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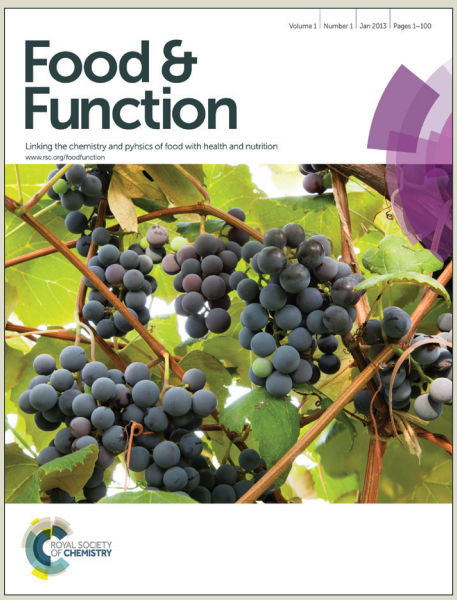

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Muscadine Grape Seed Oil as a Novel Source of Tocotrienols to Reduce Adipogenesis and Adipocyte Inflammation

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Abstract

Tocotrienols are unsaturated forms of vitamin E previously shown to reduce adipogenesis and adipose inflammation. In this study, muscadine grape seed oil (MGSO) was identified as a novel source of tocotrienols containing significant amounts of α- and γ-tocotrienol with minor seasonal changes. The aim of this study was to assess the anti-adipogenic and anti-inflammatory potential of MGSO by using primary human adipose-derived stem cells (*h*ASCs). Differentiating *h*ASCs were treated with MGSO and compared with rice bran and olive oil. Accumulation of triglyceride was significantly lower in MGSO-treated *h*ASCs than rice bran and olive oils. A tocotrienol rich fraction (TRF) from MGSO was prepared by solid phase extraction and eluted with 15% 1, 4 dioxane in hexane. The MGSOs-derived TRF treatment significantly reduced mRNA and protein expression that are crucial to adipogenesis (*e.g.*, PPARγ and aP2) in *h*ASCs. Furthermore, TRF from MGSO markedly reduced LPS-induced proinflammatory gene expression in human adipocytes and cytokine secretion to the medium (IL-6 and IL-8). Collectively, our work suggests that MGSOs are a stable and reliable natural source of T3 and MGSOs may constitute a new dietary strategy to attenuate obesity and its associated adipose inflammation.

1. Introduction

Muscadine grape is the native species of grape widely grown in the Southern States and its nutraceutical benefits have been well documented.[1] With their major use in the production of wine and juice, several thousand tons of muscadine grape pomace is generated as byproducts, which is about 10-20% of the total grape by weight.[2] Traditionally, most of this grape pomace, especially the seeds, is wasted in landfills. However, non-traditional uses of pomace from production of individual phenolic compounds as nutraceuticals to grape seed oil are providing the industry with new opportunities for value added products. As byproducts for the wine and nutraceutical industries, muscadine grape seed oil (MGSO) is receiving more and more attention.

Tocotrienols (T3) are a less known form of vitamin E with an unsaturated sidechain, which can be further classified into four isomers α, β, γ, and δ-T3. [3] T3, particularly γT3 was found to exhibit potent anti-inflammatory and anti-cancer properties by modifying multiple signaling pathways, which are unseen by tocopherol (TP) supplementation.[4] It was reported that γT3 lowers the incidence of cardiovascular diseases, [5] diabetes [6] and cancer [7] in both experimental animal and human clinical studies. Recently, it was shown that γT3 is effective in reducing adiposity, [8, 9] and improving plasma glucose and lipid profiles against high fat diet in obesity prone animal models. [10] Moreover, it was recently demonstrated that pure γT3 at a concentration as low as 1 μM was able to inhibit new fat cell formation (adipogenesis) in human adipogenic precursor cells. [11] Thus far, the evidence gained by our group and others strongly suggests that γT3 may be used as a promising dietary strategy to prevent hyperplastic obesity.

T3 are present in a limited variety of vegetable oils such as rice bran and red palm oil, but seldom exist in edible oils that are typically consumed in the American diet (*i.e.*, soybean, corn and rapeseed oils). [12] It is controversial whether grape seed oil is a significant source of T3; Crews *et al* [13] investigated thirty varieties of grape seed oils from Spain, France and Italy, and found that the total content of TPs and T3s was as high as 1,208 mg/kg comprising mostly (>50%) αT3 and γT3. Conversely, other studies conducted in Canada, Portugal, and Turkey [14-16] found that T3 amounts fluctuated significantly between grape varieties ranging from 250-1,500 mg/kg oil. However, no study has been conducted to evaluate the T3 content as well as the biological activity of grape seed oil extracted from varieties of muscadine.

In this study, it was hypothesized that MGSO is an important dietary source for T3 that could exert biological activity in the prevention and/or treatment of obesity. T3 content in five different varieties of MGSO was analyzed and compared to other edible oils. Additionally, the effectiveness of these oils in reducing fat cell formation (adipogenesis) and inflammation in human adipose stem cells (*h*ASCs) was assessed.

2. Material and Methods

2.1 Chemicals and Materials

All reagents and solvents used for analysis in this study were of HPLC grade and purchased from Thermo Fisher Scientific (Hampton, NH, USA). The standards for tocopherol and the fatty acid methyl ester (FAME) mixture were purchased from Supelco (Bellefonte, PA, USA) while standards for tocotrienols were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rice bran and olive oils were purchased from the local market in Gainesville, Florida and the cell culture supplies were purchased from Fisher Scientific. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2 Muscadine grape sampling

Five of the most widely used varieties of muscadine grape cultivars, namely Alachua, Carlos, Fry, Granny Val, and Nobel were harvested from selected vineyards at the Center for Viticulture and Small Fruit Research at Florida A&M University (Tallahassee, FL, USA). All cultivars were grown in the same geographical region in Tallahassee with similar climatic conditions and soil characteristics. All samples were fully ripe and harvested between August and September of 2012 and 2013. The collected samples were shipped to the University of Florida on the same day and stored in the cold room (4 °C). Grape seeds were obtained by manually removing the skin/flesh and subsequently freeze drying in a freeze dryer (Advantage, The Virtis Company, NY, USA). The freeze-dried samples were stored at -20 °C until analysis.

2.3 Extraction of Grape seed oil

Muscadine grape seed samples (10 g) were weighed and crushed in a grinder (Omni International, Kennesaw, GA, USA) for 2 min with 15 sec intervals. The fresh oil was extracted twice from the crushed seeds by adding 100 mL hexane in a light-prevented flask for 24 h. Then the hexane was evaporated by flushing with nitrogen. Fresh oils and their blends were analyzed for vitamin E content, fatty acid composition or stored at -20 °C for further use.

2.4 Determination of Vitamin E Content and Fatty Acid Composition

Vitamin E isomers were determined in the seed oils using a HPLC system equipped with fluorescence detector and normal-phase column (Luna, 5 μ silica 100 Å, 250×4.6 mm). Briefly, seed oils (50 mg) were

weighed and dissolved in 10 ml n-hexane. Separation and quantification was conducted with a mobile phase consisting of hexane, isopropanol, ethyl acetate, and acetic acid (97.6:0.8:0.8:0.8; v/v/v/v) at 1 mL/min flow rate according to Huang *et al.* [17] The wavelength was set at 270 nm for excitation and 330 nm for emission. For fatty acid composition, 20 mg of muscadine grape seed oil was methylated and then diluted 1:50 with hexane. Fatty acid profile of the grape seeds oil was performed on a GC HP 6890, equipped with a flame ionization detector and DB 225 MS capillary column (30 m x 0.25 mm x 0.2 μm) as previously described. [16]

2.5 Preparation of Edible oils

Rice bran oil, olive oil, different varieties of MGSOs and their blends were saponified and complexed to fatty acid free bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mM BSA stock as described previously. [18]

2.6 Cell culture and treatment

Subcutaneous adipose tissue was obtained from females with a body mass index (BMI) of ~30 during liposuction or abdominal plastic surgeries with approval from the Institutional Review Board at the University of Florida and University of Nebraska. Human adipose-derived stem cells (*h*ASCs) were isolated and cultured as in previous studies. [19] Each independent experiment was repeated at least twice using a pool of *h*ASCs from three or four subjects to avoid individual variation.

2.7 Determination of triglyceride accumulation

Triglyceride accumulation in the cells was determined by oil red O staining as previously described. [11] The *h*ASCs were seeded in 35 mm plates and treated with either vehicle (BSA) or saponified-edible oils. The next day, cultures were induced for adipogenic differentiation by adding differentiation cocktail plus oils and allowed to differentiate for 10 days. Upon day 10 of differentiation, cells were washed twice with cold HBSS, fixed and stained with oil red O dye. The images of human adipocytes with different oil treatment were visualized by an EVOS microscope (Life Technologies, Carlsbad, CA, USA). Oil red O dye in each plate was eluted and further quantified by absorbance at 500 nm (OD 500), and expressed as a percentage of the vehicle control (BSA).

2.8 Isolation of Tocotrienol Rich Fraction (TRF) by solid phase extraction (SPE)

The tocotrienol rich fraction (TRF) from muscadine grape seed oil was extracted by SPE as previously described. [20] To prepare TRF, 0.24 g of blended MGSO was weighted and dissolved in 1 ml n-hexane. The silica column (2,000 mg/15 ml volume, Thermo Fisher Scientific, Asheville, NC, USA) was conditioned with 10 ml of n-hexane before applying the oils. Initially, squalene and other components were eluted with 10 ml hexane (hexane fraction, HX). TRF was prepared by two different elution conditions. TRF was successively eluted with 10 ml of 1, 5, 10, and 15% (v/v) diethyl ether (DE) in hexane (Table 3) or it was successively eluted with 10 ml of 1, 5, 10, and 15% (v/v) 1,4-dioxane (DX) in hexane (Table 3). The collected fractions (HX, DE, or DX) were evaporated under N_2 at room temperature. The dry residues were weighted and diluted (50 times), and transferred into brown vials for HPLC analysis or storage at -20 °C. The concentration of tocotrienols in crude oil, and HX, DE, and DX fractions was detected previously by normal phase-HPLC, and the efficiency of extraction was calculated as a percentage of T3 in the fractions to that in the original oil. The TRF for the cell treatment was isolated from 10 g of MGSO using the method described above with increasing concentration of DX as eluting solvent. The 15% DX fraction was collected and used for determining T3 concentration by HPLC. Then, the TRF was dissolved in ethanol and the concentration of total T3s in the stock solution were adjusted to 1 mM, and stored at -20 °C.

2.9 The influence of MGSO on adipogenesis in *h*ASCs

The *h*ASCs were seeded in 35mm plates and treated with vehicles (BSA), 200 μM MGSO, or 5.7 μg/ml TRF (containing 1μM T3s), then induced into differentiation by an adipogenic cocktail and allowed to differentiate for 10 days. On day 10, total mRNA and protein of the cells were harvested as described previously. [21, 22] mRNA expression was determined by real-time qPCR (CFX96, Bio-Rad), and relative gene expression was normalized by the average of two reference genes, *36B4* and *GAPDH*. Gene-specific primers for qPCR were described previously. [11] To measure the protein expression, western blot analysis was performed as previously described. [23] To prepare the total cell lysates, monolayers of cell cultures

were scraped with ice cold radio immune precipitation assay (RIPA) buffer (Thermo Fisher Scientific) with protease inhibitors (Sigma) and phosphatase inhibitors (2 μM Na_3VO_4, 20 mM β-glycerophosphate and 10 mM NaF). Proteins were fractionated using 10% SDS-PAGE, transferred to PVDF membranes, and incubated with the relevant antibodies as described previously. [9] Chemiluminescence from ECL (PerkinElmer, Waltham, MA, USA) was detected with FluorChem E (Proteinsimple, Santa Clara, CA, USA). [24] Polyclonal or rabbit monoclonal antibodies targeting PPARγ (#2443), CEBPα (# 8178), aP2 (# 3544), FAS (#3180), β-actin (#4967) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.10 Determination of MGSO on adipose inflammation

To test the outcome of MGSO on adipose inflammation, *h*ASCs were differentiated into adipocytes. On day 12, cultures were starved by changing the medium with serum-free DEME/F12 for 24 h. For the treatment, the medium was spiked with either vehicle, 200 μM MGSO, or 5.7 μg/ml TRF for an additional 24 h. The cells were stimulated for inflammation by spiking 10 ng/ml LPS into the medium. After 6 h, the total cell lysates were harvested with Trizol for qPCR analysis. [21] At 24 h, the conditioned medium was collected and tested for inflammatory cytokines using Human Inflammation Array C1 (Ray Biotech, Norcross, GA, USA) according to the manufacturer's protocol. The complete blots of 32-cytokine arrays were imaged by a FluorChem E System (Proteinsimple) as previous described. [18]

2.11 Statistical analysis

The data were statistically analyzed using student's t-test or one-way ANOVA with Tukey's multiple comparison tests. All analyses were performed with GraphPad Prism 5 (Version 5.04). $P < 0.05$ is considered as statistically significant. Results are presented as mean ± SEM.

3. Results

3.1 Vitamin E Content and Fatty Acid Composition in Muscadine Grape Seed Oil (MGSO)

The concentrations of tocopherol and tocotrienol were analyzed by normal-phase HPLC (Table 1 and Fig. 1). As shown in Fig. 1A and 1B, HPLC profiles revealed that MGSOs contain high levels of γ-tocotrienol

(40.7-68.9 mg/100g oil) and α-tocotrienol (30.1-48.1 mg/100g oil), which are comparable to the contents found in commercial rice bran oil (55.1±19.5 mg/100 g oil for γ-tocotrienol and 22.6±2.3 mg/100 g oil for α-tocotrienol). In addition, a MGSO blend contains higher levels of γTP than rice bran oil (Fig. 1C). Moreover, the contents of tocotrienols in muscadine grape seed oils were stable between two seasons (in 2012 and 2013) with an average of 2.71% difference in γ-tocotrienol and 10.01% difference in α-tocotrienol (Fig. 1D). GC results (Table 2) showed that polyunsaturated fatty acids (PUFA) are most abundant (68.1-72.5%) in muscadine grape seed oils, followed by monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) ranging from 13.8-16.2% and 12.1-14.5%, respectively (Table 2). Regarding fatty acid profiles, linoleic acid (C18:2) is the predominant fatty acid (67.9-72.3%), followed by oleic (C18:1), palmitic (C16:0), and stearic (C18:0) acids ranging from 13.8-16.2%, 7.8-8.4%, and 4.0-5.9%, respectively.

3.2 Effects of MGSO on Triglyceride Accumulation

Although several constituents of edible oils (*e.g.*, polyphenols and conjugated linoleic acid) were claimed to reduce adipogenesis, [18, 25] the impact of edible oil as a whole dietary component has not been investigated. To address this issue, differentiating *h*ASCs were treated with vehicle (BSA), 200 μM of MGSO blends (T3s concentration is 0.1-0.2 μM), rice bran oil, and olive oil for 10 days. Triglyceride (TG) accumulation was measured by oil red O staining. Olive oil (OLO), which has a similar fatty acid composition to MGSO but without T3, significantly increased the oil red O accumulation in the cells compared to the vehicle control (Fig. 2A and 2B). Whereas the edible oils with high levels of T3, rice bran oil (RBO) and MGSO, did not increase TG accumulation. Compared with 200 μM OLO treatment, 200 μM and 400 μM of MGSO blends significantly reduce the TG accumulation in the differentiating human adipocyte (Fig. 2C). Moreover, MGSO extracted from five major muscadine varieties decreased TG accumulation compared to OLO treatment but was not significantly different to the vehicle control (Fig. 2D).

3.3 Isolation of tocotrienol-rich fraction (TRF) from MGSOs

To further determine the effect of MGSOs on adipogenesis, a TRF was prepared by solid phase extraction (SPE). In this study, a gradient concentration of DE/hexane and DX/hexane as eluting solvents were compared by measuring the concentration of T3 in the different fractions. From this experiment, 15% DE/hexane was the most efficient for isolating αT3 from the SPE column: 69.61% of αT3 in MGSOs could be extracted. However, the concentration of γT3 was rather low in the DE/hexane fractions: 2.63% of γT3 in MGSOs could be extracted. Interestingly, the 15% DX/hexane fraction isolated high levels of γT3 (84.4%), δT3 (66.6%), and αT3 (17.5%) (Table 3). These results indicated that DE/hexane was a better eluting solution for extracting αT3, while DX/hexane was a better solvent to extract γT3 and δT3. Furthermore, the concentration of T3s in the MGSO blends and various DE/hexane and DX/hexane fractions was analyzed by HPLC. As seen in Table 4, TRF isolated from 15% DX/hexane contains the highest concentration of γT3 (46.1 mg/g sample), in which the purity of total T3 is 7.31%. Moreover, 5.7 μg/ml MGSO-derived TRF (1 μM T3s) was shown to significantly reduce TG accumulation than vehicle control (Fig. 3A).

3.4 Effects of MGSOs and TRF on adipogenesis

MGSOs and MGSO-derived TRF were evaluated on adipogenesis in *h*ASCs, the mRNA level of the important markers involved with adipogenesis were measured. It was found that 200 μM MGSO and 5.7 μg/ml MGSO-derived TRF significantly reduce mRNA expression of PPARγ and CEBPα, which are transcription factors crucial to adipogenesis. Interestingly, TRF showed a stronger outcome than MGSOs in inhibiting the mRNA expression of the other adipocyte signature genes such as aP2 (adipocyte specific fatty acid binding protein), FAS (fatty acid synthase), and perilipin (adipose-specific lipid droplet coating protein) (Fig. 3B). Consistent with the gene expression results, the 200 μM MGSO treatment showed a trend to reduce protein expression of the adipogenic marker but there was no significant difference compared with the vehicle control. However, TRF (5.7 μg/ml) markedly reduced protein expression of CEBPα, aP2 and FAS (Fig. 4).

3.5 Effects of MGSOs and TRF on Adipose-inflammation

To test whether MGSOs and TRF reduces inflammation in adipocytes, the cultures of human adipocytes were pretreated for 24 h with either vehicle (BSA), 200 μM MGSOs, or 5.7 μg/ml TRF and then induced to acute inflammation by LPS (10 ng/ml). After 6 h of LPS treatment, LPS significantly increased the mRNA level of pro-informatory genes, IL-6, IL-8, and MCP-1. As expected, the LPS induced-inflammation was attenuated by both MGSO and TRF treatments by decreasing the mRNA levels of IL-6 (only TRF), IL-8 and MCP-1 (Fig. 5A). To further determine the cytokine secretion, the conditioned media was used for inflammatory cytokines or chemokines array. As seen in Fig. 5B, the levels of IL-6 and IL-8 secretion into the media were markedly decreased in cultures with TRF treatment compared to the LPS control.

4. Discussion

Tocotrienols (T3s) are unsaturated forms of vitamin E that exert multiple health benefits. The natural sources of tocotrienols are limited and include rice bran oil and red palm oil. However, T3 seldom exist in dietary oils in the typical American diet. In this study, we assessed whether muscadine grape seed oil (MGSO) is an ample source of T3 by using five common varieties of muscadine grapes. Our results showed that MGSO contains an average of 40.1 mg αT3/100 g oil and 50.8 mg γT3/100 g oil, suggesting that MGSO is a valuable natural source of T3. Moreover, this work confirmed the potential that MGSO is effective in attenuating new fat cell formation and adipose inflammation.

This is the first report demonstrating that MGSOs can attenuate adipogenesis and adipose inflammation in a cell model. Moreover, our study may provide scientific evidence to emphasize the importance of T3s in edible oil. Based upon the current and previous studies,[26, 27] MGSO could be considered to be a reliable source of T3s, ranking third to red palm oil and rice bran oil. Superior to palm and rice bran oils, MGSO is enriched with mono- and poly-unsaturated fatty acids, which are claimed to be healthier for one's diet.[28] In this study, the content of unsaturated fatty acids reaches 85-90% of the total fatty acids, which is consistent with the reported properties of seed oils extracted from other grape species.[29, 30] More importantly, this work discovered that MGSO contains significant amount of γT3,

which is equal to or even higher than rice bran oil (Fig. 1A). Based on the chromatogram, MGSO has a sharp symmetrical peak for γT3 while the rice bran oil, although broader, has an impurity represented by an upward shoulder in the γT3 peak. This may cause an overestimation of γT3 depending on how the peak was integrated.

Health benefits of T3 consumption have been mostly established for rice bran oil. Recent studies have demonstrated that rice bran oil and its active constituents improve blood cholesterol [31] and insulin resistance. [32] Furthermore, results from animal studies indicated that the high level of γ-oryzanol and tocotrienols in rice bran oil may be responsible for its special health-promoting functions. [33] Based on our initial results that MGSO possesses significant amounts of T3, we hypothesized that MGSO may be a better source of T3 than rice bran oil and may offer an alternative solution to attenuate high fat diet-mediated obesity. The first aspect investigated was to compare the effects of various edible oils on the formation of new fat cells from *h*ASCs. The oil red O staining results revealed that the cells treated with olive oil (devoid of T3) increased TG accumulation compared with the vehicle control (Fig. 2). This was consistent with other studies [34, 35] and supported the notion that unsaturated fatty acid would facilitate adipogenesis by binding with the transcription factors that are crucial to adipogenesis, such as PPARγ.[36] However, no increases in the TG accumulation were observed, under RBO and MGSOs treatment, even at a higher concentration of 400 μM MGSOs. Given the fact that MGSO contains a high profile of unsaturated fatty acids similar to oleic acid, these results indicate that the inhibition of T3 on adipogenesis may override fatty acid-derived new fat cell formation. [37]

To further clarify the impacts of MGSOs on adipogenesis, an isolated tocotrienol fraction from MGSOs using a SPE column was prepared. SPE is a convenient method to separate different chemical classes from a mixture according to their polarities.[20, 5] In previous studies, TPs and T3s were well-eluted by 1 to 10% (v/v) diethyl ether in hexane using a silica column or chromatography. [20, 38] However, our results revealed that 15% (v/v) diethyl ether in hexane is better at extracting αT3 (69.61%), but not for the more polar tocotrienols (e.g., γT3 and δT3). Interestingly, better extraction of γT3 and δT3 was achieved

using hexane with a relatively strong polar modifier 1, 4-dioxane, which is consistent with the results observed in normal phase HPLC. [39] This may be due to the different polarities that T3 isomers have depending on the number of methyl groups carried in the chromanol ring. [39] For instance, αT3 with one methyl group has the lowest polarity, whereas γT3 and δT3 has higher polarities with two or three methyl groups. [40] Thus, using gradient concentrations of 1, 4-dioxane as the eluting solution with silica SPE columns, T3s may be eluted in the following order: αT3 > γT3 > δT3. Moreover, the results indicate that dioxane/hexane may be the better method to extract the TRF, because the major T3s eluted (*e.g.,* γT3 and δT3) have been demonstrated to be more effective at inhibiting adipogenesis than αT3. [11]

In this study, MGSO was able to reduce the mRNA expression of two major transcription factors of adipogenesis, *i.e.*, PPARγ and CEBPα, and to decrease the mRNA and protein expression of the downstream targets of adipogenesis (Fig. 3B and 4). It was plausible to assume that the TRF derived from MGSOs would have a stronger result than MGSO itself by eliminating the compounding adipogenic effects from fatty acids in the oils. In support of this notion, the results revealed that the MGSO-derived TRF could significantly reduce the expression of not only the transcription factors but also their downstream targets for adipogenesis. In this study, the MGSO-derived TRF was effective in attenuating adipose-inflammation induced by LPS (Fig. 5). These results indicated that MGSO-derived TRF may have equal or higher biological activity as a TRF derived from other sources. [41] Moreover, these observations may provide scientific evidence for a clinical study that revealed grape seed oil, but not sunflower oil, attenuated the inflammation in overweight or obese subjects. [42] However, a weaker response than TRF in reducing the expression of pro-inflammatory genes (*e.g.*, little effects on IL-6) were seen after treatment with MGSOs in inflamed adipocytes. This indicated that T3s in MGSOs are crucial in reducing adipose-inflammation but end up being minimal due to the influence of other components in the complex alimentary matrix (*e.g.*, n-6 fatty acid). [43]

The consumption of T3s in a daily diet is relatively low compared with TPs. [44] For instance, the daily T3 intake in the Japanese population was estimated around 2 mg/day/person compared to

296 approximately 8-10 mg/day/person intake for TPs. [45] As increasing healthy benefits are reported, T3 tends
297 to be recognized as an important daily supplement by consumers. In this study, we demonstrated that
298 MGSOs are an alternative source of T3 and effective in reducing adipogenesis and inflammation in
299 primary cultures of human adipocytes. Further research is warranted to determine the efficacy of MGSO
300 in humans. As a unique source of T3 in the favorable formulation of mono- and poly-unsaturated fatty
301 acids, MGSOs would be a valuable addition to the market of edible oils. In addition, it is anticipated that
302 MGSOs fortified with T3s could be developed to maximize their benefits in attenuating obesity and its
303 associated metabolic complications.

304 **Conflict of interest**

305 The authors declare that they have no conflict of interest.

306 **Acknowledgement**

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444

445 **Table 1** Vitamin E concentration of five varieties of muscadine grape seed oil harvested in two seasons

Variety	Year	αTP[2]	βTP	γTP	δTP	αT3[3]	γT3	δT3
Alachua	2012	17.07±0.11[1]	-[4]	42.87±0.49	-	33.94±0.40	41.18±0.41	-
	2013	21.41±0.09	-	63.75±0.46	-	48.14±0.34	47.34±0.29	-
Carlos	2012	18.49±0.06	-	45.74±0.28	-	33.67±0.20	56.36±0.42	-
	2013	14.97±0.40	-	34.97±1.52	-	36.65±1.78	68.92±0.36	-
Fry	2012	23.07±0.30	-	115.72±1.91	-	30.11±0.39	42.87±0.61	-
	2013	15.26±0.01	-	43.36±0.16	-	31.58±0.05	46.48±0.07	-
Granny Val	2012	18.53±0.40	-	56.56±1.45	-	35.71±0.79	43.44±0.89	-
	2013	14.75±0.01	-	39.63±0.09	-	40.18±0.01	44.91±0.09	-
Nobel	2012	16.64±0.10	-	61.43±0.69	-	39.63±0.39	40.73±1.05	1.82±0.01
	2013	16.38±0.13	-	62.18±0.87	-	44.15±0.57	46.36±0.49	1.92±0.02

446 [1], all the data represents means (n=4) ± SEM, and expressed as mg/100g oil. [2], TP, tocopherol; [3], T3,
447 tocotrienol; [4], not detected.

448

449 **Table 2** Fatty acid composition in muscadine grape seed oil

Variety	Year	C16:0 (%)	C18:0 (%)	C18:1 (%)	C18:2 (%)
Alachua	2012	8.14±0.06[1]	4.67±0.03	16.0±0.03	69.3±0.13
	2013	7.84±0.05	5.92±0.02	14.9±0.04	71.4±0.09
Carlos	2012	8.08±0.04	5.49±0.04	14.6±0.05	69.8±0.05
	2013	8.13±0.04	5.22±0.01	13.8±0.01	70.9±0.06
Fry	2012	8.19±0.05	4.33±0.01	16.2±0.02	68.4±0.02
	2013	8.42±0.06	4.45±0.02	16.6±0.02	67.9±0.03
Granny Val	2012	8.16±0.05	5.85±0.02	15.6±0.02	69.2±0.02
	2013	8.08±0.05	5.71±0.01	13.8±0.01	70.2±0.02
Nobel	2012	8.07±0.04	6.43±0.01	14.6±0.03	71.3±0.05
	2013	8.09± 0.05	4.05±0.02	14.1±0.02	72.3±0.04

450 [1], all the data represents means (n=4) ± SEM, and expressed as a percentage of individual fatty acid to
451 total fatty acids.

452

453 **Table 3.** The extraction efficiency of T3 in different SPE fractions

SPE Fraction	Efficiency of T3 extraction (%)			
	αT3	δT3	γT3	Total
Hexane	-	-	-	-
1% DE[2]	-	-	-	-
5% DE	-	-	-	-
10% DE	8.60[1]	-	-	6.02
15% DE	69.61	-	2.65	49.48
1% DX[3]	- [4]	-	-	-
5% DX	-	-	-	-
10% DX	55.93	-	-	39.16
15% DX	17.48	66.62	85.44	37.42

454 [1], all the data represents means of triplicates, and expressed as percentage of T3 in each SPE eluted
455 fraction to that in the original oils. [2], 1%-15% DE, the fraction eluted by 1% to 15% (v/v) Diethyl ether in
456 hexane; [3], 1-15% DX, the fraction eluted by 1% to 15% (v/v) 1,4 dioxane in hexane. [4], not detected.

457

458

459 **Table 4**. Concentration of tocotrienols in muscadine grape seed oil and different SPE fractions

Grape seed oil /SPE fraction	Tocotrienol (mg/g of sample)				Purity of T3 (%)
	αT3	δT3	γT3	Total	
Grape seed oil	0.361[1]	0.019	0.485	0.865	0.09
10% DE[2]	11.77	- [4]	-	11.77	1.18
15% DE	95.23		1.43	96.66	9.67
10% DX[3]	76.51	-	-	76.51	7.65
15% DX	23.92	3.04	46.14	73.09	7.31

460 [1], all the data represents means of triplicates, and expressed as mg/g sample. Grape seed oil, an average
461 blending of MGSOs from five varieties; [2], 10%-15% DE, the fraction eluted by 10% or 15% (v/v) Diethyl
462 ether in hexane; [3], 10-15% DX, the fraction eluted by 10% or 15% (v/v) 1,4 dioxane in hexane. [4], not
463 detected.

464

Figure Legends

Fig. 1 The content of vitamin E isomers in muscadine grape seed oil (MGSO). HPLC chromatography for vitamin E isomers in MGSO blend (A) and commercial rice bran oil (B). The content of vitamin E isomers (C) were compared between MGSOs blends and commercial rice bran oil. (D) The content of γT3 were compared between the samples harvested in two seasons (in year 2012 and 2013) among five major varieties of muscadine. All data represent means (n=4) ± SEM. *, P<0.05; **, P<0.01, ***, P<0.001.

Fig. 2 Effects of MGSOs on triglyceride accumulation in differentiating *h*ASCs (day 10). Image of oil red O staining (A) in differentiating *h*ASCs treated with vehicle control (BSA), 200 μM of olive oil (OLO), rice bran oil (RBO), and blended MGSOs from five varieties. (B) Oil red O staining in differentiating *h*ASCs were quantified and compared between different treatments of edible oils. (C) Oil red O staining in differentiating *h*ASCs treated with different doses of MGSOs (50, 100, 200, and 400 μM) were quantified and compared, using 200 μM of olive oil (OLO) and vehicle as controls. (D) Oil red O staining in differentiating *h*ASCs treated with MGSOs extracted from five major varieties of muscadine. All data represent means (n=4, or 5) ± SEM. Each independent experiment was repeated at least twice using a mixture of cells. Values not sharing a common letter differ significantly by one-way ANOVA.

Fig. 3 Effects of MGSOs and MGSO-derived TRF on triglyceride accumulation and the mRNA expression of adipogenic markers. (A) Oil red O staining in differentiating *h*ASCs treated with BSA and 5.7 μg/ml MGSO-derived TRF were quantified and compared. (B).The differentiating *h*ASCs were treated with BSA (control), 200 μM MGSO blends, 5.7 μg/ml MGSO-derived TRF for 10 days. The mRNA expression of adipogenic markers were measured by qPCR. All data represent means (n=4, or 5) ± SEM. *, P < 0.05; **, P < 0.01, ***, P < 0.001.

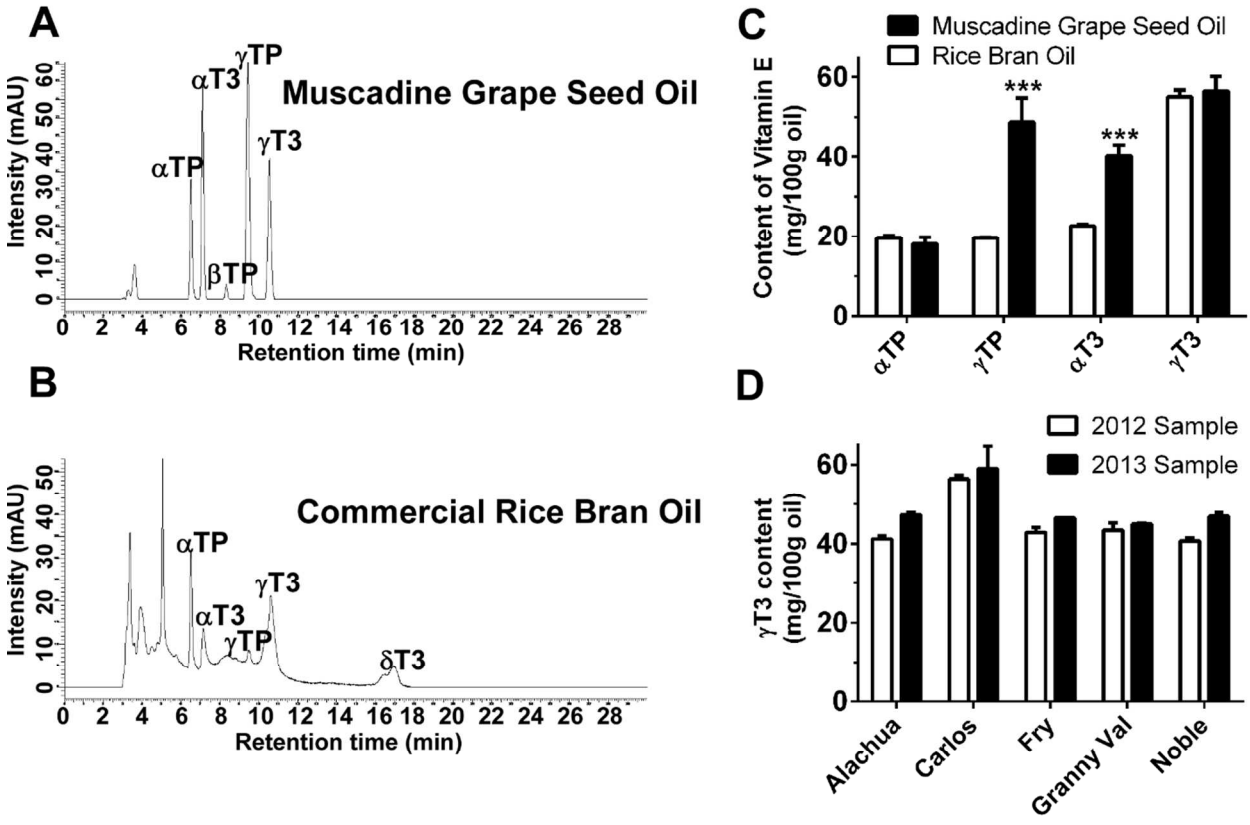
Fig. 4 Effects of MGSOs on the protein expression of adipogenic markers. The differentiating *h*ASCs were treated with BSA (control), 200 μM MGSO blends, 5.7 μg/ml MGSO-derived TRF for 10 days. The protein expression of adipogenic markers were measured by western blotting. The intensity of individual

489 marker in western gel were quantified and compared. All data represent means (n=4) ± SEM. *, P<0.05;
490 **, P<0.01.

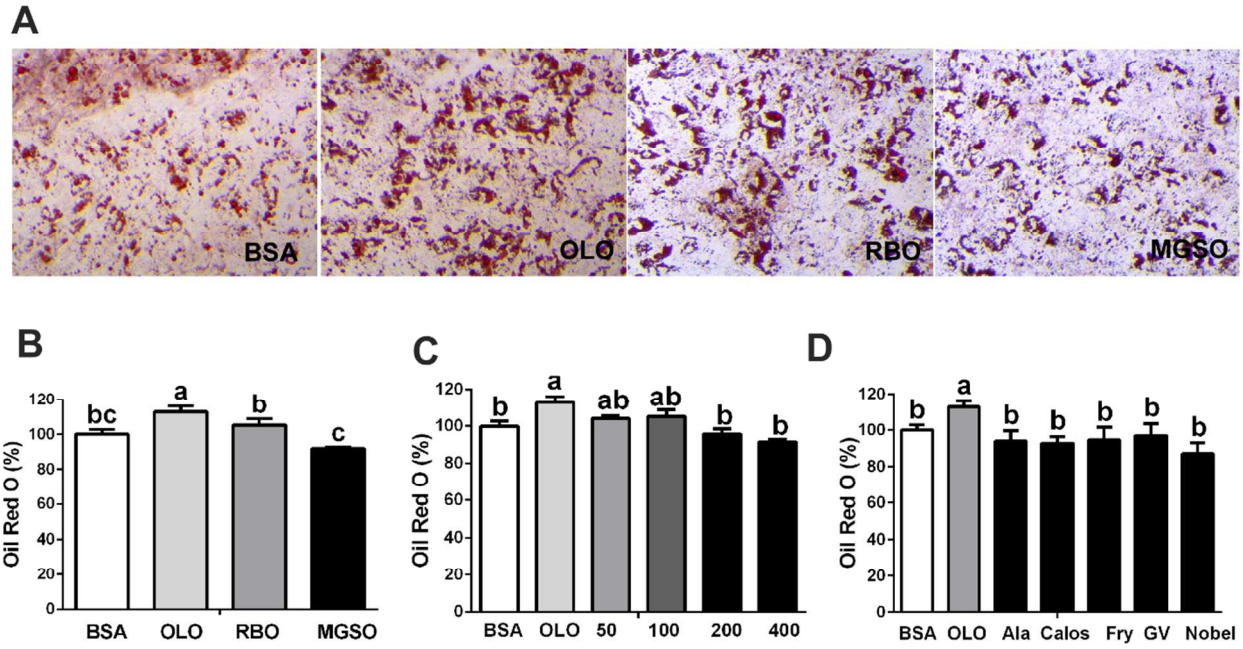
491 **Fig. 5** Effects of MGSOs on the LPS-induced inflammation in adipocytes. Differentiated human
492 adipocytes (12 days) were pretreated with vehicle (BSA), 200 μM MGSO blends, 5.7 μg/ml MGSO-
493 derived TRF for 24h, and stimulated with 10 ng/ml LPS for 6 h or 24 h. At 6h, the mRNA expression of
494 pro-inflammatory markers (A) were measured by qPCR. After 24 h of LPS treatment, multiple
495 inflammatory cytokines (B) secreted in the medium were detected by Human Cytokine Array C1. All data
496 represent means (n=4-5) ± SEM. Each independent experiment was repeated at least twice using a
497 mixture of cells. Values not sharing a common letter differ significantly by one-way ANOVA.

498

Fig. 1



514 # Fig. 2



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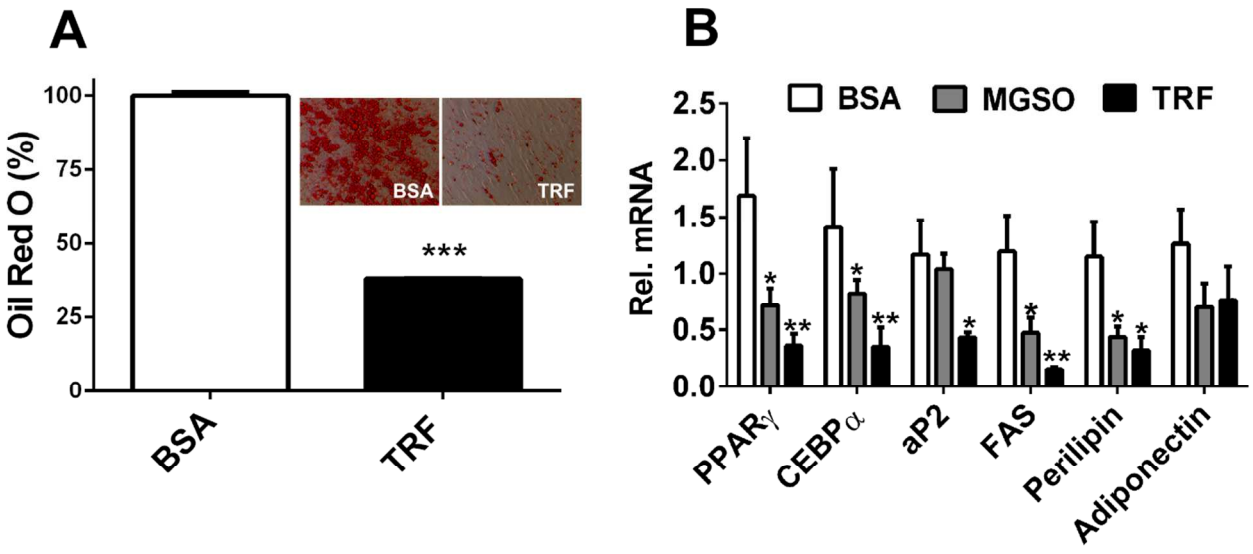
518 # Fig. 3



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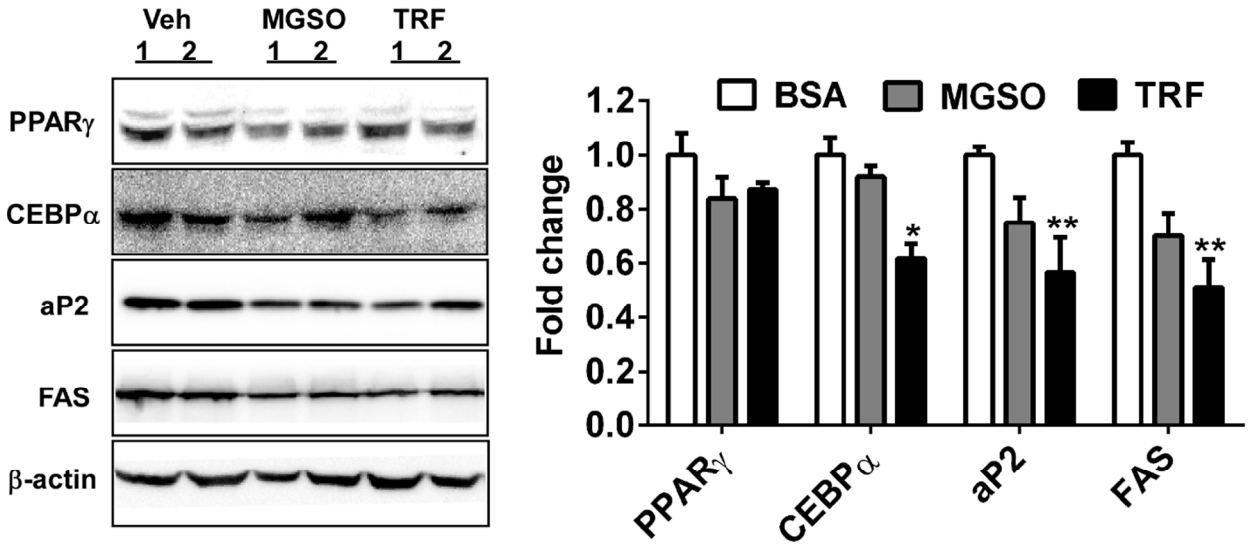
521 **Fig. 4**



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524 **Fig.5**

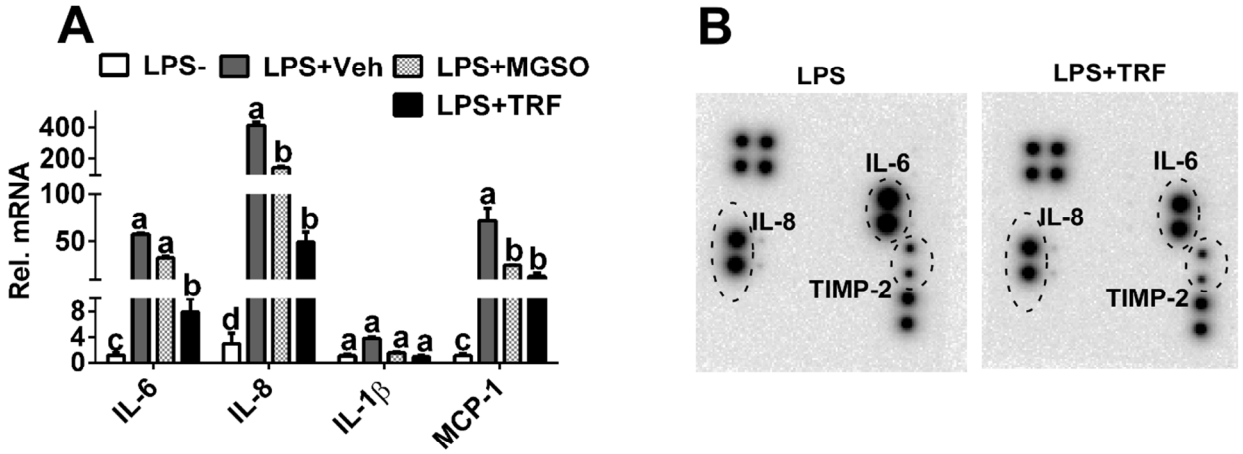

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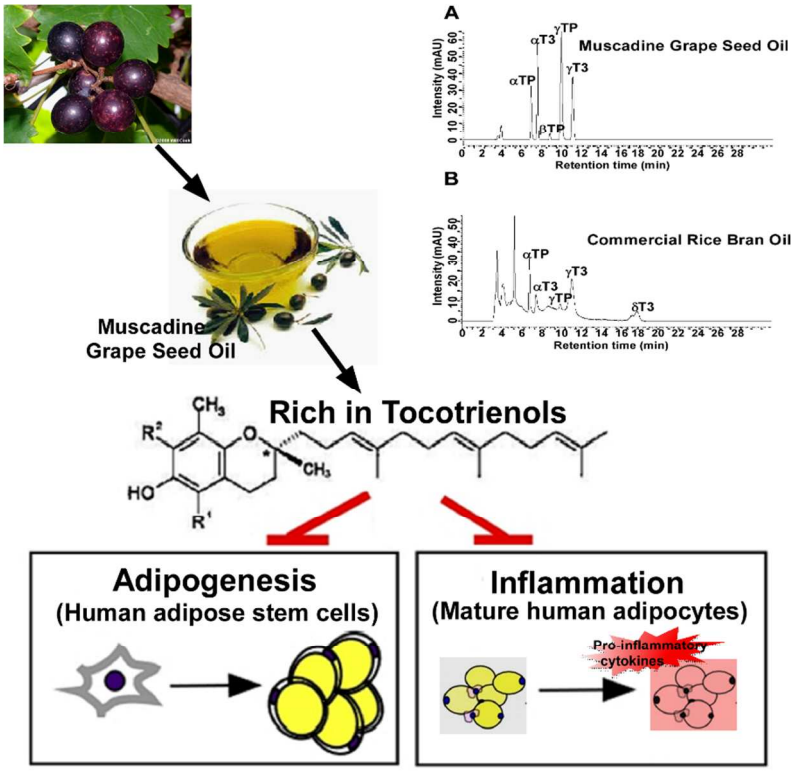
Table of Content



This is the first report showing that muscadine grape seed oil can attenuate obesity-associated metabolic diseases in a cell model.