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Physiological and Morphological Effects of Different Concentrations of Usnic Acid on Culture Lipoma Cells

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Abstract: Usnic acid (UA) is a thermogenic secondary metabolite of lichen. This study was designed to investigate UA local effects on lipoma cell line. Two groups of cultured cells were studied, G1: served as control, G2: divided into 5 subgroups, each treated with UA in different concentrations (0.15, 0.45, 0.75, 1, 1.5µM). Cell count, viability and morphology were evaluated after 1, 3, 6, 12, 24 and 48 hours of incubation. Leptin, adiponectin, tumor necrosis factor (TNF)-α and lipoprotein lipase (LPL) were analyzed using ELISA kits. Cell count and viability studies revealed that UA had an anti-proliferative effect on culture lipoma cells which was concentration and time dependent. UA treated cells showed apoptosis at low concentrations (0.15-0.45µM) and necrosis at high concentration (0.75 µM). Increased levels of leptin, adiponectin, TNF-α and LPL in UA treated samples were observed. Morphological studies showed deformed shrunken cells. Ultrastructure showed features of apoptosis. These changes may be via local auto/paracrine interaction of leptin, adiponectin, TNF-α and LPL. In conclusion: low concentrations of UA could be a promising local therapeutic agent for lipoma lesion/ future use in controlling adipocytes growth should be tried.

Key words: Adiponetin; Leptin; Tumor necrosis factor-α

INTRODUCTION

Adipocytes constitute the main components of fatty tissues. Recently, adipocytes were considered among cells of highly metabolic activity that are capable of acting as an endocrine organ [1-3]. Hormones and adipocytokines secreted by adipocytes were proved to have an impact on brain activities related to feeding behavior [4].

Type 2 diabetes, hypertension, cardiovascular diseases and accelerated aging are chronic illness in which obesity was considered the main risk factor [5-8]. The increase in adipose tissue mass is either due to increase cell number (hyperplasia) or increase cell size (hypertrophy) or both [9].

Park and coworkers [10] reviewed the use of many herbal derivatives in treatment of a number of clinical diseases including cancer and obesity. Usnic acid (UA) is known as a a secondary metabolite of lichen *Usnea* [11]. UA has an anti-inflammatory, antioxidant and anti-proliferative activities [12, 13]. Usnic acid was classified as a fat burner and incorporated in many weight loss agents [14] but most were hindered by its systemic side effects mainly hepatotoxicity [15].

Based on previous data, the primary objective of this research was conducted to study the local effects of different concentrations of usnic acid on the morphology of cultured lipoma cells, which proved to simulate adipocytes proliferation *in vivo*. Also, this study aimed to analyze some active adipose tissue-derived hormones and cytokines as leptin, adiponectin, tumor necrosis factor (TNF)-α and lipoprotein lipase (LPL) enzyme in order to define any local mechanism by which UA could affect adipocytes activity thus can open the way for using a new UA formulation for local treatment of people with obesity thus avoiding any systemic effects.

MATERIALS AND METHODS

Chemicals: (+)-Usnic acid (molecular weight: 344.32, Assay: 98%, optical activity: [α] 25/D +488°, c = 0.4 in chloroform, mp: 201-203 °C) prepared from *Usnea*

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dasypoga, 2, 6-Diacetyl-7, 9-dihydroxy-8, 9*b*-dimethyldibenzofuran-1, 3 (2*H*,9*bH*) dione ($C_{18}H_{16}O_7$) was purchased from Sigma-Aldrich (Chemie Gmbh, Munich, Germany).

Effect of Different Concentration of UA on Lipoma Cells: Human lipoma cells were obtained from tissue culture laboratory, molecular biology unit, Assiut University, Assiut, Egypt. The first trail was done using different concentrations of UA dissolved in dimethyl sulfoxide (DMSO) (5, 25 & 500 µg/ml) according to Sung and coworkers [16]. The control group was prepared for comparison. The initial count of cells was 1.2 X 10^7 cells/ml. After incubation for 48 hours, trypan blue assay revealed death of all cells while the control group was still alive. Incubation from 12 to 24 hours was then tried with an initial cell count of 37 X 10^6 cells/ml which revealed similar results as those observed after 48 hours.

Based on a study done by Pramyothin and colleagues [17] on the effects of UA on hepatocytes, the following concentrations of UA (0.15, 0.45, 0.75, 1 and 1.5 µM) were tried to study its effect of on lipoma cells. UA was freshly prepared and further diluted in compatible medium to obtain the needed concentrations.

Lipoma cells were subcultured in Dulbecco's modification of Eagles's minimal essential medium (DMEM) [45 ml of DMEM + 5ml Fetal-Calf-Serum-(FCS)] with 10 µM of amphotrysine B (as antifungal), 250 µM penicillin and 250 µM streptomycins. Cells were counted in random flasks to determine the appropriate cell count for carrying out the experiment. Subculture flasks were seeded with lipoma cells (16 x 10^4) and were divided into control and UA treated groups. All flasks incubated at 37 with 5% CO_2 throughout the experiment. For control and each UA concentration, triplicate samples were used.

Cells Counting: Cells count was done using the hemocytometer. The media was discarded; the cells were washed with phosphate-buffered saline (PBS). A trypsin/EDTA 10% solution (PBS, without Ca^{2+}, Mg^{2+}) was added to both control and experimental flasks for 3:4 minutes in the incubator at 37C°. Cells were then re-suspended in 5:10 ml complete medium, centrifuged at 320Xg for 10 minutes. The pellets were washed with PBS and the supernatant was removed and replaced by 3ml complete medium, centrifuged at 320Xg for 10 minutes. 5ml of complete medium was added to suspend cell pellet then take 1:1 cell suspension and trypan blue for counting [18].

Cells Viability Assay: The percentage of cell viability equal the viable cells/ total cell count X100.

N.B. the total cells count equal to the viable cells count plus the dead cells count.

Cells Morphology: Cells from all flasks, after 3 hours culturing, were photographed using an inverted light microscope and assessed for morphological changes.

Preparation of Cells for Electron Microscope Examination: Culture cells (0.15 µM UA after 24 hours; as about 45.45% of cells are still viable) were treated with trypsin, then suspended in phosphate buffer (pH 7.4), centrifuged at 320Xg for 10 minutes. After discard of supernate, the cells were fixed in 1ml of 2.5% gultraldehyde in phosphate buffer pH 7.4 till further processing for both scanning and transmission electron microscopy [19].

Biochemical Analysis: Supernatant media of both control and treated cells culture were analyzed using ELISA kits for determined the levels of leptin, adiponectin and lipoprotein lipase enzyme (Glory Science Co., Ltd, TX 78840, USA) and TNF-α (Assaypro, Saint Charles, Missouri, USA).

Statistical Analysis: Statistical Package for Social Science (SPSS) version 20 for windows program was applied to analyze the present data. The data were expressed as means +/- standard deviation (SD). Comparison of variables between groups was performed using One-way analysis of variance (ANOVA) or Student's *t*-test as appropriate. Statistical significance was considered at *P*-value < 0.05.

RESULTS

Cell Counting and Cell Viability Table (1) and Figure (1) showed the effect of different concentrations of UA (0.15, 0.45, 0.75, 1 and 1.5 µM) on lipoma cell viability from one to 48 hours. It was observed that UA decreased cell viability in a concentration and time dependent manner. The viable lipoma cell counts decreased with increasing the concentration of UA. The percentage of viable cells at concentration of 0.15 µM was 92.31, 75.86, 60.34, 57.14, 50.99 and 45.45% after 1, 3, 6, 12, 24 and 48 hours respectively. While at concentrations of 0.45 and 0.75 µM, the cells viability after 1 hour were 73.33 and 37.50% respectively and after 3 hours were 57.69 and 22.58%, respectively and no viable cells after 6 hours. Also, no viable cells were encountered at higher concentrations (1 µM and 1.5 µM).

Table 1: Description of cell counts (viable and dead cells) at different times and concentrations.

Parameters	Conc. 0.15 µM	Conc. 0.45 µM	Conc. 0.75 µM	Conc. 1 µM	Conc. 1.5 µM
After 1 hour					
Viable cells	15.00±4.97	5.50±0.58	3.00±0.82		
	(11.00-22.00)	(5.00-6.00)	(2.00-4.00)		
Dead cells	1.25±0.50	2.00±1.83	5.00±0.82		
	(1.00-2.00)	(0.00-4.00)	(4.00-6.00)		
Cells viability x10^4	92.31%	73.33%	37.5%		
After 3 hour					
Viable cells	16.50±5.80	7.500±1.73	3.50±3.87		
	(12.00-25.00)	(5.00-9.00)	(0.00-9.00)		
Dead cells	5.25±0.96	5.50±3.42	12.00±6.27		
	(4.00-6.00)	(1.00-9.00)	(5.00-20.00)		
Cells viability x10^4	75.86%	57.69%	22.58%		
After 6 hour					
Viable cells	8.75±2.87	0.00±0.00	0.00±0.00		
	(5.00-11.00)	(0.00-0.00)	(0.00-0.00)		
Dead cells	5.75±1.71	0.75±1.71	5.25±0.96		
	(4.00-8.00)	(0.00-2.00)	(4.00-6.00)		
Cells viability x10^4	60.34%	0.00%	0.00%		
After 12 hour					
Viable cells	25.00±5.35				
	(20.00-31.00)				
Dead cells	18.75±2.63				
	(15.00-21.00)				
Cells viability x10^4	57.14%				
After 24 hour					
Viable cells	19.25±1.71				
	(17.00-21.00)				
Dead cells	18.50±2.52				
	(16.00-22.00)				
Cells viability x10^4	50.99%				
After 48 hour					
Viable cells	16.25±2.63				
	(14.00-19.00)				
Dead cells	19.50±0.57				
	(19.00-20.00				
Cells viability x10^4	45.45%				

Data are expressed as mean +/-SD, minimum- maximum

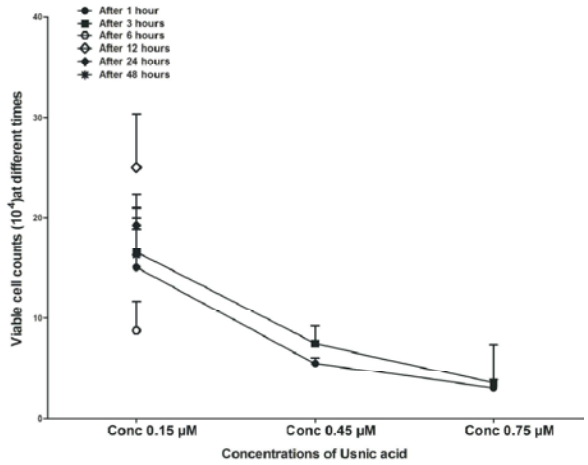

Fig. 1: Description of viable cell counts at different times and concentrations of Usnic acid.

Cell Morphology by Light Microscopy: Examination of cultured cells before and after addition of different concentration of UA showed that cells underwent both apoptotic and necrotic changes in concentration dependent manner. At lower concentrations, cells were deformed and shrunken (signs of apoptosis), while at higher concentrations cells showed necrosis with loss of cellular outlines and extrusion of lipid droplets to the media (Fig. 2).

Scanning Microscopy: Scanning microscopy provided the three dimensional features of lipoma cells. The addition of UA at low concentration (0.15 µM) resulted in marked shrinkage and irregularity of cell surface compared to non-treated cells (Fig. 3).

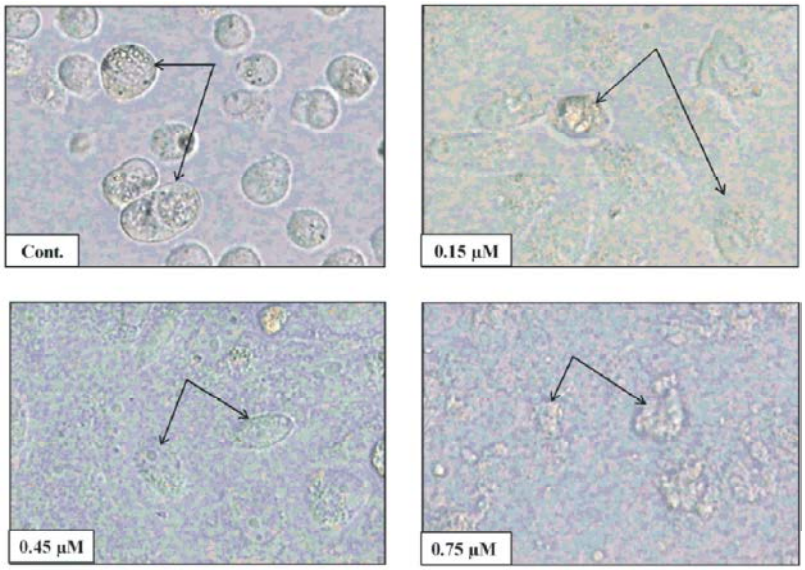

Fig. 2: Showing concentration dependent effect of Usnic acid (UA) (3 hours culture). Notice that low concentrations produced shrinkage and deformity of lipoma cells, while high concentration resulted in necrosis and loss of cellular outlines, releasing of cellular contents to nearby media (arrows). (X 400)

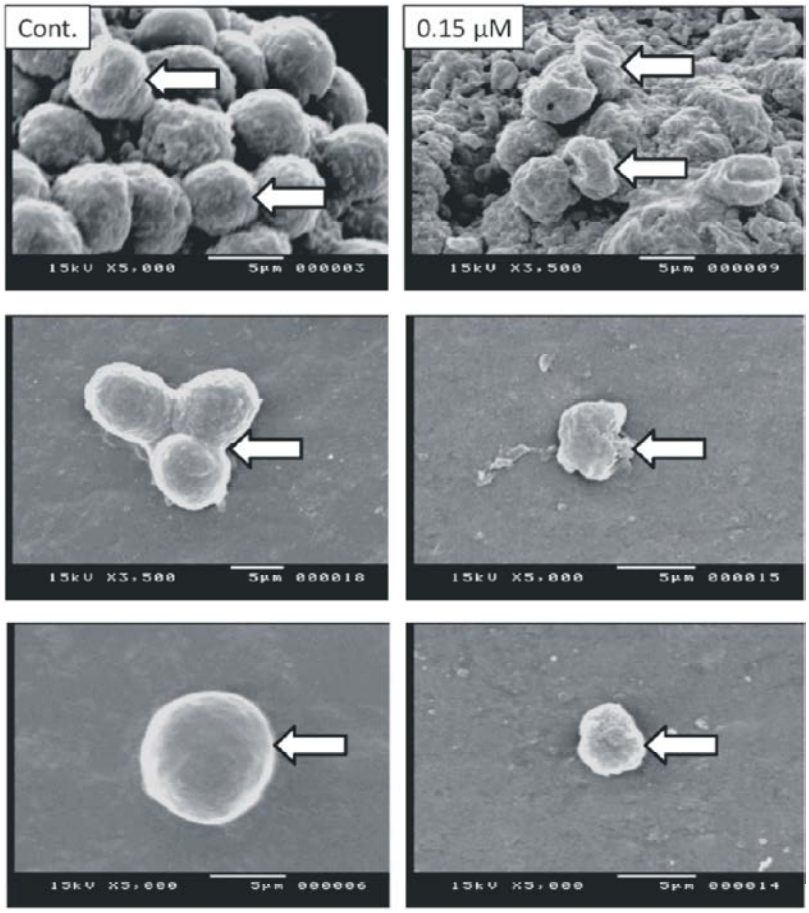

Fig. 3: Scanning microscopic pictures of lipoma cell culture showed marked shrinkage and irregularity of cell surface in Usnic acid (UA) treated cells compared to non-treated cells (control).

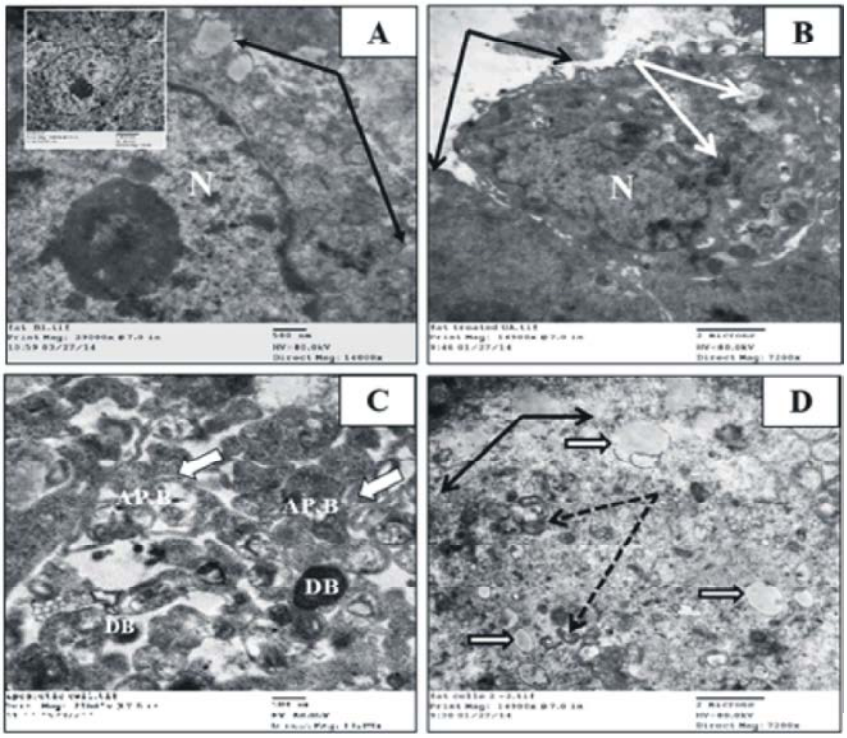

Fig. 4: Transmission electron microscope of lipoma cells of (A): control; showed euchromatic nucleus (N) with a prominent nucleolus. The cytoplasm contains fat droplets (arrows). (B) At low concentration of UA (0.15 µM), apoptotic lipoma cells had preserved outlines (black arrows), electron dense cytoplasm and irregular nuclei with increased heterochromatin content (N). The cytoplasm showed dense lamellated bodies, few lipid droplets and vacuoles (white arrows). (C) A magnified part from an apoptotic lipoma cells showing apoptotic bodies (AP.B) degenerated organelles, dense bodies (DB) and numerous lamellated bodies (white arrows). (D) Necrotic lipoma cells are swollen with ill-defined outlines, karyolysis and showed disrupted organelles (black arrows), dense bodies (dotted arrows) and lipid droplets (white arrows).

Transmission Electron Microscope: Transmission electron microscopy provided more confirmation of the UA effects on lipoma cells that was depend on different concentrations. At lower concentration of UA (0.15 µM), apoptotic lipoma cells looked shrunken with electron dense cytoplasm, increased nuclear heterochromatin and formation of apoptotic bodies. Meanwhile, necrosis of cells was observed in the form of swelling, cell membrane damage, karyolysis and disruption of cellular contents (Fig. 4).

Biochemical Studies: After 1 hour of incubation, levels of leptin in the supernatant media of cultured lipoma cells was insignificantly increased at UA concentrations (0.15-0.45 and 0.75 µM), while it was significantly increased at UA concentrations of 1 µM and 1.5 µM compared with control (P =0.017 and P =0.0001, respectively). After 3 hours of incubation, levels of leptin in the supernatant media of lipoma cultured cells was significantly increased at UA concentrations of 0.75 and 1 µM compared with control (P =0.002 and P =0.0001, respectively). After 6 hours of incubation, levels of leptin in the supernatant media of cultured lipoma cells was also significantly increased at UA concentrations of 0.75 and 1 µM compared with control (P =0.003 and P =0.0001, respectively) (Table 2).

After 1 hour of incubation, levels of adiponectin in the supernatant media of cultured lipoma cells was significantly increased at UA concentrations of 1 µM and 1.5 µM compared with control (P =0.002 and P =0.0001, respectively). After 3 hours of incubation, levels of adiponectin in the supernatant media of cultured cells was significantly increased at UA concentration of 1 µM compared with control (P =0.005), meanwhile, the level of UA concentration of 0.75 µM was significantly decreased compared to its levels after 1 hour (P =0.043). After 6 hours, levels of adiponectin in the supernatant media of cultured cells was significantly increased at UA concentration of 1 µM compared with control (P =0.007) (Table 3).

Table 2: Description of Leptin in different times and concentration.

Parameters	Control	Conc. 0.15 µM	Conc. 0.45 µM	Conc. 0.75 µM	Conc. 1 µM	Conc. 1.5 µM
After 1 hour	1.74±0/44 (1.15-2.46)	2.51±1.30 (1.41-3.94) *P* =0.248	2.39±0.39 (1.94-2.63) *P* =0.328	2.72±1.52 (1.04-4.00) *P* =0.150	3.46±1.14 (2.19-4.26) *P* =0.017	5.23±0.65 (4.59-5.88) *P* =0.0001
After 3 hours	1.74±0/44 (1.15-2.46)	0.99±0.81 (0.05-1.49) *P* =0.120; **P* =0.103	2.52±0.34 (2.26-2.90) *P* =0.223; **P* =0.660	3.87±1.27 (2.84-5.29) *P* =0.002; **P* =0.279	4.44±0.60 (3.80-4.99) *P* =0.0001; **P* =0.091	
After 6 hours	1.74±0/44 (1.15-2.46)	1.35±0.42 (0.87-1.66) *P* =0.226; **P* =0.173	1.88±0.62 (1.17-2.28) *P* =0.949; **P* =0.424	3.30±0.88 (2.75-4.31) *P* =0.003; **P* =0.429	4.88±0.40 (4.43-5.19) *P* =0.0001; **P* =0.188	
After 12 hours	1.74±0/44 (1.15-2.46)	1.55±0.19 (1.36-1.74) *P* =0.239; **P* =0.364				
After 24 hours	1.74±0/44 (1.15-2.46)	1.66±0.16 (1.48-1.76) *P* =0.435; **P* =0.365				
After 48 hours	1.74±0/44 (1.15-2.46)	1.57±0.14 (1.47-1.73) *P* =0.260; **P* =0.360				

Data are expressed as mean +/-SD, minimum- maximum

P: significance versus control; *P: versus after 1st hour.

Table 3: Description of adiponectin in different times and concentration.

Parameters	Control	Conc. 0.15 µM	Conc. 0.45 µM	Conc. 0.75 µM	Conc. 1 µM	Conc. 1.5 µM
After 1 hour	5.97±1.33 (4.70-7.85)	8.60±1.27 (7.14-9.50) *P* =0.071	6.10±1.38 (5.29-7.69) *P* =0.928	7.90±3.05 (5.85-11.41) *P* =0.174	11.02±2.94 (9.15-14.40) *P* =0.002	20.00±1.20 (18.80-21.19) *P* =0.0001
After 3 hours	5.97±1.33 (4.70-7.85)	5.64±1.18 (4.90-7.00) *P* =0.968; **P* =0.171	5.66±1.94 (4.41-7.90) *P* =0.981; **P* =0.322	6.82±2.79 (5.19-10.50) *P* =0.436; **P* =0.043	10.55±2.37 (7.88-12.41) *P* =0.005; **P* =0.763	
After 6 hours	5.97±1.33 (4.70-7.85)	6.29±1.85 (4.68-8.31) *P* =0.744; **P* =0.319	7.25±3.46 (5.14-11.24) *P* =0.395; **P* =0.438	8.96±2.88 (7.02-12.27) *P* =0.089; **P* =0.090	11.38±2.81 (9.10-14.52) *P* =0.007; **P* =0.388	
After 12 hours	5.97±1.33 (4.70-7.85)	5.44±0.75 (4.63-6.12) *P* =0.763; **P* =0.108				
After 24 hours	5.97±1.33 (4.70-7.85)	5.57±0.45 (5.19-6.07) *P* =0.873; **P* =0.093				
After 48 hours	5.97±1.33 (4.70-7.85)	5.30±0.22 (5.09-5.52) *P* =0.621; **P* =0.059				

Data are expressed as mean +/-SD, minimum- maximum. P: significance versus control; *P: versus after 1st hour

After 1 hour of incubation, levels of TNF-α in the supernatant media of cultured cells was significantly increased at UA concentration of 1.5 µM compared with control (P =0.031). At UA concentration of 0.15 µM, levels of TNF-α in the supernatant media of cultured cells was significantly increased compared with control after 12, 24 and 48 hours of incubation (P =0.046, P =0.029 and P 0.06, respectively) (Table 4).

Table 4: Description of tumor necrosis factor alpha in different times and concentration.

Parameters	Control	Conc. 0.15 µM	Conc. 0.45 µM	Conc. 0.75 µM	Conc. 1 µM	Conc. 1.5 µM
After 1 hour	0.02±0.01 (0.02-0.03)	0.02±0.01 (0.02-0.03) P =0.667	0.03±0.02 (0.02-0.06) P =0.305	0.02±0.003 (0.02-0.02) P =0.980	0.02±0.002 (0.02-0.02) P =0.899	0.04±0.025 (0.02-0.09) P =0.031
After 3 hours	0.02±0.01 (0.02-0.03)	0.02±0.003 (0.02-0.02) P =0.856; *P =0.332	0.03±0.016 (0.02-0.05) P =0.226; *P =0.851	0.03±0.021 (0.02-0.06) P =0.114; *P =0.330	0.02±0.003 (0.02-0.02) P =0.856; *P =1.000	
After 6 hours	0.02±0.01 (0.02-0.03)	0.02±0.006 (0.02-0.03) P =0.987; *P =0.252	0.02±0.003 (0.02-0.02) P =0.936; *P =0.379	0.05±0.063 (0.02-0.14) P =0.078; *P =0.432	0.02±0.00 (0.02-0.02) P =0.873; *P =0.391	
After 12 hours	0.02±0.01 (0.02-0.03)	0.04±0.032 (0.02-0.07) P =0.046; *P =0.371				
After 24 hours	0.02±0.01 (0.02-0.03)	0.03±0.01 (0.02-0.04) P =0.029; *P =0.475				
After 48 hours	0.02±0.01 (0.02-0.03)	0.08±0.059 (0.02-0.14) P =0.009; *P =0.171				

Data are expressed as mean +/-SD, minimum- maximum

P: significance versus control; *P: versus after 1st hour

Table 5: Description of lipoprotein lipase in different times and concentration.

Parameters	Control	Conc. 0.15 µM	Conc. 0.45 µM	Conc. 0.75 µM	Conc. 1 µM	Conc. 1.5 µM
After 1 hour	4.84±1.67 (3.52-6.69)	5.03±1.55 (3.24-5.98) P =0845	6.50±0.27 (6.31-6.69) P =0.316	5.63±0.99 (5.00-6.78) P =0.578	9.15±1.34 (7.60-9.98) P =0.008	11.25±0.44 (10.81-11.69) P =0.0001
After 3 hours	4.84±1.67 (3.52-6.69)	3.44±0.52 (3.02-4.02) P =0.360; *P =0.313	5.24±0.48 (4.71-5.66) P =0.417; *P =0.213	7.65±1.23 (6.31-8.73) P =0.008; *P =0.042	8.41±0.91 (7.86-9.46) P =0.002; *P =0.373	
After 6 hours	4.84±1.67 (3.52-6.69)	3.41±3.54 (0.02-7.09) P =0.562; *P =0.411	6.62±1.75 (5.71-8.60) P =0.200; *P =0.609	6.77±1.21 (5.71-8.08) P =0.174; *P =0.055	9.81±2.14 (7.34-11.17) P =0.007; *P =0.746	
After 12 hours	4.84±1.67 (3.52-6.69)	3.96±1.00 (2.83-4.73) P =0.693; *P =0.154				
After 24 hours	4.84±1.67 (3.52-6.69)	4.09±1.23 (2.69-4.97) P =0.795; *P =0.143				
After 48 hours	4.84±1.67 (3.52-6.69)	4.74±0.61 (4.13-5.17) P =0.781; *P =0.195				

Data are expressed as mean +/-SD, minimum- maximum

P: significance versus control; *P: versus after 1st hour.

After 1 hour of incubation, levels of lipoprotein lipase (LPL) in the supernatant media of cultured lipoma cells was significantly increased at UA concentrations of 1 µM and 1.5 µM compared with control (P =0.008 and P =0.0001, respectively). After 3 hours of incubation, levels of LPL in the supernatant media of cultured lipoma cells was significantly increased at UA concentrations of 1 µM and 1.5 µM compared with control (P =0.008 and P =0.002, respectively); meanwhile, level at concentration of 0.75 µM was significantly increased compared to its levels after 1 hour of incubation (P =0.042). After 6 hours of incubation, levels of LPL in the supernatant media of cultured lipoma cells was significantly increased at UA concentration of 1 µM compared with control (P =0.007) (Table 5).

DISCUSSION

Lipolysis in adipocyte models was observed upon using natural compounds such as curcumin, conjugated linoleic acid and epigallocatechin gallate (EGCG) [20, 21]. Lipoma is a benign hyperplasia of subcutaneous adipocytes. It is characterized by enhanced adipogenesis compared with normal adipose tissue [22]. So, it may resemble proliferating cells in case of people with obesity [23].

In the present study, an anti-proliferative effect of UA was tested against human lipoma cell line. A concentration dependent decreased in cell viability was observed. Light microscopic examination of unstained cultured lipoma cells (3 hrs. cell culture) showed enhanced apoptosis at low concentration of UA (0.15 µM) and cell necrosis at high concentration (0.75 µM). In this respect, Sung *et al*. [16] reported that *Lethariella cladonioides* (LC) which belongs to lichen family exerted anti-proliferative activity and had a concentration dependant inhibitory effect on *3T3-L1* preadipocytes via suppression of lipid accumulation and enhancing triglyceride hydrolysis. Burlando and coworkers [24] reported the anti–neoplastic effect of UA against *MM98* malignant mesothelioma cells and *A431* vulvar carcinoma cells. Koparal [25] reported the anti-proliferative effect of UA against *HepG2* hepatocarcinoma cells. UA was classified as uncouplar of oxidative phosphorylation [11] and may promote energy wasting and reduce lipoma cell size in a similar way of uncoupling protein-1 [26].

A diverse members of adipokines were reported to be secreted by adipocytes and were known to exert regulatory functions as energy homeostasis, insulin sensitivity, appetite and bone mass [27]. In the present study, hormones and cytokines levels in the supernatant media of control and treated cultures were measured to correlate structural alteration with secretory functions of lipoma cells in response to different concentrations of UA treatment. Leptin, a hormone secreted primarily by adipocytes [28], was found in this study to be increased in media of lipoma cell culture treated with UA in concentration and duration dependent pattern. Leptin was reported to be implicated in adipocyte apoptosis [29]. Leptin was also reported to induce lysis of adipocytes either in tissue explants or cultured cells [30]. In this respect, Jaubert and coworkers [31] reported that leptin inhibited glycerol synthesis, reduced fatty acid re-esterification in adipocytes with a subsequent decreased in lipid accumulation in the cells via suppression of lipogenesis, an increased in triglyceride hydrolysis and fatty acid and glucose oxidation. Harris [32] found that administration of leptin to mice reduced body fat stores by inhibiting cell proliferation *in vivo*. These results could explain the finding reported by this study that usnic acid may stimulate local leptin release from lipoma cells via paracrine/ autocrine manner resulted in either apoptotic or necrotic changes in cultured lipoma cells depending on UA concentrations [33]. Further studies are needed to search for the role of leptin receptors on lipoma cells in controlling lipoma cell proliferation. On contrary to the results of the present study, William and coworkers [34] reported that leptin inhibited de novo fatty acid synthesis from glucose in isolated rat adiposcytes. Elimam and coworkers [33] reported that although adipocytes possess leptin receptors, no lipolytic effect was reported when adipocytes were incubated with increasing concentrations of leptin.

Adiponectin is a hormone secreted by adipocytes. It is linked to insulin sensitivity and inflammatory responses associated with obesity [35]. In the present study, addition of UA to culture lipoma cells resulted in significant concentration and duration dependent increase in the level of adiponectin in the supernatant media of cultured cells. In this respect, Dietze-Schroeder and coworkers [36] reported that in obese persons, the degree of obesity is negatively correlated with adiponectin. Its increase in the media may via autocrine action exerts anti proliferative effect on lipoma cells that result in decrease in lipoma cells viability and size. Suga and colleagues [22] reported that adiponectin level was down- regulated in lipoma cell culture. The degree of adiposity was reported to be inversely related to adiponectin levels [37, 38].

Matsuzawa and coworkers [39] found that increased visceral adipose tissue mass is associated with a decrease in adiponectin plasma levels. Administration of adiponectin was found to improve adipose tissue metabolic function in rats fed high fat diet [40]. Lazra and coworkers [41] described an autocrine/paracrine effect of globular adiponectin (10?nM) with decreasing lipid accumulation and metabolism in *3T3-L1* adipocytes. So, UA may have dual action, first it may stimulate secretion and release of those two specific cytokines (leptin and adiponectin) which upon increase in the vicinity of culture cells stated to exert its anti-proliferative effect on lipoma cells observed in this study that may act via an autocrine/paracrine pathway on lipoma cells inhibiting their growth.

In the present study, it was observed that addition of UA to lipoma cell line culture resulted in significant increase in TNF-α levels in lipoma cell culture media compared with the control that was concentrations and time dependent. TNF-α is 26-kDa; a transmembrane protein that has many impact regulating roles on adipose tissue. TNF-α was postulated to be linked to leptin production and adipocyte differentiation. *In vitro*, it was reported to stimulate lipolysis and impair preadipocyte differentiation [42, 43]. In view of such data, one can suggest that the increase of TNF-α observed herein may play a role in the decrease of cell viability upon addition of UA.

An anti-adipogenic effects of TNF-α was reported in the literature [44, 45]. Prins and coworkers [42] reported that TNF-α in different concentrations induced apoptosis *in vitro* of human preadipocytes and adipocytes. Based on such data, the increased of TNF-α and leptin levels observed in the present study after UA treatment may be implicated in the observed decrease in cell viability either through autocrine / paracrine anti-proliferating effect or via inducing lipoma cell apoptosis [28] or a direct effect of TNF-α inducing cell necrosis [46]. Leptin – TNF-α interaction and their effects on controlling lipoma as well as normal adipocyte cell growth have to be thoroughly studied.

Lipoprotein lipase exerts important role in lipid transport in many body organs including white and brown adipose tissue [47]. Any disorder of LPL enzyme expression can affect transport or storage of fatty acids with further impact on body mass and initiation and or development of obesity [48]. Masuno and coworkers [49] reported that LPL enzyme was produced by *3T3-L1* adipocytes which could explain its presence in culture media of lipoma cells in the present study. The addition of UA in different concentrations to lipoma cells resulted in an increase of LPL concentration in culture media that was concentrations and duration dependent. This increase could point either to direct effect of UA that enhancing LPL enzyme release from lipoma cells which via autocrine / paracrine action promotes triglyceride hydrolysis [50] or as a result of lipoma cell necrosis induced by TNF-α and subsequent release of LPL into culture media. More work is needed to confirm which mechanism is involved in the increase of LPL enzyme provoked by the thermogenic UA.

CONCLUSIONS

In conclusion, usnic acid decreased culture lipoma cell size, lipid content and induced cell apoptosis at low concentrations and necrosis at high concentrations. These effects might be via local production of leptin, adiponectin, TNF-α and LPL enzyme by the adipocytes that in turn exerted antiproliferative effect. More investigations at molecular level are needed to confirm such events. So, low concentrations of UA could be tried for local induction of similar cell apoptosis in obese adipocytes as therapeutic treatment for people with obesity to avoid systemic its side effects.

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