www.roavs.com EISSN: 2223-0343
RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Effects of *Vaccinium arctostaphylos* on lipolysis and adipogenesis in diabetic rats

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Abstract

Several experimental and clinical studies support hypolipidemic effect of *Vaccinium arctostaphylos* fruits in diabetic subjects. However, no study has yet evaluated the possible direct action of this plant on adipose tissue. Therefore, the present work was performed to investigate the effects of hydroalcoholic extract of *V. arctostaphylos* fruits on adipogenesis and lipolysis. Preadipocytes were isolated from retroperitoneal adipose tissue of diabetic rats and differentiated to adipocyte in the presence of this extract. The effect of *V. arctostaphylos* extract on lipolysis was evaluated with organ culture method. The extract had no significant effect on lipid droplet accumulation as evaluated with Oil Red O staining. Incubation of preadipocytes with the extract for 24 h did not change the cell viability. The extract, even at high concentrations (up to 1000 µg/ml) had virtually no significant effect on lipolysis. In conclusion, the present data demonstrated that *V. arctostaphylos* fruit has no effects on fat tissue adipogenesis and lipolysis in diabetic rats.

Keywords: Adipogenesis; Diabetes; Lipolysis; *Vaccinium arctostaphylos*

To cite this article: Ghorbani A, M Soukhtanloo, Z Rajaei and MAR Hadjzadeh, 2014. Effects of *Vaccinium arctostaphylos* on Lipolysis and adipogenesis in diabetic rats. Res. Opin. Anim. Vet. Sci., 4(8): 437-441.

Introduction

Adipose tissue has crucial role in the regulation of body metabolism, endocrine functions and immune processes. Many pathologic conditions are associated with alterations in adipose tissue functions. For example, dysfunction of adipose tissue is observed in obesity, metabolic syndrome, diabetes, familial combined hyperlipidaemia and polycystic ovarian syndrome (Kershaw and Flier, 2004; Van de Woestijne et al., 2011).

The mass of fat tissue is determined by the number and the size of adipocytes (Ghorbani et al., 2009; Smith and Ravussin, 2006). Obesity is the result of body fat mass expansion which increases the risk of diabetes mellitus, metabolic syndrome and cardiovascular diseases (Lavie et al., 2009; Chiang et al., 2011). The mass of adipose tissue can be reduced by reduction of adipocytes number through inhibiting preadipocytes differentiation and inducing adipocyte apoptosis, or by decrease of adipocytes size through increasing lipolysis and inhibiting adipogenesis (Rayalam et al., 2008). Several medicinal plants have been reported to affects adipocyte life cycle, preadipocytes differentiation and lipolysis (Rayalam et al., 2008; Andersen et al., 2010; Ghorbani et al., 2014a).

Vaccinium arctostaphylos, a member of *Vaccinium* genus (blueberry), has a long history of medical uses in traditional medicine. The plant fruits are rich in phenolic compounds such as gallic, protocatechuic, gentisic, chlorogenic, caffeic, ferulic, salicylic, and *trans*-cinnamic acids (Ayaz et al., 2005). Also, *V.*

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arctostaphylos contains anthocyanins which have several biological activities including anti-obesity, anti-hypertensive, anti-diabetic and cardioprotective effects (Kianbakht et al., 2013).

Pharmacological studies have shown that *V. arctostaphylos* induces antimicrobial, hypotensive and antioxidant activity (Mahboubi et al., 2013). Results of experimental and clinical studies indicate that fruit of *V. arctostaphylos* decreases blood glucose in alloxan-diabetic animals and diabetic patients (Abidov et al., 2006; Feshani et al., 2011; Kianbakht et al., 2013). Fruit extract also decreases serum lipids in hyperlipidemic patients and diabetic animals (Feshani et al., 2011; Kianbakht et al., 2014; Soltani et al., 2014). However, in our knowledge, no study has yet evaluated the possible direct actions of *V. arctostaphylos* on adipose tissue.

The present work was performed to investigate the effects of hydroalcoholic extract of *V. arctostaphylos* fruits on adipogenesis and lipolysis in diabetic rats.

Materials and Methods

Chemicals and Reagents

Fatty acid-free bovine serum albumin fraction V, 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-Diphenyl-2H-tetrazolium bromide (MTT), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES), glycerol assay reagent, isoproterenol and type-II collagenase were purchased from Sigma. Dimethyl sulfoxide and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Fluka. Indomethacin and human insulin were kindly provided by Exir Company (Iran). Dulbecco's Modified Eagles Medium (DMEM) and fetal bovine serum were purchased from Gibco. Streptozotocin (STZ) was prepared from Enzo Life Sciences (USA).

Preparation of extracts

The *V. arctostaphylos* fruits were purchased from Golchay Company (Iran). The macerated extract was prepared by suspension of 100 g of the fruit powder in 700 ml of 70% ethanol and incubation for 72 h at 37°C. The extract was then filtered and dried on a water bath at 40°C. The yield of the dried extract related to the weight of the dried fruit was 45%. A stock solution was prepared by dissolving 100 mg of dried extract at 1 ml of dimethyl sulfoxide.

Animals

Male Wistar rats (250-300 g) were housed in a room with controlled temperature (22±2°C) and lighting (12 h light/12 h darkness). The animals were given standard diet and water *ad libitum*. Experiments were done according to the ethical guidelines of the animal care of the Mashhad University of Medical Sciences, Iran. For induction of diabetes, a single dose of STZ (55 mg/kg, ip) was injected intraperitoneally (Ghorbani et al., 2010; Shafiee-Nick et al., 2012). The animals were considered to be diabetic if they had fasting blood glucose concentration of 250 mg/dl or higher.

Lipolysis assay

Effect of *V. arctostaphylos* on lipolysis was studied using an *ex-vivo* organ culture method (Ghorbani et al., 2011; Ghorbani et al., 2013). Seven days after injection of STZ, the retroperitoneal fat pad was removed from diabetic rats. The fat tissue was minced into uniform small pieces of about 5 mg. Then, the tissue pieces were distributed into 24-well culture plate (100 mg/well) and bathed with 1 ml Krebs-Ringer bicarbonate (KRB) buffer supplemented with 25 mM HEPES, 5.5 mM glucose and 2% (w/v) bovine serum albumin. To make up one batch of KRB, 10 ml of NaCl 0.154 M was added to 0.4 ml of KCl 0.154 M, 0.3 ml of $CaCl_2$ 0.11 M, 0.1 ml of KH_2PO_4 0.154 M, 0.1 ml of $MgSO_4$ 0.154 M, and 2.1 ml of $NaHCO_3$ 0.154 M. The tissues were left untreated (basal lipolysis) or treated with isoproterenol (stimulated lipolysis) in the absence or presence of *V. arctostaphylos* extract. The tissues were incubated at 37°C in a humidified chamber under constant shaking for 90 min. At the end of the incubation, glycerol concentration in the KRB was measured by an enzymatic method using glycerol assay reagent.

Preadipocyte preparation and adipogenesis assay

Retroperitoneal adipose tissue from diabetic rats was sliced into small pieces and washed with phosphate-buffered saline (PBS). Then, the tissue pieces were digested in PBS containing collagenase (2 mg/ml) at 37°C under 60 cycles/min shaking. After centrifugation (2000 rpm for 5 min), the cellular pellet was suspended in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were cultured in T25-flask and incubated in a humidified 5% CO_2 incubator until they reached confluence. Then, the cells were harvested using trypsin and seeded in 12-well plates (10^4 cells/well). After 24 h, the culture medium was changed into differentiation medium (DMEM supplemented with 3% fetal bovine serum, 250 µM IBMX, 34 µM d-panthothenate, 1 µM dexamethasone, 0.2 µM insulin and 5 µM indomethacin). After 3 days, the cells were exposed to the adipocyte maintenance medium (DMEM supplemented with 3% fetal bovine serum, 34 µM d-panthothenate, 1 µM dexamethasone and 0.2 µM insulin). The cells were further cultured for 6 days and the adipocyte maintenance medium was changed every 3 days (Ghorbani, 2013). To evaluate the effects of *V. arctostaphylos* extract on adipogenesis, varying

concentrations of the extract or vehicle (1% dimethyl sulfoxide) was added to differential medium and adipocyte maintenance medium during 9 days of differentiation.

Oil Red O staining

Oil Red O was used to stain accumulated intracellular triglycerides in differentiated adipocytes. After 9 days of differentiation, the cells were fixed with 10% formalin and then stained by 200 µl of Oil Red O solution. The stained cells were washed three times with distilled water. Then, the stain was eluted from adipocytes by 200 µl isopropanol and its optical density was read at 545 nm using a StatFAX303 plate reader (Yu et al., 2011; Ghorbani et al., 2014b).

Cell proliferation assay

Effect of *V. arctostaphylos* on viability of isolated rat preadipocytes and L929 mouse fibroblast cells was evaluated by MTT colorimetric method (Hadjzadeh et al., 2006; Liu et al., 2013). The cells were seeded in 96-well culture plates containing DMEM supplemented with 10% FBS and penicillin/streptomycin. After 24 h, the medium was changed by fresh one containing different concentrations of *V. arctostaphylos* extract. The cells were further incubated in a humidified 5% CO_2 incubator for 24 h. Then, 10 µl of 5 mg/ml MTT solution was added to each well and the plate was placed at 37°C for 2 h. Then, the resulting formazan was dissolved by adding 200 µl dimethyl sulfoxide to each well and absorbance was read at 545 nm against 630 nm as background.

Statistical analysis

The values were analyzed using the one-way analysis of variance followed by Tukey's post hoc test. Results were considered to be statistically significant, if the p-values were under 0.05. The results are expressed as the mean ± standard error.

Results

Effect of *V. arctostaphylos* on lipolysis

Effect of *V. arctostaphylos* extract on basal and stimulated lipolysis was demonstrated in Table 1. The extract at 100 µg/ml had no significant effect on basal glycerol release (98±3.4 and 120±11% for vehicle and *V. arctostaphylos*, respectively). To examine the effect of *V. arctostaphylos* on stimulated lipolysis, the lipolytic activity was also tested in the presence of isoproterenol, a nonselective beta adrenergic receptor agonist. As expected, isoproterenol led to a significant elevation in lipolysis (348±76% of control, P<0.001). The extract at concentrations of 10, 100 and even 1000 µg/ml had virtually no significant effect on the stimulated lipolysis.

Effect of *V. arctostaphylos* on viability of preadipocytes

Incubation of preadipocytes in the presence of *V. arctostaphylos* extract had no significant effect on cell viability (Table 2). In the presence of 25, 50, 100, 200 and 400 µg/ml of the extract, viability of the cells was 104±1.5, 105±1.3, 108±1.7, 104±2 and 99±2.6%, respectively. Similarly, the extract exhibited no cytotoxicity up to concentration of 800 µg/ml on L929 cells.

Effect of *V. arctostaphylos* on adipogenesis

Exposure of differentiating cells to the *V. arctostaphylos* extract had no significant effect on lipid droplet accumulation as evaluated with Oil Red O staining (Table 3). The level of lipid droplet at the presence of 100, 200 and 400 µg/ml of the extract was 139±16, 109±9.5 and 94±7% of untreated cells (100±9%), respectively.

Discussion

Mass of adipose tissue is determined by the level of preadipocytes differentiation into mature adipocytes, the rate of preadipocytes proliferation, the balance between lipogenesis and lipolysis within mature adipocytes, and the rate of adipocyte apoptosis (Smith and Ravussin, 2006). The present study showed that hydroalcoholic extract of *V. arctostaphylos* has no significant effect on the level of preadipocytes differentiation into adipocytes. Formation of new adipocytes has positive correlation with increase of body fat mass and risk of metabolic syndrome or obesity. Therefore, *V. arctostaphylos* can be consumed by type-2 diabetic patients for its hypoglycemic and

Table 1: Effects of macerated extract of *V. arctostaphylos* on basal and stimulated lipolysis in diabetic rats (n=6)

	Vehicle	Isoproterenol (1 µM)	*V. arctostaphylos* (100 µg/ml)	Isoproterenol (1 µM) + *V. arctostaphylos* (10 µg/ml)	Isoproterenol (1 µM) + *V. arctostaphylos* (100 µg/ml)	Isoproterenol (1 µM) + *V. arctostaphylos* (1000 µg/ml)
Basal lipolysis (% of Control)	98±3.4[b]	348±76[a]	120±11[b]	-	-	-
Stimulated lipolysis (% of Isoproternol)	-	100±22	-	104±12	107±17	141±22

The data are presented as means ± SEM of 6 independent experiments. Values (mean ± SEM, *n* = 3) with different superscripts in a row differ significantly (P<0.05).

Table 2: Effects of macerated extract of *V. arctostaphylos* on viability of preadipocytes isolated from diabetic rats (*n* = 4) and L929 mouse fibroblast cells (*n* = 8)

	Vaccinium arctostaphylos (µg/ml)						
Cell type	Vehicle	25	50	100	200	400	800
Preadipocytes	100±1.3	104±1.5	105±1.3	108±1.7	104±2	99±2.6	-
Fibroblast cells	100±1.4	96±1.2	94±1.2	94±2.8	95±3	95±1.2	98±1

Table 3: Effect of macerated extract of *V. arctostaphylos* on lipid droplet accumulation in differentiating preadipocyte isolated from diabetic rats

Lipid accumulation (% of Vehicle)	*Vaccinium arctostaphylos* (µg/ml)			
	Vehicle	10	100	1000
	1000±9	139±16	109±9.5	94±7

hypolipidemic effects without any concern about unwanted effects on adipose tissue. This recommendation is supported by the results of MTT assay which showed *V. arctostaphylos* do not increase proliferation of preadipocytes.

Our data also showed that hydroalcoholic extract of *V. arctostaphylos* do not reduce surviving of fibroblast cells. The L929 fibroblast cells are routinely used for testing if an agent or drug has cytotoxic effect (Mortazavian et al., 2012). Therefore, it seems that *V. arctostaphylos* can be considered as a safe medicinal plant at the concentration range up to 400 µg/ml.

Lipolysis is a highly regulated process and control of this process is important for maintaining body homeostasis and prevention of metabolic diseases. Although a variety of factors affect lipolysis, catecholamines and insulin are the main pro-lipolytic and anti-lipolytic hormones, respectively (Large et al., 2004). In diabetes, insufficiency in insulin secretion or resistance to the action of insulin allows glucagon and catecholamine to stimulate lipolysis. Increase lipolysis enhances fatty acid delivery to liver which may lead to ketoacidosis, a life-threatening condition (Perilli et al., 2013). We showed previously that *V. arctostaphylos* acts directly on the Langerhans islets to stimulate insulin release (Shafiee-Nick et al., 2011). Also, several studies have reported that its fruits, like insulin, decrease blood glucose in diabetic subjects (Abidov et al., 2006; Feshani et al., 2011; Kianbakht et al., 2013). To test whether hydroalcoholic extract of *V. arctostaphylos* has also insulinomimetic action in fat tissue, lipolysis was investigated in adipose tissue incubated with this extract. This investigation was performed with *ex vivo* organ culture method which has certain advantages over isolated adipocyte because it retains paracrine, autocrine, cell-cell and cell-matrix interactions (Ghorbani and Abedinzade, 2013). Our data showed that *V. arctostaphylos* has no effect on basal or catecholamine (isoproterenol)-stimulated lipolysis in adipose tissue isolated from diabetic rats. It means that *V. arctostaphylos* did not show insulin like effect on adipocyte. Therefore, the previous reported insulin like effects (e.g. hypoglycemia) for this plant is most probably achieved by its indirect effects on other tissues. Yet, it is reasonable to assume again that this plant is safe because it do not increase the risk of ketoacidosis.

In conclusion, the present study demonstrated that macerated extract of *V. arctostaphylos* fruit has no unwanted effects on fat tissue adipogenesis and lipolysis in diabetic rats.

Acknowledgments

The authors declare that they have no conflict of interest. This work was supported by a grant from Research Council of Mashhad University of Medical Sciences, Mashhad, Iran.

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