, Torrance, CA, USA) at 520 nm, and the operating conditions were as follows. The mobile phase solvents used were (A) HCOOH-H_2O (1:9) and (B) HCOOH-MeOH-H_2O (1:5:4), with (A) 100% in the 0 - 2 min period, with (A) 30% and (B) 70% in the 2 - 20 min period, with (B) 100% in the 20 - 22 min period, and with (A) 100% in the 22 - 24 min period. Before performing HPLC, the samples were filter using 0.45 μm filter, and the flow velocity was set to 0.8 mL/min.

2.4. Cell Culture and Adipocyte Differentiation Induction

Mouse 3T3-L1 preadipocytes were purchased from the Korean Cell Line Bank (Seoul, Korea). 3T3-L1 cells were cultured in growth medium (GM; DMEM supplemented with 100 mL/L BCS, 100,000 U/L penicillin, and 100 mg/L streptomycin) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. To induce adipocyte differentiation, at 2 days post-confluence (referred to as day 0), 3T3-L1 cells were exposed to differentiation medium (DM; DMEM with 100 mL/L FBS, containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin) for 2 days. On day 2, the medium was replaced with DM (DMEM with 100 mL/L FBS, containing 5 μg/mL insulin only). On day 4, the medium was replaced with DMEM supplemented with 100 mL/L FBS and cells were incubated for the next 4 - 8 days to fully differentiate. To examine the effects of AM-Ex on adipocyte differentiation, the cells were incubated in D-M in the presence or absence of various concentration of AM-Ex at 2 day intervals when the medium was replenished.

2.5. Cell Viability Assay

Cell viability was determined by the MTT assay, as described previously [26]. In brief, 3T3-L1 cells were plated in 24-well plates at a density of 3×10^4 cells/well. After incubation for 24 h, the cells were treated with AM-Ex at concentrations ranging from 0 to 2000 μg/mL and incubated for 72 h. At the end of the treatment period, the media were removed and 1 mg/mL MTT solution was added, and the cells were incubated at 37˚C for 2 h. After incubation, MTT solution was removed, 0.5 mL of isopropanol was added to dissolve formazan crystals, and the absorbance was measured at 570 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Oil Red O Staining and Lipid Accumulation Quantification

3T3-L1 cells were induced differentiation and treated with various concentration of AM-Ex as described above. Eight days after induction of differentiation and treatment, the fully differentiated 3T3-L1 cells were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 h, washed with PBS, then stained with Oil red O solution for 1 h. After removing excess staining solution, the stained cells were rinsed with water and dried. The stained cells were visualized by light microscopy (AxioImager, Carl Zeiss, Jena, Germany). Intracellular lipid accumulation was analyzed by dissolving the stained lipid droplets in 100% isopropanol and measuring the absorbance at 520 nm.

2.7. Measurement of Cellular Triglyceride Contents

Following differentiation and treatment with AM-Ex, total lipids in cells were extracted, according to the conventional extraction method [27], with minor modification. In brief, the cells were collected, homogenized immediately and chloroform/methanol (2:1) solution was added. The cell lysate was mixed, incu-

bated at 37˚C for 30 min, and centrifuged at 5000 rpm for 10 min. The bottom organic layer was collected and dried using a high-efficiency concentrated centrifuge. The lipid pellet was reconstituted in 20 μL chloroform. Triglyceride contents were assayed by the TG-S kit (Asan Pharmaceutical, Hwaseong, Korea), according to the manufacturer's instruction.

2.8. Quantitative Real-Time RT-PCR

Eight days after induction of differentiation and treatment with AM-Ex, total RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The content and purity of total RNA were estimated using a micro-volume spectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). Complementary DNA was synthesized from 1 μg of total RNA and 1 μM of Oligo-dT primer using HyperScript™ RT master mix (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instruction. Quantitative real-time polymerase chain reaction (PCR) was conducted using a Rotor-gene 3000 PCR (Corbett Research, Mortlake, Australia) and Rotor-Gene™ SYBR Green kit (Qiagen) according to the manufacturer's instruction. The sequences of the primers used in this study are shown in Table 1. PCR amplification of cDNA was carried out at 94˚C for 3 min, followed by 40 cycles as follows: 95˚C for 10 s, 60˚C for 15 s, and 72˚C for 20 s. The results were analyzed with Rotor-Gene 6000 Series System Software program, version 6 (Corbett Research), and normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 1. Gene-specific primers used for real-time PCR analysis.

Primer		Sequence (5' - 3')
ACC1	Forward	GGAGATGTACGCTGACCGAGAA
	Reverse	ACCCGACGCATGGTTTTCA
ACL	Forward	TGGATGCCACAGCTGACTAC
	Reverse	GGTTCAGCAAGGTCAGCTTC
aP2	Forward	GGATTTGGTCACCATCCGGT
	Reverse	TTCACCTTCCTGTCGTCTGC
C/EBP-α	Forward	TGGACAAGAACAGCAACGAGTAC
	Reverse	GCAGTTGCCCATGGCCTTGAC
FAS	Forward	AGGGGTCGACCTGGTCCTCA
	Reverse	GCCATGCCCAGAGGGTGGTT
LPL	Forward	CCAATGGAGGCACTTTCCA
	Reverse	CACGTCTCCGAGTCCTCTCTCT
PPAR-γ	Forward	CAAAACACCAGTGTGAATTA
	Reverse	ACCATGGTAATTTCTTGTGA
SREBP-1c	Forward	CACTTCTGGAGACATCGCAAAC
	Reverse	ATGGTAGACAACAGCCGCATC
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCAGG
	Reverse	CCACCACCCTGTTGCTGTAGCCA

2.9. Statistical Analysis

Results are presented as the mean ± SEM. Statistical analyses were performed using the Student's t-test to test the differences between the undifferentiated group (GM—treated group) and the differentiated group (DM—treated group). Analysis of variance (ANOVA) test, followed by Duncan's multiple comparison test was performed to determine whether AM-Ex has significant effects on the differentiated group. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Quantification of C-3-Gal in AM-Ex

HPLC chromatograms of AM-Ex and C-AM-Ex are shown in **Figure 1**. The standard peak of C-3-Gal was compared with the peaks of C-3-Gal in AM-Ex and C-AM-Ex. Higher amoumts of C-3-Gal were estimated in AM-Ex than in C-AM-Ex, 2408 mg/100g versus 1049 mg/100g for AM-Ex and C-AM-Ex, respectively. These HPLC measurements of C-3-Gal content in AM-Ex indicate that AM-Ex produced by the high pressure homogenizing method contain a large amount of C-3-Gal.

3.2. Effect of AM-Ex on Cell Viability of 3T3-L1 Preadipocyte

To investigate the concentration of AM-Ex that was not cytotoxic, we determined the effect of AM-Ex on 3T3-L1 cell viability by conducting the MTT assay. Concentrations of AM-Ex between 500 to 2000 μg/mL showed significant reductions in the viability of 3T3-L1 cells. However, the viability of cells treated with lower doses of AM-Ex (1 to 400 μg/mL) were not significantly different compared to non-treated cells (**Figure 2**). Therefore, we used it at concentrations of 0 - 400 μg/mL in subsequent experiments to exclude the possibility that the inhibitory effect of AM-Ex on adipogenesis is due to its cytotoxic effect on 3T3-L1 cells.

3.3. AM-Ex Inhibits Adipocyte Differentiation in 3T3-L1 Preadipocytes

3T3-L1 preadipocytes undergo morphologic changes from a spindle-like to round shape and accumulate intracellular lipids after addition of differentiation inducing reagents [28]. To investigate the effect of AM-Ex on adipocyte differentiation and adipogenesis, post-confluent 3T3-L1 cells were induced by DM with or without the various concentration of AM-Ex. Accumulated lipid droplets in the differentiated 3T3-L1 adipocyte were visualized and quantified by Oil Red O staining. As shown in **Figure 3(a)**, on day 8 after differentiation, 3T3-L1 adipocytes accumulated intracellular lipid droplets. AM-Ex significantly decreased the accumulation of lipid droplets. Cells treated with 400 μg/mL AM-Ex showed markedly reduced accumulation of lipid droplets (37.2% reduction) compared to non-AM-Ex-treated control cells (**Figure 3(b)**). These results demonstrated that AM-Ex inhibited adipocyte differentiation and adipogenesis

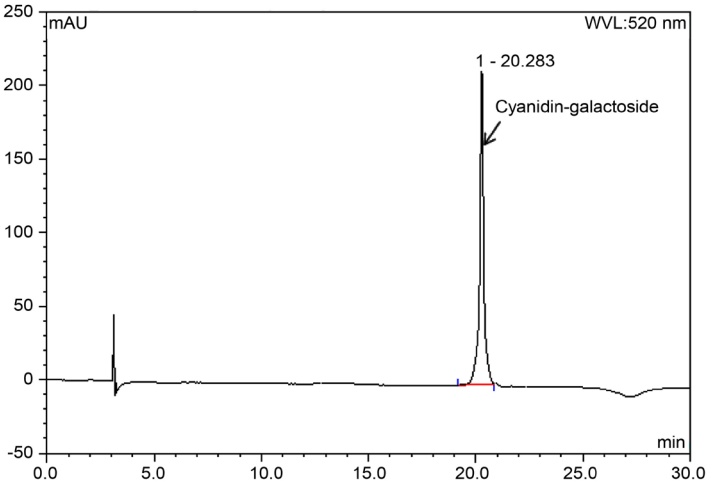

Figure 1. High-performance liquid chromatography chromatograms of AM-Ex and C-AM-Ex. (a) Standard, 1 mg/mL cyanidin-3-O-galactoside. (b) 1 mg/mL AM-Ex. (c) 1 mg/mL C-AM-Ex. AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract; C-AM-Ex, control 70% ethanol *Aronia melanocarpa* Nero extract.

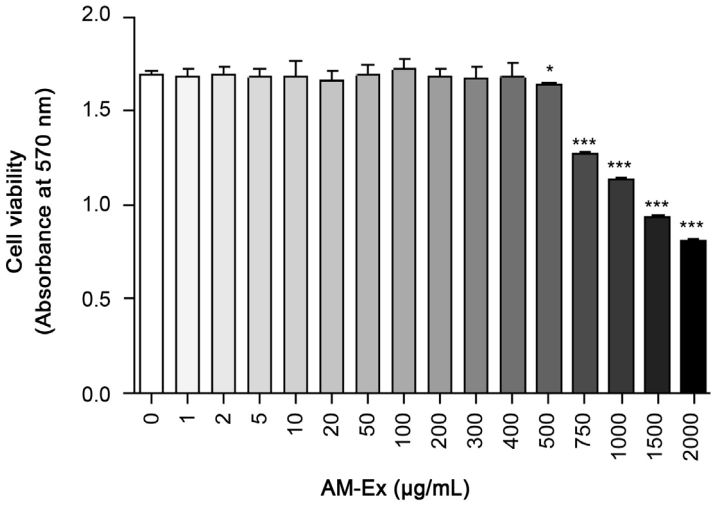

Figure 2. Effect of AM-Ex on cell viability in 3T3-L1 preadipocyte. 3T3-L1 cells were plated at 3×10^4 cells/well and incubated for 24 h. After incubation for 24 h, the cells were incubated for 48 h in medium containing concentrations of AM-Ex ranging from 0 to 2000 μg/mL. Cell viability of 3T3-L1 cells was measured by the MTT assay. Values are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ significantly different from that of non-AM-Ex treated cells. AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract.

in 3T3-L1 preadipocytes.

3.4. AM-Ex Inhibits Triglyceride Accumulation in 3T3-L1 Adipocytes

To investigate the effect of AM-Ex on lipogenesis, we determined intracellular triglyceride accumulation in 3T3-L1 adipocytes. AM-Ex at concentrations of 100, 200 or 400 μg/mL reduced the triglyceride levels in 3T3-L1 adipocytes by 24.4%, 28.6%, and 36.2%, respectively, compared to the non-AM-Ex-treated control (**Figure 4**). These results indicate that AM-Ex suppressed lipogenesis in 3T3-L1 adipocyte.

3.5. AM-Ex Inhibits the Expression of Adipogenic Transcription Factors

Adipogenesis is the process by which the differentiation from preadipocytes to mature adipocytes occurs. Several transcription factors such as CCAAT/enhancer binding proteins (C/EBPs), peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding protein 1c (SREBP-1c) are involved in adipogenesis [29]. Thus, we next examined whether AM-Ex suppressed the expression of adipogenic transcription factors. Compared to the non-DM-treated (undifferentiated) cells, the DM-treated (differentiated) cells exhibited dramatically increased C/EBP-α, PPAR-γ, and SREBP-1c mRNA expression. AM-Ex significantly decreased C/EBP-α, PPAR-γ, and SREBP-1c mRNA expression (**Figure 5**). These results suggest that AM-Ex inhibited

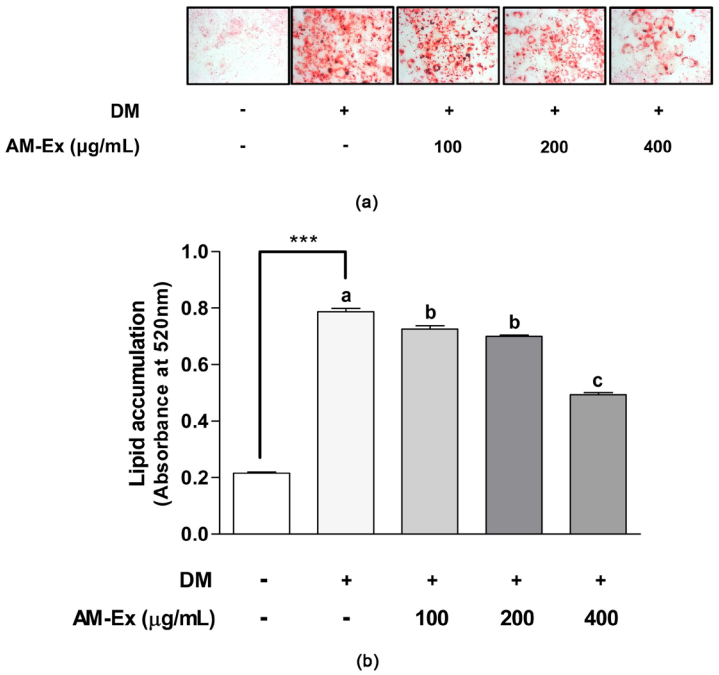

Figure 3. Effect of AM-Ex on adipocyte differentiation in 3T3-L1 cells. (a) 3T3-L1 cells were plated at 3×10^4 cells/well and induced differentiation of adipocyte in medium containing various concentrations of AM-Ex. The differentiated 3T3-L1 cell were stained with Oil red O solution and observed by light microscopy. (b) The accumulated lipid droplets in stained 3T3-L1 adipocyte with Oil red O solution were quantified. Values are expressed as mean ± SEM. ***$P < 0.001$ significantly different from that of non-DM-treated (undifferentiated) cells. Means with different letters are significantly different ($P < 0.05$). AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract.

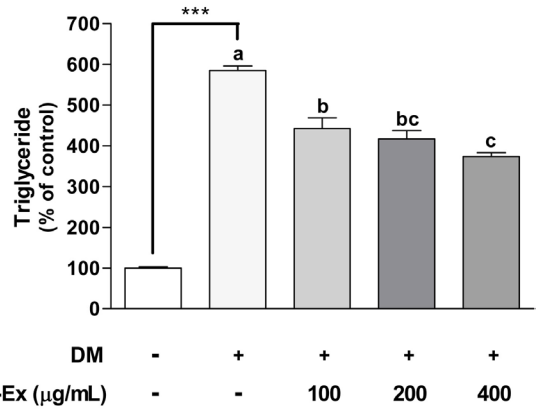

Figure 4. Effect of AM-Ex on intracellular triglyceride accumulation in 3T3-L1 cells. 3T3-L1 cells were plated at 3×10^4 cells/well. 3T3-L1 cells were differentiated and treatment with AM-Ex. The intracellular triglyceride accumulation in differentiated 3T3-L1 adipocyte was measured. Values are expressed as mean ± SEM. ***$P < 0.001$ significantly different from that of non-DM-treated (undifferentiated) cells. Means with different letters are significantly different ($P < 0.05$). AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract.

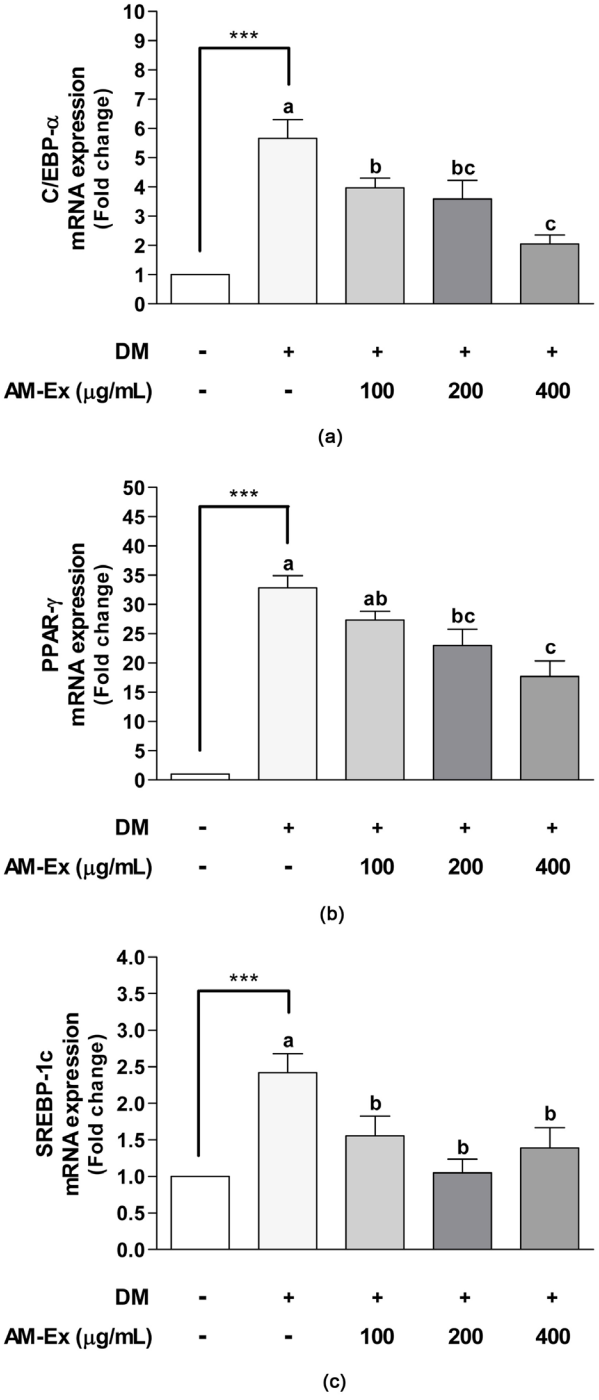

Figure 5. Effect of AM-Ex on the expression of adipogenic transcription factors in 3T3-L1 cells. 3T3-L1 cells were plated at 3×10^4 cells/well. Eight days after induction of differentiation and treatment with AM-Ex, total RNA was isolated. Real-time RT-PCR of C/EBP-*α*, PPAR*γ*, and SREBP-1c was performed. Values are expressed as mean ± SEM. ***$P < 0.001$ significantly different from that of non-DM-treated (undifferentiated) cells. Means with different letters are significantly different ($P < 0.05$). AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract; C-3-Gal, cyanidin 3-*O*-galactoside; C/EBP-*α*, CCAAT/enhancer binding protein-*α*; PPAR-*γ*, peroxisome proliferator-activated receptor-*γ*; SREBP-1c, sterol regulatory element-binding protein-1c.

adipocyte differentiation and adipogenesis through down-regulation of transcription factor, including C/EBP-α, PPAR-γ, and SREBP-1c.

3.6. AM-Ex Attenuates the Expression of Adipogenesis and Lipogenesis-Related Genes in 3T3-L 1 Cells

Adipogenic transcription factors, including C/EBP-α, PPAR-γ, and SREBP-1c, cooperatively induce the expression of specific genes involved in adipogenesis and lipogenesis [30]. Since C/EBP-α, PPAR-γ, and SREBP-1c were down-regulated by AM-Ex, we examined the gene regulation for adipogenesis and lipogenesis in AM-Ex-treated 3T3-L1 adipocytes using quantitative real-time RT-PCR. The mRNA expression of adipocyte protein 2 (aP2) and lipoprotein lipase (LPL), specific adipogenesis-related genes, were decreased by AM-Ex treatment. At 400 μg/mL, AM-Ex considerably reduced mRNA expression of aP2 and LPL by 76.2% and 34.8%, respectively, compared to the non-AM-Ex-treated control (Figure 6). As shown in Figure 7, AM-Ex significantly down-regulated the

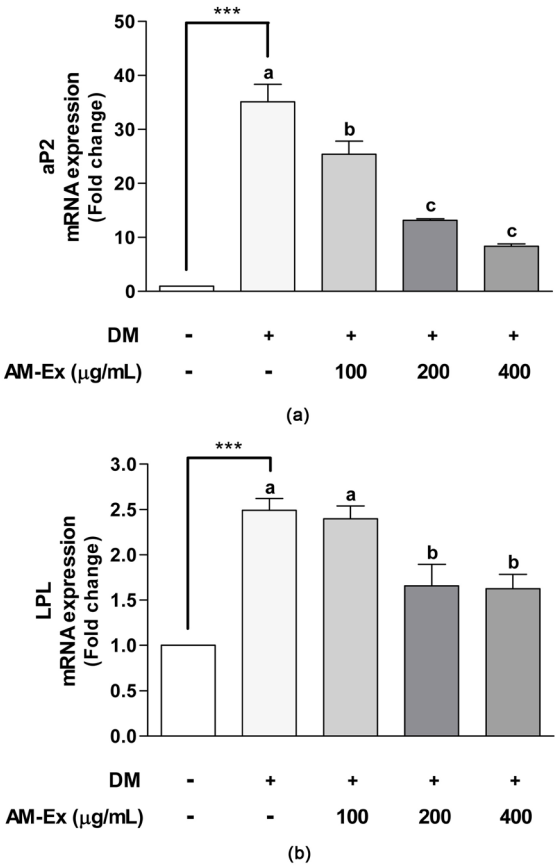

Figure 6. Effect of AM-Ex on the expression of adipogenesis-related genes in 3T3-L1 cells. 3T3-L1 cells were plated at 3×10^4 cells/well. Eight days after induction of differentiation and treatment with AM-Ex, total RNA was isolated. Real-time RT-PCR of aP2 and LPL was performed. Values are expressed as mean ± SEM. ***$P < 0.001$ significantly different from that of non-DM-treated (undifferentiated) cells. Means with different letters are significantly different ($P < 0.05$). AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract; aP2, adipocyte protein 2; LPL, lipoprotein lipase.

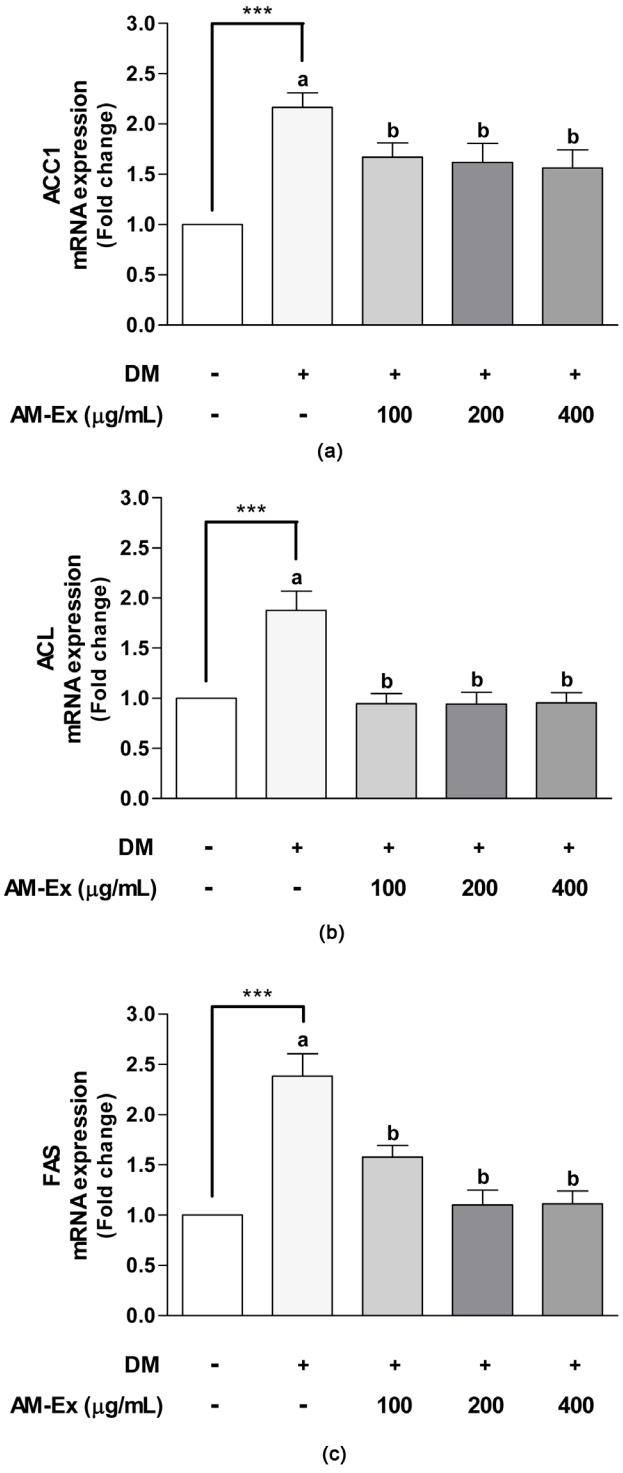

Figure 7. Effect of AM-Ex on the expression of lipogenesis-related genes in 3T3-L1 cells. 3T3-L1 cells were plated at 3×10^4 cells/well. Eight days after induction of differentiation and treatment with AM-Ex, total RNA was isolated. Real-time RT-PCR of ACC1, ACL and FAS was performed. Values are expressed as mean ± SEM. ***$P < 0.001$ significantly different from that of non-DM-treated (undifferentiated) cells. Means with different letters are significantly different ($P < 0.05$). AM-Ex, cyanidine-3-O-galactoside enriched *Aronia melanocarpa* Nero extract; ACC1, acetyl-CoA carboxylase 1; ACL, ATP-citrate lyase; FAS, fatty acid synthase.

mRNA expression of lipogenesis-related genes, including acetyl-CoA carboxylase 1 (ACC1), ATP-citrate lyase (ACL), and fatty acid synthase (FAS). However, there was no significant difference in the expression of ACC1, ACL, and FAS at AM-Ex concentrations of 100, 200, or 400 μg/mL (**Figure 7**). These results suggest that AM-Ex effectively suppressed adipogenesis and lipogenesis by down-regulating expression of major adipogenic and lipogenic target genes.

4. Discussion

Aronia melanocarpa (Chokeberry, AM) is a rich source of polyphenols, especially anthocyanins present as different forms of cyaniding-glycosides, procyanidins, and flavonoids. Numerous health promoting effects of AM, related to antioxidant activity have been reported [31]. Cyanidine glycosides are known to be effective in preventing obesity associated with metabolic diseases [32]. Anthocyanins also have some beneficial effects similar to other polyphenols including anti-inflammatory, anti-obesity, anti-diabetic, and anti-hypertensive effects. However, the mechanisms of its anti-obesity effect are still unclear compared to other AM functions. In the present study, we examined the effect of AM-Ex, an AM extract with a high content of C-3-Gal, on adipogenesis and lipogenesis in vitro using 3T3-L1 adipocytes.

Increased adipose tissue and adipocyte dysfunction related to obesity have been shown to be associated with abnormal adipogenesis regulation [33]. Adipocyte hypertrophy and hyperplasia both increase in adipose tissue, leading to obesity. Gene expression is tightly controlled and regulated by multiple components of various molecular circuits. In this process, transcription factors play an important role in different biological processes, such as differentiation, developmental process, and response to external and internal stimuli [30]. The process by which preadipocytes differentiate into mature adipocytes is regulated by hundreds of downstream protein-coding genes responsible for adipogenesis and by long noncoding RNAs (lncRNAs). This means that a large network of transcription factors acting together directly or indirectly, control the differentiation of adipocytes and the phenotypic characteristics of mature adipocytes [30] [34] [35] [36].

PPARγ and C/EBPα are crucial transcription factors in adipogenesis. PPAR is considered a critical factor in glucose and energy metabolism [37] [38]. C/EBPα plays an important role in adipogenesis, which directly induces diverse adipocyte genes. These two transcription factors play essential roles in determining the fate of differentiating preadipocytes [34] [39]. However, C/EBPα alone cannot induce adipogenesis without PPARγ. [37]. When PPARγ was overexpressed in mature 3T3-L1 adipocytes, both the adipocyte size and intracellular triglyceride content were increased [40]. Therefore, finding a functional food ingredient that can regulate PPARγ activity may be a complementary treatment for obesity-related diseases [41]. SREBP1c is also a critical factor that mediates induction

of lipid biosynthesis in adipocytes by increasing gene expression of major lipogenesis genes [42]. Many other transcription factors have been shown to exert a positive or negative effect on adipogenesis. For example, EBF1, KLF4, KLF5, KLF6, KLF15, EGR2, CEBPB, CEBPG and ARNTL promote adipogenesis. In contrast, GATA2, GATA3, KLF2, KLF3, IRF3, and IRF4 inhibit adipogenesis [34].

In the present study, we observed that AM-Ex treatment decreased PPARγ, C/EBPα, and SREBP1c mRNA expression in 3T3-L1 cells (**Figure 5**). These transcription factors regulate aP2, LPL, ACC, ACL, and FAS genes that control adipogenesis in the adipose tissue. For example, aP2, also called fatty acid binding protein 4 (FABP4) is postulated to be an early marker of the metabolic syndrome. Blocking this protein may represent a treatment for heart disease [43], diabetes [44], asthma [45], obesity [46], and fatty liver disease [47]. LPL acts as a key enzyme in the catabolic pathway of triglyceride-rich lipoproteins. LPL is synthesized and secreted by adipocytes and muscles, and is transported to capillary endothelial cells to hydrolyze the triglyceride core of circulating very low density lipoprotein and chylomicrons into fatty acids and monoglyceride. The hydrolysis products are taken up by the tissue. Depending on the energy state, LPL causes fatty acids to be transported to fat tissue for storage, and in the fasting state, fatty acids are broken down in muscles for use as fuel.

When adipose LPL is increased, free fatty acid produced by hydrolysis of lipoprotein stimulates PPAR transcription factor. In adipose tissue, LPL stimulates PPARγ and the LPL gene exhibits a mutually positive feedback loop that is stimulated by PPARγ [48]. Cinnamon and green tea polyphenols also have been reported to inhibit LPL and mRNA expression of other lipogenic genes [49] [50]. ACC regulates the metabolism of fatty acids. When ACC is activated, malonyl-CoA is formed to synthesize new fatty acids and inhibit the β-oxidation of fatty acid in mitochondria by inhibiting the transfer of fatty acyl groups from acyl CoA to carnitine with carnitine acyltransferase. In mammals, two main isoforms of ACC are expressed, ACC1 and ACC2, which differ in both tissue distribution and function. Although ACC1 is found in the cytoplasm of all cells, it is abundant in lipogenic tissue, such as adipose tissue and lactating mammary glands, while ACC2 is found in many oxidative tissues such as skeletal muscle and heart [51]. ACC1 and ACC2 are highly expressed in the liver where both fatty acid oxidation and synthesis are important [52]. This difference in tissue distribution means that ACC1 is involved in the regulation of fatty acid synthesis and ACC2 is involved in the regulation of fatty acid oxidation. ACL is an enzyme that catalyzes the hydrolysis of ATP and the conversion of citrate and CoA to acetyl-CoA and oxaloacetate [53]. Acetyl-CoA is used as a precursor in several important biosynthetic pathways, including triglyceride and cholesterol production [54]. FAS is a key enzyme in de novo lipogenesis. Metabolism and homeostasis of FAS are transcriptionally regulated by Upstream Stimulatory Factors (USF1 and USF2) and SREBP-1c in response to feeding/insulin in living animals

[55]. FAS mRNA and protein levels were increased in obese Zucker rats [56], and polyphenols of cinnamon [49] and dietary green tea [50] were reported to inhibit FAS mRNA expression in diet-induced insulin-resistant animals. Recently, aronia juice or extract intake in animal models and humans was reported to prevent or treat obesity. Qin and Anderson [22] reported that chokeberry extract intake lowered blood glucose, triglyceride, cholesterol, LDL-cholesterol, and epididymal fat pads in Wister rats fed fructose-rich diet. And also the expression of PPARγ and adiponectin mRNA were up-regulated and aP2, FAS, and LPL mRNA levels were inhibited by chokeberry extract consumption. Takahashi *et al.* [23] reported that aronia fruit consumption inhibited hyperglycemia as well as visceral fat accumulation in high-fat diet-induced diatomic obese rats. Yamane *et al.* [16] reported that aronia juice had a beneficial effect on diabetes and obesity by decreasing dipeptidyl peptidase and a-glucosidase activity. Kardum *et al.* [57] reported that aronia juice was effective in improving obesity in abdominally obese women aged 45 - 65 years.

Studies on the mechanism of the molecular regulation associated with the anti-obesity effects of AM have recently begun. Kowalska *et al.* [58] reported that various combinations of berries, including AM, in 3T3-L1 adipose cells reduced adipogenesis and oxidative stress. In this study, cells treated with 100 μg/mL of mixed berry extract down-regulated PPARγ (67%), C/EBPα (72%), SREBP1 (62%), aP2 (24%), HSL (39%), and PLIN1 (32%). In the present study, the data showed that AM-Ex treatment decreased aP2, LPL, ACC1, ACL, and FAS. In particular, aP2 expression was decreased by AM-Ex treatment in a dose-dependent manner (**Figure 6** and **Figure 7**).

5. Conclusion

Our results showed that the anti-obesity effect of AM-Ex occured through down-regulation of the transcription factors PPARγ, C/EBPα, SREBP-1c, and adipogenesis and lipogenesis-related genes, aP2, LPL, ACC1, ACL, and FAS. These results demonstrate that AM-Ex can be used as a preventive or therapeutic agent for obesity. Future, animal and human studies are needed to further investigate the mechanism and proper concentration of AM to be used as anti-obesity agents.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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