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Antiadipogenic effects of açai seed extract on high fat diet-fed mice and 3T3-L1 adipocytes: A potential mechanism of action

Patricia Leticia Trindade[a], Elaine dos Ramos Soares[a], Elisa Bernardes Monteiro[a], Ângela Castro Resende[b], Nathalia Moura-Nunes[a], Vanessa Souza-Mello[c], Danielly Cristiny Ferraz[a], Julio Beltrame Daleprane[a,*]

[a] Laboratory for Studies of Interactions Between Nutrition and Genetics, LEING, Department of Basic and Experimental Nutrition, Rio de Janeiro State University, Rio de Janeiro, Brazil
[b] Laboratory of Cardiovascular Pharmacology and Medicinal Plants, Department of Pharmacology, Rio de Janeiro State University, Rio de Janeiro, Brazil
[c] Laboratory of Morphometry, Metabolism and Cardiovascular Diseases, Department of Anatomy, Rio de Janeiro State University, Rio de Janeiro, Brazil.

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ABSTRACT

Body adiposity is an important risk factor for the development of chronic non-transmissible diseases. Studies on the process of adipogenesis have been extensively performed *in vivo* and *in vitro* models to describe the molecular and cellular bases of adipose tissue development and the effect of natural products in this process. The açai seed extract (ASE) has been evidenced as a potential regulator of body mass. In our work high-fat diet–fed mice treated with ASE (300 mg/Kg/d) (HFD-ASE) showed a lower adipose index (−32.63%, $p < 0.001$) than the high-fat diet–fed mice group (HFD) and the adipocytes from the HFD group were considerably enlarged ($p < 0.001$) compared to those in the control group (CG) and HFD-ASE group (+175% and +123%, respectively). We also evaluated the effects of ASE on the modulation of adipogenesis in 3T3-L1 cells. ASE exposure (25 and 100 μg/mL) led to a decrease of 26.6 ($p < 0.05$) in proliferation and also inhibited pre-adipocyte differentiation through the decreasing expression ($p < 0.05$) of transcription factors and adipogenic proteins such as PPARɣ, SREBP-1, and FAS. These results show that the ASE reduce adipogenesis and suppress lipid accumulation in the *in vivo* model and in 3T3-L1 adipocytes and reinforce ASE as a potential strategy to modulate adipogenesis.

1. Introduction

Obesity is a chronic non-transmissible disease that affects distinct age, ethnic and social classes. It has recently been shown that overweight and obesity affect 52% of the worldwide population and its prevalence has increased over recent decades [1]. Adipogenesis requires the activation of several transcriptional factors [2]. Among all, peroxisome proliferator-activated receptor ɣ (PPARɣ) is known as a major regulator of adipogenesis and is at the center of the adipogenic cascade. PPARɣ is highly adipocyte- specific with its expression rapidly increasing after hormone-induced adipocyte differentiation [3]. Sterol regulatory element binding protein (SREBP) is a key lipogenic transcription factor that is nutritionally regulated by glucose and insulin [4,5]. Insulin can function upstream to PPARɣ by activation of SREBP-1 and fatty acid synthase (FAS) [6,7]. Both SREBP-1 and FAS take part in lipogenesis and are essential for the formation of mature adipocytes. Therefore, adipogenesis is the key to understand the molecular mechanisms involved in obesity genesis. Diverse cellular model systems are used to study the molecular pathways of adipogenesis and adipocyte function *in vitro*. 3T3-L1 cell lines are preferred because they develop a homogeneous population of mature adipocytes that are morphologically and biochemically similar to adipocytes *in situ* [8–10].

Considering the great relevance of this issue, several studies have evaluated the influence of bioactive compounds on obesity and obesity-related diseases. Açai (*Euterpeoleracea Mart*) is largely diffused in the Amazon region, Brazil, and its fruits are rich in polyphenolic content [11]. Açai seed extract (ASE) is rich in catechin and polymeric proanthocyanidins [12,13] and has shown potential biological activities in different *in vitro* and *in vivo* experimental models, such as induced endothelium-dependent vasodilatation, anti-inflammatory properties, antihypertensive and antioxidant effects in experimental hypertension and oxidative stress [12–14], beneficial effect on metabolic syndrome and hypoglycemic actions, as well as promotion of body weight reduction [15]. These discoveries propound a favorable use of ASE as a medicinal plant extract with helpful therapeutic properties. However, the mechanisms of the antiobesity effect of ASE still remain unclear,

* Corresponding author at: Rua São Francisco Xavier, 524, Pavilhão João Lyra Filho, 12° andar, Sala 12.150, Bloco F, 20550-900 Rio de Janeiro, RJ, Brazil.
E-mail address: beltrame@uerj.br (J.B. Daleprane).

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and no studies have determined the molecular functions of ASE on adipogenesis. Thus, the aim of this study was to investigate the effects of ASE on the modulation of adipogenesis on high fat diet-fed mice and in 3T3-l1 cells to elucidate the possible molecular mechanism implicated in that process.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco, Life Technologies (Grand Island, NY, USA). Monoreagent triglycerides K117 were purchased from Bioclin (Belo Horizonte, MG, Brazil). 3-Isobutyl-1methylxanthine (IBMX), insulin, dexamethasone, annexin V-FITC assay and oil red O powder were purchased from Sigma Aldrich (St. Louis, MO, USA). AlamarBlue® reagent and the antibiotic mixture (penicillin-streptomycin) were purchased from Thermo Fisher Scientific Inc. (Barrington, IL, USA). Antibodies against PPARγ, FAS, SREBP-1 and P53 were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). All chemicals were commercially available and of the highest purity grade.

2.2. Animals and diet

Forty male C57BL/6 mice were kept under controlled conditions (temperature 21 ± 2 °C, humidity 60 ± 10%, 12/12 h light/dark cycle) and group housed in pathogen-free cages with free access to food and water. All procedures in this study were in accordance with the conventional guidelines for experimentation with animals (National Institutes of Health Publication No. 85–23, revised in 1996) and approved by the Animal Ethics Committee of State University of Rio de Janeiro (Protocol Number CEUA/042/2016). Twenty mice were submitted to a high-fat diet (HFD: 14% of energy as protein, 50% as fat (lard) and 36% as carbohydrates, total energy 21 KJ/g). Simultaneously, other ten mice received a control diet (CD: 14% of energy as protein, 10% as fat, and 76% as carbohydrates, total energy 15 KJ/g). Both diets followed the recommendations by the AIN-93 M for rodents [16], manufactured by PragSolucoes (Jau, Sao Paulo, Brazil), and this protocol lasted ten weeks. Subsequently, the treatment started and the HF group was divided into two groups (n = 10 each group), as follows: a) CD group fed the control diet throughout the experiment; b) HFD group–untreated, fed the high-fat diet throughout the experiment; c) HFD-ASE group fed the high-fat diet throughout the experiment and treated with ASE (300 mg/kg/d), based on a previous study [15]. The treatments lasted four weeks by gavage. Food intake was measured daily, and body mass (BM) was measured weekly. Energy intake (in KJ) was obtained as the product of food consumption and the energy content of the diet.

2.3. Euthanasia and tissue collection

The animals were starved for 6 h on the day of sacrifice and were deeply anesthetized (intraperitoneal sodium pentobarbital, 150 mg kg^{-1}). We cautiously dissected and weighed the subcutaneous (inguinal), and intra-abdominal (epididymal and retroperitoneal) fat pads, a fragment of retroperitoneal fat pad was fixed in a freshly prepared fixative solution (4% formaldehyde w/v, 0.1 M phosphate buffer, pH 7.2) for 48 h for light microscopy. Also, we determined the adiposity index as the ratio between the sum of the intra-abdominal and subcutaneous fat divided by body weight and multiplied by one hundred [17].

2.4. Cell culture

Mouse 3T3-L1 cells (CL-173, ATCC®) were maintained in proliferation medium containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% of penicillin/streptomycin at 37 °C with 5% CO_2, humidified atmosphere. After cells had grown to confluency, cells were stimulated in differentiation medium (DM) (10% FBS, 10 µg/mL insulin, 1 mM dexamethasone, and 0.5 mM IBMX). 72 h following DM stimulation, the media was changed to insulin media (10% FBS and 10 µg/mL insulin) and changed after every 48 h until the end of differentiation. Cells were then maintained in DMEM containing 10% FBS, and media was changed every 48 h.

Table 1
Food behavior, body mass gain and adipose index of high fat diet-fed mice treated or not with açai.[1]

Parameters	Experimental groups[2]		
	CD	HFD	HFD-ASE
Food intake (g/day)	3.4 ± 0.4	3.1 ± 0.3	3.2 ± 0.4
Energy intake (kJ/day)	51.5 ± 4.6[b]	65.1 ± 3.6[a]	67.2 ± 1.6[a]
Adipose index (%)	2.1 ± 0.4[b]	4.6 ± 1.2[a]	2.7 ± 0.7[b]

Significant differences are indicated by different superscript letters (One-way ANOVA followed by Holm–Sidak *post hoc* test, $p < 0.05$).
[1] Values are expressed as means ± standard deviation.
[2] CD: control; HFD: high-fat diet; HFD-ASE, high-fat diet treated with 300 mg/Kg/d of açai seed extract (ASE).

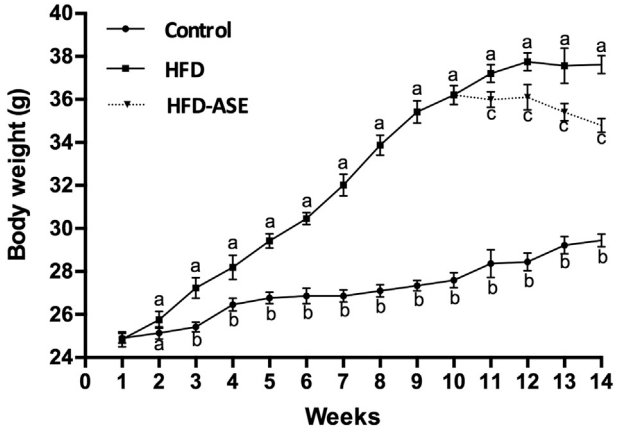

Fig. 1. Body mass evolution. Values are expressed as mean ± standard deviation, n = 10 per group. Groups: control diet (CD); high-fat diet (HFD) and high-fat diet treated with ASE (300 mg/Kg/d) (HFD-ASE). Significant differences are indicated by different letters (Oneway ANOVA followed by Holm–Sidak *post hoc* test, $p < 0.05$).

2.5. Experimental design

To describe the action of ASE on the process of adipogenesis in 3T3-L1 cells, two experimental designs were applied. Forty-eight hours after achieving confluence (day 0), cells were divided into two groups: Pre Treatment groups (PTTM) – In proliferation medium, cells were incubated with different concentrations of ASE (0, 10, 25, 50 and 100 µg/mL), after that, the medium was removed, and replaced with DM for 72 h. Posteriorly, the medium was changed to insulin medium and changed after every 48 h until the end of differentiation; Treatment group (TTM) – After stimulation in DM, cells were incubated with different concentrations of ASE (0, 10, 25, 50 and 100 µg/mL) concomitantly with insulin media and changed after every 48 h until the end of differentiation.

2.6. Cell viability assay

The viability of 3T3-L1 was determined using AlamarBlue® reagent according to the manufacturer's instructions. The cell viability

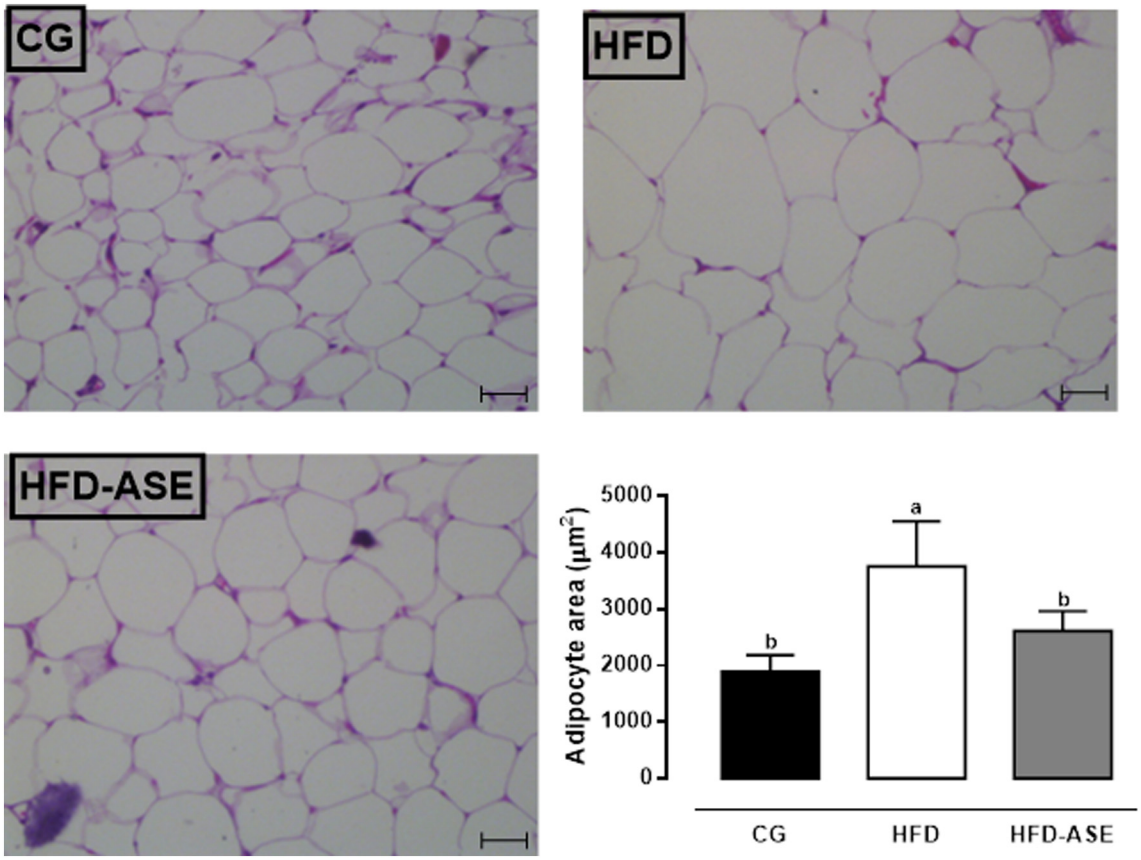

Fig. 2. Representative findings of retroperitoneal adipose tissue of mice fed control diet (CD), high-fat diet (HFD) and high-fat diet treated with ASE (300 mg/Kg/d) (HFD-ASE) for 8 weeks. Data are presented as mean ± standard deviation (SD) and different letters mean significant difference between the groups, $p < 0.05$, bar = 100 µm.

evaluation was performed at the end of 11 days of differentiation, in both groups (PTTM and TTM), in triplicate. Furthermore, a confluent fibroblast viability test was performed, with the addition of different concentrations of ASE for 48 h, to determine the ASE toxicity in another cell than the adipocyte. The fluorescence of each well was determined at 570 nm (excitation) and 600 nm (emission) on a Chameleon Multilabel Plate Detection Platform (Hidex, Oy, Turku, Finland).

2.7. Oil Red O staining

Cells were washed with PBS, fixed in 3.7% formaldehyde for 60 min, and then washed 3 times with distilled water and dried at room temperature. Then the cells were stained with Oil Red-O (6 parts of 0.3% saturated Oil Red-O in isopropanol and 4 parts of water) for 15 min. Excess dye was removed and cells were washed with 70% ethanol for 5 or 6 times. Thereafter, the stained cells were washed with water and dried at room temperature. Photographic records of cells stained at 20× magnification were made. The absorbance was evaluated on a microplate reader at 490 nm.

2.8. Quantification of triglycerides in 3T3-L1 cells

The quantification of triglycerides was carried out at the end of 11 days of differentiation. Cells were lysed and centrifuged for 15 min at 4 °C. After centrifugation, the lipid fraction was separated and then quantified following the instructions of the analysis kit (mono reagent triglycerides K117). The absorbance was later evaluated in a microplate reader at 500 nm. For the quantification calculations, the absorbance of the samples was multiplied by a constant, indicated by the kit manufacturer, and its result divided by the absorbance of the standard. The results were expressed in milligrams per deciliters (mg/dL).

2.9. Western blotting

Proteins involved in adipogenesis were evaluated by Western Blot. Concisely, cells treated as indicated were washed with ice-cold PBS, scraped off, lysed in 100 µL of lysis buffer and centrifuged (15,000*g*, 15 min). The amount of protein was quantified using DC protein assay kit (BioRad, Hercules, California, USA) and 50 µg of total protein was loaded on 10% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Nonspecific binding was blocked with 5% (w/v) defatted milk powder in Tween-20 tris-buffered saline (TTBS) for 2 h followed by incubation with PPARɣ, SREBP-1, FAS, P53 (1:1000) and b-actin (1:500) in TTBS overnight at 4 °C. Blots were then incubated with anti-goat or anti-mouse antibodies (1:1000 in 2% milk/TTBS) followed by enhanced chemiluminescence detection (ChemiDocTM XRS device, Bio-Rad) using the Imagelab software, V4.0, Build 16 (Bio-Rad). Band intensities were quantified by densitometric analysis using ImageJ 1.3 software (NIH, USA).

2.10. Statistical analysis

All results were expressed as the mean ± standard deviation from triplicate measurements performed in at least 3 independent experiments. Statistical analysis was performed by 1-way ANOVA followed by Holm-Sidak's multiple comparisons test. Probability values were considered significant at $p < 0.05$. All data were analyzed in GraphPad Prism version 6 software (GraphPad software, La Jolla, USA).

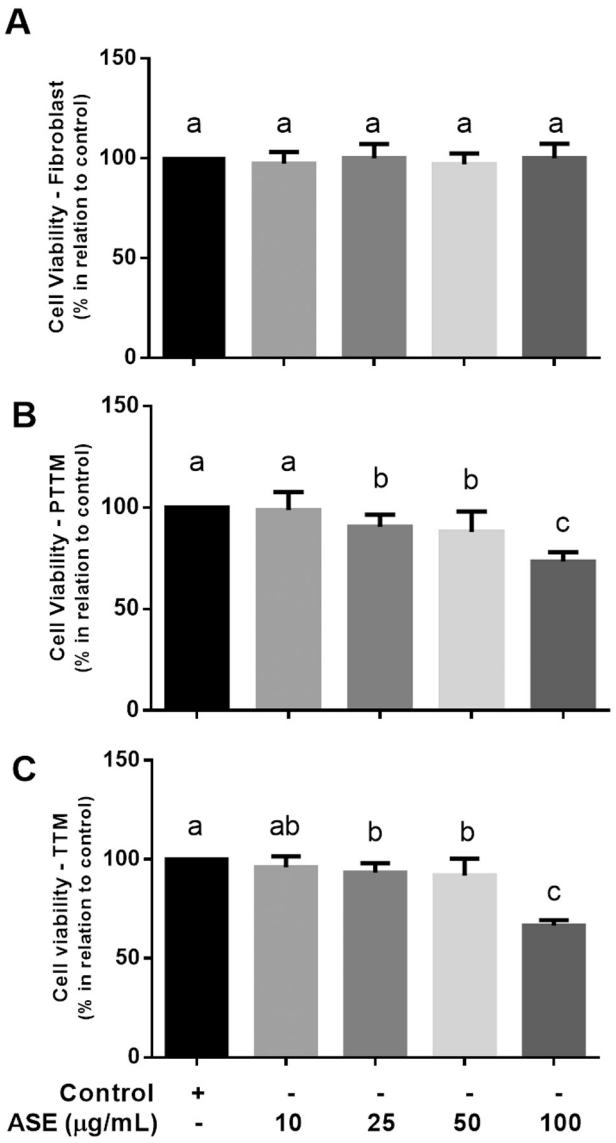

Fig. 3. Cell Viability assay in fibroblasts (A), PTTM (B) and TTM (C). Cytotoxicity of 3T3-L1 cells was expressed as the percentage of the result obtained for the control group. Data are presented as mean ± standard deviation (SD) and different letters mean significant difference between the groups, $p < 0.05$. Abbreviations: ASE (açai seed extract), PTTM (Pretreatment group) and TTM (Treatment group).

3. Results

3.1. ASE protects weight gain and adiposity in high-fat diet–fed mice

At the end of the four-week treatment period, the average weekly food intake was not significantly different among the four groups (Table 1). Nevertheless, the energy intake was higher in the HF groups compared to the CD group (+ 27.6%, $p < 0.05$, in the HFD group; +31.7%, $p < 0.05$, in the HFD-ASE group). Moreover, the HFD group showed a greater body mass gain throughout the experiment compared to the other groups (+66.9% compared to CD, $p < 0.001$; and +44.9% compared to HFD-ASE group, $p < 0.05$) (Fig. 1). The HF group had a higher adiposity index (+219.1%, $p < 0.001$) than the C group. However, the HFD-ASE showed a lower adiposity index (−32.63%, $p < 0.001$) than the HFD group, but higher than the CD group (+28.6%, $p < 0.001$) (Table 1). The retroperitoneal white adipose tissue histology demonstrated a unilocular cellular conformation (Fig. 2). Adipocytes from the HFD group were considerably enlarged ($p < 0.001$) compared to those in the CG and HFD-ASE groups (+175% and +123%, respectively).

3.2. Effect of ASE on viability during adipocyte differentiation

Cell cytotoxicity was evaluated at the end of the differentiation process in both experimental models as well as in non-induced fibroblasts. We observed that there was no change in fibroblast viability, remaining 100% viable (Fig. 3A). In the PTTM group all tested-ASE concentrations led to a significant decrease ($p < 0.05$) in the feasibility of the treated cells (Fig. 3B) with major viability reduction on 26.58% compared to control. Higher results were obtained in the TTM group (Fig. 3C), both of which presented a dose-dependent reduction in viability, with a larger reduction on 33.50% compared to control.

3.3. Effect of ASE on intracellular lipid accumulation

The role of ASE in adipogenesis was determinate through evaluation of cell differentiation analysis, cell morphology and the concentration of intracellular triglycerides at the end of cell differentiation period. We observed a decrease in adipogenesis, with a reduction of the intracellular triglyceride concentration in cells pretreated with ASE (Fig. 4A) and preadipocytes submitted to ASE treatment until their complete differentiation (Fig. 4B). Such decline was observed in a dose-dependent manner in both groups, with statistical significance ($p < 0.05$) in all treatments, when compared to the control group (untreated cells). After obtaining these data, it was decided to standardize the ASE concentrations to be used in the future experiments. In this sense, the concentrations of 0, 25 and 100 µg/ml were chosen.

3.4. ASE inhibited adipogenesis through PPARγ, FAS, and SREBP-1 expression in 3T3-L1 adipocytes

To investigate and characterize the action of ASE on adipocyte differentiation, we evaluated key proteins inherent to this process. The results obtained by the Western Blotting analysis demonstrate decreased protein expression of the PPARɣ receptor (Fig. 5A and B) when 3T3-L1 cells were pretreated or treated with concentrations of 25 and 100 µg/mL of ASE. Similar results were observed for FAS (Fig. 5C and D) and SREBP-1 expression (Fig. 5E and F) in cells treated under the same conditions above and had statistical significance ($p < 0.05$) in all treatments, when compared to the control group (untreated cells).

4. Discussion

Nutritional and pharmacological studies have been performed with açai demonstrating the presence of important phenolic compounds with biological activities, including anti-obesity effects [13,15]. In the present study, treatment with ASE prevented body mass gain and adiposity in high-fat diet–fed mouse. Other recent studies, using the same food matrix of the present study or another berry like *Euterpe edulis* Mart, support our findings. The inclusion of those fruits in the diet of HF-fed mice prevented body weight gain, adiposity, and significantly improved plasma and tissue markers of obesity, suggesting that even low doses of these compounds may have a protective role against body weight gain and consequently obesity development [18,19]. In order to evaluate the possible mechanism involved in açai extract adipogenesis modulation we perform experiments with preadipocytes 3T3-L1. The differentiation of preadipocytes into adipocytes is regulated by different transcription factors that initiate and regulate the entire process of cell differentiation. Given the background, the study of phenolic compounds has demonstrated beneficial effects on human health by acting on the possible modulation of the metabolic pathways involved with adipogenesis [20,21]. The 3T3-L1 cell line, which is widely used as an *in vitro* model, has provided support for adipocyte research over several decades. Although the results obtained by this model have limitations and the

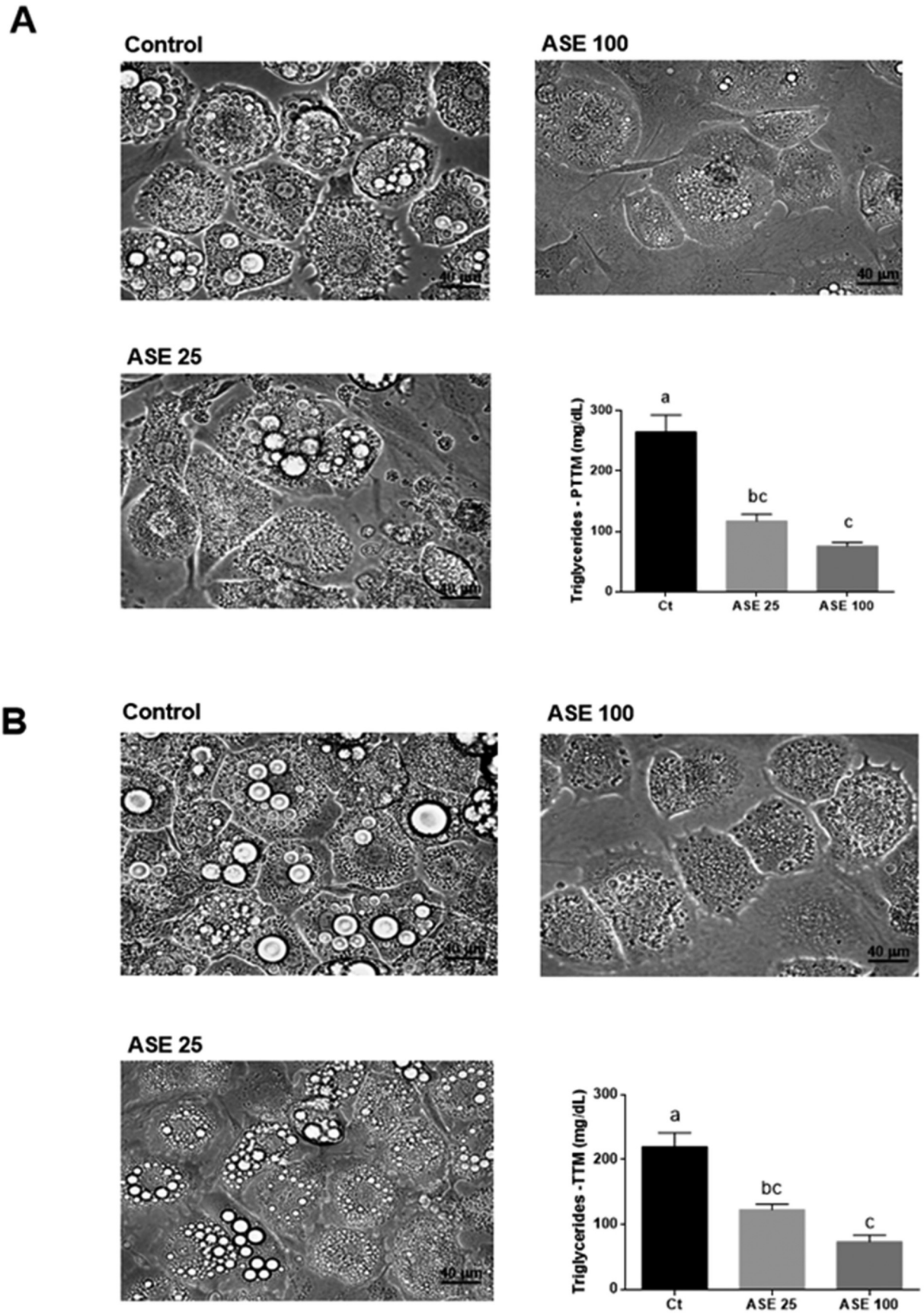

Fig. 4. Effects of ASE on lipid accumulation in 3T3-L1 adipocytes. (A) Photomicrographs of PTTM 3T3-L1 adipocytes stained with oil red, followed by the graph that represents the quantification of lipid accumulation in each group. (B) Photomicrographs of TTM 3T3-L1 adipocytes stained with oil red followed by the graph that represents the quantification of lipid accumulation in each group. Data are presented as mean ± standard deviation (SD) and different letters mean significant difference between the groups, $p < 0.05$. Abbreviations: ASE (açai seed extract), PTTM (Pretreatment group) and TTM (Treatment group).

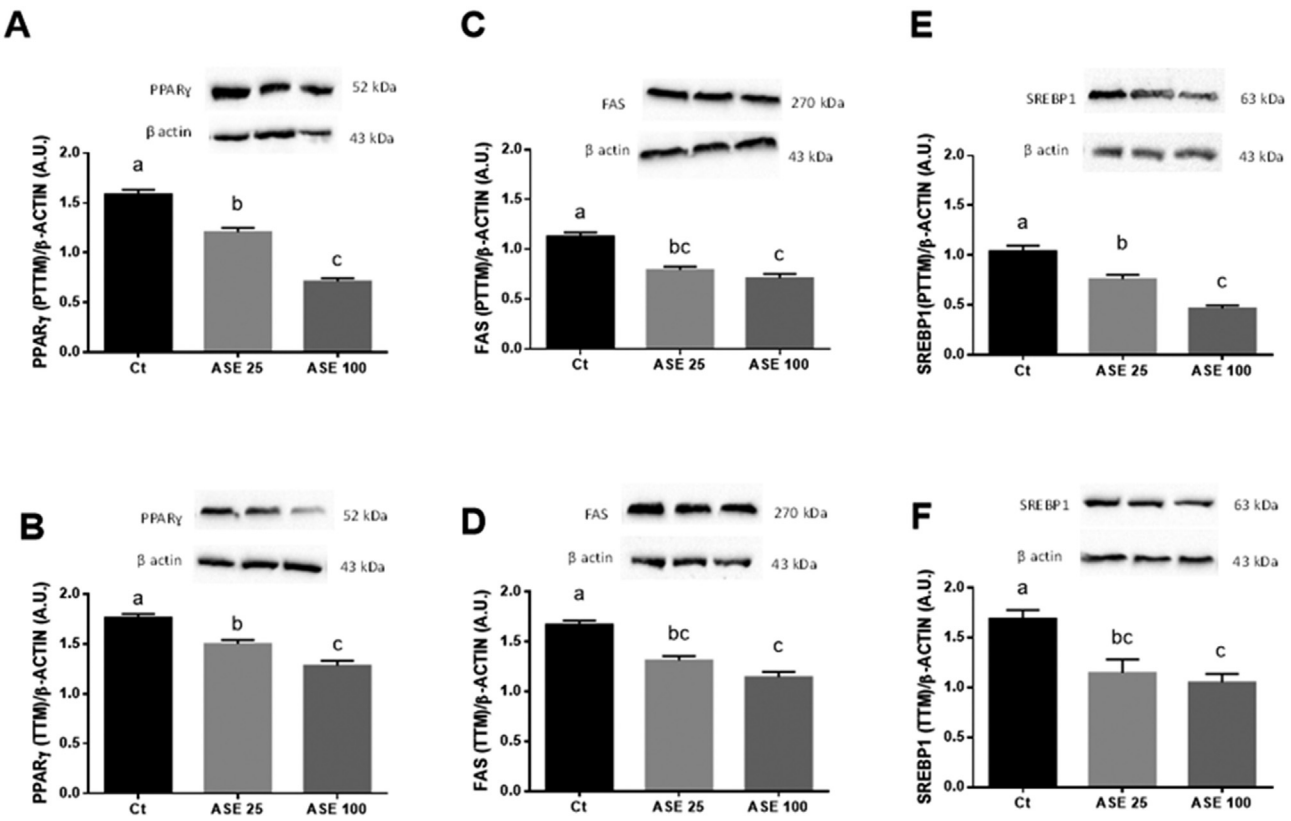

Fig. 5. Effects of ASE on adipogenic and lipogenic proteins expression in 3T3-L1 adipocytes. Expression levels of PPARγ protein PTTM (A) and PPARγ protein TTM (B), FAS protein PTTM (C) and FAS protein TTM (D), and SREBP-1 protein PTTM (E) and SREBP-1 protein TTM (F) analyzed using western blotting. Data are presented as mean ± standard deviation (SD) and different letters mean significant difference between the groups, $p < 0.05$. Abbreviations: ASE (açai seed extract), PTTM (Pretreatment group), TTM (Treatment group), PPAR (peroxisome proliferator-activated receptor), FAS (fatty acid synthase) and SREBP (Sterol regulatory element-binding proteins).

extrapolation of these results to *in vivo* conditions must be carried out with caution, this is still the most indicated and used way to provide a homogeneous cell population, allowing the realization of cultures indefinitely and thus enabling its reproducibility [22–24].

In this study, we aimed to identify the possible modulating role of ASE on the process of adipogenesis *in vitro*. In relation to the ASE toxicity, both experimental groups promoted a progressive reduction of cell viability, as the concentrations of ASE were increased in the respective assays. As in our study, other researches that used epigallocatechin gallate (EGCG) and resveratrol reported anti-adipogenic effect, by reducing the viability of the preadipocytes, in addition to expressing results consistent with a decrease in cell proliferation [25–27], endorsing our findings and reinforcing our hypothesis of the effect of ASE as a potential modulator in the process of adipogenesis. It is noteworthy that the toxicity exerted by ASE could only be observed in the preadipocytes and adipocytes, which make us believe in the selectivity of this extract, since the non-induced fibroblasts were exposed to ASE did not present a reduction of feasibility.

The incorporation and quantification of triglycerides were also affected in a dose-dependent manner, in both experimental models applied in this work. Studies using phenolic compounds expressed results that resemble our findings, such as inhibition of preadipocyte proliferation, suppression of adipocyte differentiation and accumulation of intracellular triglycerides, suppression of lipogenesis and concomitant downregulation of lipogenic genes as PPARγ and FAS. Such results in these studies, as herein, are elucidated by the modulating effect of these compounds on PPARγ signaling [25,28–32].

Once observed the decrease in the accumulation of triglycerides in 3T3-L1 adipocytes submitted to ASE treatments, we investigated the possibility of alteration of the expression of the specific genes involved in adipogenesis, in search of the best elucidation of the molecular pathways involved in this process. Confirming our conjecture, we observed in our tests these reductions, conditioned to the increase of ASE concentrations (0.25 and 100 μg/ml), in both experimental models, of PPARɣ, SREBP -1 and FAS. Our results confirm data from the literature showing that studies with compounds such as arginine, genistein, gingerol and resveratrol, in different concentrations, may act blocking the conversion of preadipocytes into adipocytes, likewise reducing and/or inhibiting the expression of SREBP-1, FAS and PPARɣ [33–36].

It is known that the PPAR-γ receptor plays an important role in the regulation of triglyceride metabolism in adipocytes and control of lipid mobilization in these cells, playing an essential role in the maintenance of the adipocyte phenotype [37,38]. Likewise, the sterol regulatory element binding protein 1 (SREBP-1) is a key regulator of adipocyte differentiation and maturation. SREBP-1 is able to produce an endogenous ligand that reinforces the PPARγ activity [39]. Hence, SREBP-1 interacts with PPARγ and exerts an important role in the adipocyte gene expression, regulating the expression of FAS, an enzyme responsible for the formation of mature adipocytes, which also regulates important genes involved in fatty acid metabolism [30,31]. Therewith, in this context, our results demonstrate that ASE exerts antiobesogenic effects by inhibiting adipogenesis through the downregulation of adipogenic transcription factors and their target genes.

5. Conclusion

From the results obtained in this work, we can conclude that the ASE demonstrated the ability to modulate the adipogenic process, acting on the regulation of adipocyte differentiation and maturation, and consequently inhibition of adipogenesis process. Finally, we

suggest that ASE may be an important tool for prevention or treatment of obesity.

Declarations of interest

None.

Acknowledgments

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