Original Article

Effect of capsaicin on transcription factors in 3T3-L1 cell line

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Abstract. Capsaicin is a spicy ingredient of *Capsicum annuum* and a lipophilic, crystalline, odorless and colorless alkaloid. Although the effect of capsaicin on adipocyte differentiation is well-known, the role of capsaicin on transcription factors while adipocyte differentiation is not clear. The aim of this study is thus to identify and characterize the transcription factors in the process of adipocyte differentiation after the capsaicin treatment. In this study, concentration of 0, 50, 100, 150, 200 and 250 μM capsaicin were treated to 3T3-L1 pre-adipocytes in cell culture. MTT cell cytotoxicity, cell viability with trypan blue staining, Lactate Dehydrogenase (LDH) enzyme assay, triglyceride content assay, Glycerol-3-Phosphate Dehydrogenase (GPDH) activity, Oil Red O staining and mRNA levels of transcription factors (PPARγ, C/EBPα and SREBP-lc) were investigated in capsaicin induced 3T3-L1 preadipocyte cell line. Capsaicin treatment decreased cell population growth of 3T3-L1 preadipocytes, assessed with trypan blue staining, MTT test and rising of LDH release proportion. Capsaicin inhibited GPDH activity and intracellular triglyceride content in 3T3-L1 adipocytes in all treated groups in a dose-dependent manner. Oil Red O staining indicated that capsaicin inhibited adipocyte differentiation in 3T3-L1 adipocytes in all treatment groups. In this study, it was revealed that exposing 3T3-L1 preadipocytes and differentiating postconfluent preadipocytes to different doses of capsaicin decreased PPARγ, C/EBPα and SREBP-1c mRNA levels as compared with their controls without treatment in dose dependent manner. Although, reduction of PPARγ mRNA level was statistical significant, this decrease was not significant in C/EBPα and SREBP-1c mRNA levels. This study demonstrated that capsaicin treatment inhibited the adipogenesis through the down-regulation of transcription factors, especially PPARγ. Alternative mechanisms may involve cell cycle arrest and the induction of apoptosis. Since capsaicin is the main component found in hot pepper, the consumption of hot pepper may contribute to the maintenance of body weight and prevent the development of obesity.

Key words: Capsaicin, 3T3-L1, adipocyte, transcription factors, PPARγ, C/EBPα, SREBP-lc

1. Introduction

Obesity is characterized by the hyperplasia and hypertrophy of adipose tissues (1,2). Pluripotent stem cells have the potential to differentiate into different somatic cells including adipocytes. The differentiation process of converting preadipocyte to adipocyte involves a series of tightly regulated events of interplay between mediators of cell-cycle functions and differentiation-associated factors (3). Various preadipose cell lines have been developed in the past two decades to meet the incremental needs of researchers for studying the molecular and cellular events that take place during adipocyte differentiation (4). 3T3-L1 was first established by Green and Kehinde (5) to be a committed murine preadipocytes cell line. Some phenolic acids including gallic acid, epigallocatechin gallate, o-coumaric acid, m-coumaric acid, chlorogenic acid and capsaicin caused an improved inhibition of cell population growth and induction of apoptosis in 3T3-L1 preadipocytes (6).

Capsaicin is a spicy ingredient of *Capsicum annuum* and a lipophilic, crystalline, odorless and colorless alkaloid (7). Capsaicin is capable of increasing total energy use and carbohydrate oxidation, while slowing down lipid oxidation for a short period of time (8). Hsu and Yen showed the induction in apoptosis of adipocytes and inhibition in adipocyte differentiation, adipogenesis and adipocytic triglyceride content (9).

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The adipocyte differentiation process is very complex and tightly controlled. Changes in gene expression levels occur within a precise time-frame (10). Conversion of preadipocytes into mature adipocytes involves changes in expression of nearly 300 genes/proteins during adipogenesis, out of which around 100 proteins are up-regulated during the differentiation process (11,12). The differentiation of preadipocytes into adipocytes is regulated by an elaborate network of transcription factors that coordinate expression of hundreds of proteins responsible for establishing the mature fat-cell phenotype (13). Three important transcription factors play crucial roles in lipid metabolism and are considered as the major regulators of adipogenesis. These transcription factors are Peroxisome proliferator activated receptor gamma (PPARγ), CCAAT/enhancer binding protein α (C/EBPα) and Sterol regulatory element binding protein-lc (SREBP-lc) (14). All of these transcription factors are highly expressed in 3T3-L1 mature adipocytes during adipogenesis. PPARγ promotes preadipocyte determination and also stimulates the process of terminal differentiation (15-17). C/EBPα binds to and stimulates transcription of genes which are plentifully expressed in the mature adipocyte (18-20). SREBP-1c is primarily involved in governing the expression of enzymes responsible for fatty acid and triglyceride synthesis such as fatty acid synthase and lipropotein lipase in the presence of insulin stimulation (21-23).

Although the effect of capsaicin on adipocyte differentiation is well-known, the role of capsaicin on transcription factors while adipocyte differentiation is not clear (24). The aim of this study is thus to identify and characterize the transcription factors in the process of adipocyte differentiation after the capsaicin treatment.

2. Materials and methods

2. 1. Materials

Mouse embryonic fibroblast cells (3T3-L1) were obtained from the American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and L-glutamine were from Gibco, Invitrogen Corporation (Carlsbad CA). Penicillin and streptomycin were purchased from Cellgro, Mediatech (Manassas, VA). Trizol, DNase I kit, Superscript III First Strand cDNA synthesis kit were obtained from Invitrogen. Oil Red O powder, Accustain Harris Hematoxylin solution, dexamethasone (D8893), insulin from bovine pancreas (16634), 3-isobutyl-1-methyl xanthine (EBMX) (17018) and Capsaicin (M2028) were purchased from Sigma-Aldrich (St. Louis MO).

2. 2. Cell Culture

The mouse embryo 3T3-L1 cells were propagated and maintained in DMEM containing 10% (v/v) calf serum. After four days, cells reached confluent. After two-day postconfluent (designated Day 0) growth-arrested 3T3-L1 preadipocytes were induced to differentiate by Student et al (25). Briefly, cells were fed with DMEM containing 10% (v/v) FBS, 1 µg/mL insulin (I), 1 µM dexamethasone (D), and 0.5 mM 3-isobutyl-1-methylxanthine (M) until day 2. The cells were fed with DMEM supplemented with 10% FBS and 1 µg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Capsaicin was dissolved into serum free media. Culture was treated with capsaicin and retreated after eight hours on the same day for a specific period (25).

2. 3. MTT cell cytotoxicity

The MTT assay was performed according to the method of Mosmann (26). Cells were seeded at 2.500 cells/well in 96-well plate. After 24 hours, cells were incubated with capsaicin and DMEM containing 0.1% BSA for 72 hours. The treatment media were removed and cells were washed with sterile PBS twice. Cells were replaced with 100 µL MTT working solution (0.5 mg/mL) and incubated for further four hours. The unreacted dye is removed and the insoluble formazan crystals were dissolved and mixed thoroughly in acid-isopropanol (100 µL of 0.04 N HCl in isopropanol/well). The dye solution was measured at a test wavelength of 570 nm with a reference wavelength of 620 nm by a 96 microplate reader within 30 minutes. To calculate the absorbance values at each dose, the mean absorbance of the eight blank wells without cells were subtracted from the mean absorbance of eight wells containing cells. The optical density (OD) value of normal control group was represented as 100% while the ODs of all other treatment groups were expressed as a percentage of the normal control group (26).

2. 4. Cell counting

The ability of 3T3-L1 preadipocytes to exclude trypan blue upon staining was measured according to Sanford's method (27). Culture flasks were inoculated with $6xl0^5$ cells/25 cm^2 flask in 5 mL growth medium. The cells were incubated for 24 h to allow attachment to the substratum. The medium was then aspirated and replaced with fresh growth medium containing 0, 50, 100, 150, 200 and 250 µM capsaicin. Cells were then released from the substratum with 1

mL trypsin 72 h after capsaicin administration (96 h post inoculation). Duplicate 0.5 mL aliquots of each sample were diluted (1:1) with 0.5 mL of 4 g/L trypan blue in 8.5 g/L saline solution. The solution was pipetted into a hemacytometer and the number of viable unstained (excluding trypan blue) and non-viable stained cells was counted. Between 150 and 250 cells were counted per sample aliquot. Viability was calculated by dividing the number of viable unstained cells by the total number of cells counted and converting to a percentage (27).

2. 5. Lactate dehydrogenase enzyme assay

Preadipocytes were inoculated at $1.0x10^5$ cells/25 cm^2 flask and allowed to attach to the substratum for 24 h. Capsaicin and control treatments were administered after the 24 h attachment period. Samples (0.5 mL) of medium from each treatment flask were removed 96 h post inoculation (72 h post capsaicin administration). Duplicate 50 µL samples were added to cuvettes along with 2.5 mL of 0.2 mmol/L NADH in 80 mmol/L tris, 200 mmol/L NaCl buffer, and 0.5 mL of 1.6 mmol/L pyruvate in tris-NaCl buffer. Lactate dehydrogenase (LDH) enzyme activity was measured by analyzing the absorbance across a 10 min interval at wavelength of 340 nm at spectrophotometer (28). NADH absorbance was measured at 340 nm wavelength, indicating the amount of NADH consumed by the reaction converting pyruvate to lactate, catalyzed by LDH. The absorbance across time is a measure of LDH activity. Lactate dehydrogenase activity was expressed in nmol NADH consumed/(min. mL medium) (28).

2. 6. Triglyceride content assay

Triglyceride content was performed according to Ramírez-Zacarías et al (29). Briefly, cells in 24-well plate were rinised by PBS twice. The cells were fixed with cold 10% formaldehyde in isotonic solution for one hour. A 0.5% Oil red O isopropanol solution was diluted with 1.5 volume of water, filtered, and added to the fixed adipocyte monolayer for 1 hour. Cells were then washed with water. The stained triglyceride droplets were extracted with 1 ml isopropanol and the absorbance was read at 510 nm. The triglyceride content is standardized by triolein (29).

2. 7. Oil Red O staining

Cells were grown on plastic coverslip in 24-well plate. Cells were fixed and stained with the same method as triglyceride content assay. For each coverslip, five images were taken by camera-mounted microscope at magnification x100 (29).

2. 8. Glycerol-3-Phosphate Dehydrogenase enzyme assay

The activity of glycerol-3-phosphate dehydrogenase (GPDH), a specific marker of terminal differentiation, was measured from the cell extracts. The cell extracts were collected after 72 hour incubation. After two washes with PBS, the cell extracts were collected by scraping the cell cultures with a cell scraper into 0.3 mL (per well of 6-well plates) lysing buffer containing 50 mM Tris, 1 mM EDTA and 1 mM β-mercaptoethanol at pH 7.5. The harvested cells, after passing through a pipette several times, were then sonicated with a microtip for 10 seconds at 40W. After centrifugation at 15000g for 5 minutes at 4°C, the supernatants were assayed for GPDH according to the method of Wise and Green (30) and for the amount of total protein by Bradford method (31) against BSA. Enzyme activity was expressed as units of activity/mg protein.

2. 9. RNA Extraction

For RNA isolation from 3T3-L1 cells, 10^6-10^7 cells were lysed in 1ml Trizol reagent by passing the cell lysate several times through a pipette until no visible cell pellet was observed, 0.2 mL chloroform was added and then mixed by vortexing 30 seconds, kept in room temperature for 3 min, centrifuged at speed of 12.000 g for 15 min at 4°C. 0.45 mL supernatant was took and added same amount isopropanol to precipitate RNA (store in -20°C for 20 min), then centrifuged at speed 12.000 g for 10 min at 4°C). The precipitation was washed with 1 mL 70% ethanol and then dissolved in 50 µl pure water (32). RNA was quantified using absorption of light at 260 and 280 nm and sample integrity was checked by 1.5% agarose gel electrophoresis (32).

2. 10. cDNA synthesis

For reverse transcription of RNA to cDNA, 0.8 µg of total RNA from each sample was used for reverse transcription reaction using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions. Synthesized cDNA was stored at -20°C for later use.

2. 11. Real time polymerase chain reaction (RT-PCR)

For quantitative-polymerase chain reaction analysis, portions of double stranded cDNA were subjected to amplification with the specific primers into PCR products in optical 96-well plate. Amplification plots were analyzed using LightCycler 480 software (Roche Diagnostics). Real-time PCR amplification of PPARγ (33),

Table 1. Gene specific primers used for Real-time PCR

Gene name	Primers	Size (bp)
PPARγ	Forward: 5'- ACC CCC TGC TCC AGG AGA T -3'	84
(33)	Reverse: 5'- TGC AAT CAA TAG AAG GAA CAC GTT -3'	
C/EBPα	Forward: 5'- CGC AAG AGC CGA GAT AAA GC -3'	81
(33)	Reverse: 5'- GCG GTC ATT GTC ACT GGT CA -3'	
SREBP-lc	Forward: 5'- GAC GCT CAT TGG CCT GG -3'	205
(23)	Reverse: 5'- CTC TGG AGG CAG ACG ACA AG -3'	
β-actin	Forward: 5'- AGG TCA TCA CTA TTG GCA AC -3'	370
(34)	Reverse: 5'- ACT CAT CGT ACT CCT GCT TG -3'	

C/EBPα (33), SREBP-lc (23) and β-actin (as a house-keeping gene) (34) were performed on the LightCycler 480 (Roche Diagnostics) using LightCycler 480 Probes Master (Roche Diagnostics). Quantification of a given gene, expressed as relative mRNA level compared with a control, was calculated after normalization to β-actin. The level of PPARγ, C/EBPα and SREBP-lc detected in 3T3-L1 that has undergone different treatment was expressed in terms of the fold-difference. Mouse β-actin was used as the normalization control. Individual CT values are means of duplicate measurements with reported values representing the mean of at least three experiments. The forward and reverse primers used are displayed in Table 1. To ensure that no false positive PCR fragments were generated from contaminating genomic DNA, all primer sequences were designed to span intron regions.

2.12. Statistical analysis

Statistical analysis was performed using Student's t-test. It was used to examine the differences between the control and capsaicin-treated groups. Results were expressed as means ± standard error of values from at least three independent experiments in triplicate determinations. Statistical significance was set at $p < 0.05$.

3. Results

The effect of capsaicin on 3T3-L1 preadipocyte viability during proliferation. The effect of varied concentrations of capsaicin on cell viability was determined by analyzing the ability of cells to exclude trypan blue upon staining in the cell media after 72 hours exposure to treatments. The addition of capsaicin to the culture medium of the 3T3-L1 preadipocytes during proliferation decreased the cell viability in all treated groups ($p<0.05$). This decline started at 50 μM capsaicin applied group and then, cell viability continued to decrease on dose-dependent manner but these reduction was not statistically significant comparing with 50 μM capsaicin applied group ($p>0.05$). Overall, the percentages of cells excluding trypan blue comparing with the control group were 45.62 ± 2.43%, 43.21 ± 1.45%, 41.38 ± 2.73%, 38.91 ± 2.87% and 37.94 ± 2.56% for the 50, 100, 150, 200 and 250 μM capsaicin treatments respectively (Figure 1).

MTT analysis was carried out to detect the effect of berberine on the viability of 3T3-L1 cells during proliferation and differentiation. 3T3-L1 cells were treated with various concentrations of capsaicin (50, 100, 150, 200 and 250 μM) during each stage. Capsaicin had a similar effect on cell viability as trypan blue exclusion assay. Capsaicin treatment on 3T3-L1 preadipocytes during proliferation decreased the cell viability in all treated groups ($p<0.05$). This reduction started at 50 μM capsaicin applied group and then, cell viability continued to reduce on dose-dependent manner but these decline was not statistically significant comparing with 50 μM capsaicin applied group ($p>0.05$). The percentages of cell viability comparing with the control group were 49.22 ± 2.04%, 46.34 ± 2.07%, 45.54 ± 2.29%, 43.18 ± 2.36% and 41.94 ± 1.37% for the 50, 100, 150, 200 and 250 μM capsaicin treatments respectively (Figure 2).

As it is known, exposure to cytotoxic agents in 3T3-L1 cells causes LDH release. In current study, capsaisin had a reverse effect on LDH release compared with trypan blue exclusion assay and MTT assay. Capsaicin treatment on 3T3-L1 preadipocytes during proliferation increased LDH release in all treated groups ($p<0.05$). This increase started at 50 μM capsaicin applied group and then, LDH release continued to arise on dose-dependent manner but these increase was not statistically significant comparing with 50 μM capsaicin applied group ($p>0.05$). The percentages of LDH release comparing with the control group were 160.27 ± 11.27%, 164.07 ± 13.79%, 166.44 ± 10.83%, 169.46 ± 16.91% and 174.07 ± 21.43% for the 50, 100, 150, 200 and 250 μM capsaicin treatments respectively (Figure 3).

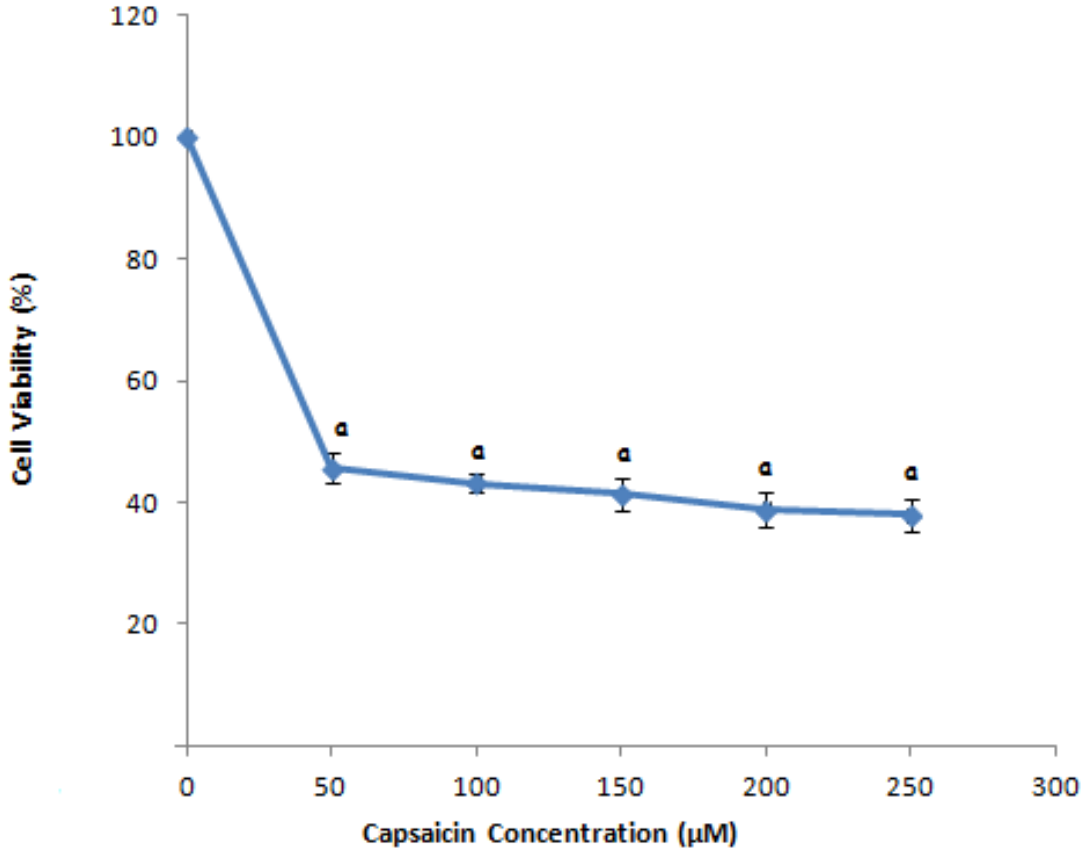

Fig. 1. The effect of capsaicin during proliferation on cell viability as assessed by the ability of cells to exclude trypan blue. Values are means ± SE, n = 3. [a]The differences were statistically significant comparing with the control group ($p<0.05$)

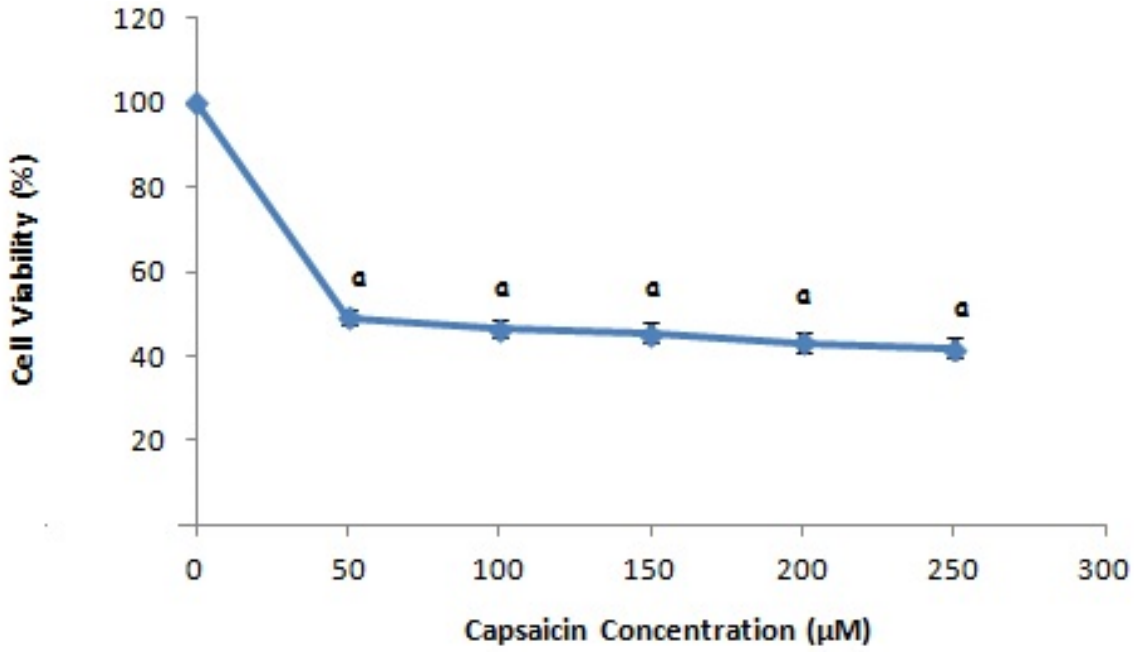

Fig. 2. The effect of capsaicin during proliferation on cell viability via MTT assay. Values are means ± SE, n = 3. [a] The differences were statistically significant comparing with the control group ($p<0.05$)

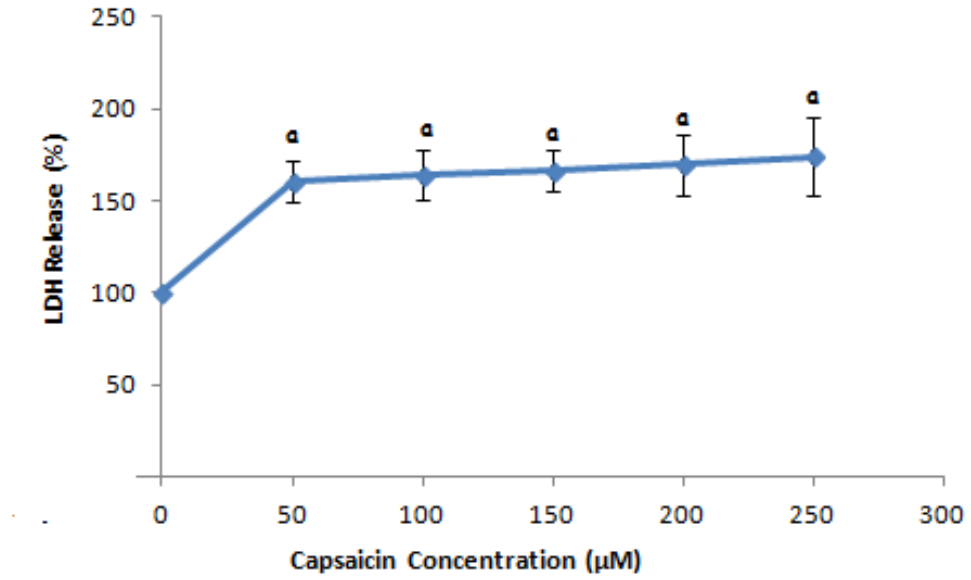

Fig. 3. The activity of LDH in the cell medium as an indicator of cell viability during proliferation. Values are means ± SE, n = 3. [a]The differences were statistically significant comparing with the control group ($p<0.05$).

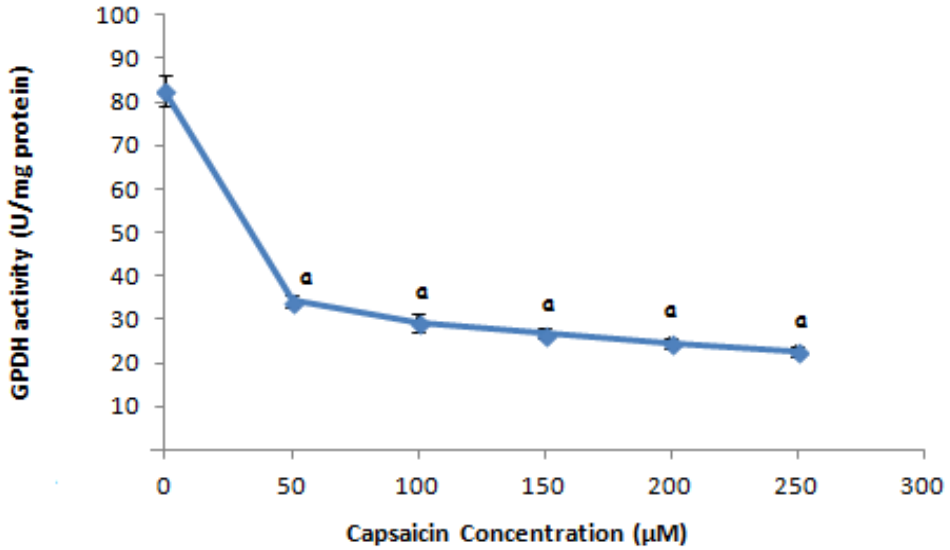

Fig. 4. The effect of capsaicin on the inhibition of GPDH activity. Values are means ± SE, n = 3. [a] The differences were statistically significant comparing with the control group ($p<0.05$).

GPDH activity was used as a marker of differentiation in 3T3-L1 cell line since GPDH activity is expressed in terminally differentiated, mature fat cells but not in preadipocytes. Capsaicin inhibited GPDH activity in 3T3-L1 adipocytes in all treated .groups ($p<0.05$). This inhibition started at 50 µM capsaicin applied group and addition of capsaicin to 3T3-L1 cells resulted in a marked decrease of GPDH activity in a dose-dependent manner. GPDH activity was 82.63 ± 3.56, 34.25 ± 1.46, 29.24 ± 1.93, 26.72 ± 1.23, 24.63 ± 1.09 and 22.63 ± 1.37 U/mg protein for the 0, 50, 100, 150, 200 and 250 µM capsaicin treatments respectively (Figure 4).

Intracellular triglyceride content was inhibited after the capsaicin treatment in all study groups. The results demonstrated that the inhibition of intracellular triglyceride in 3T3-L1 adipocytes occurred in a dose-dependent manner when cells were exposed to capsaicin ($p<0.05$). Intracellular triglyceride content was 0.65 ± 0.021, 0.18 ± 0.005, 0.16 ± 0.003, 0.15 ± 0.003, 0.13 ± 0.004 and 0.11 ± 0.005 mg/mg protein for the 0, 50, 100, 150, 200 and 250 µM capsaicin treatments respectively (Figure 5).

Oil Red O is a fat soluble dye which stains intracellular triglycerides. Histological findings of 3T3-L1 cell line demonstrated the intracellular

triglyceride content with the capsaicin treatments. Capsaicin inhibited adipocyte differentiation in 3T3-L1 adipocytes in all treatment groups (Figure 6). Oil Red O staining showed that 3T3-L1 cell count and lipid contents dramatically decreased after 50 µM capsaicin treatment as compared with control group. Furthermore, addition of capsaicin decreased 3T3-L1 cell count and lipid contents in a dose-dependent manner.

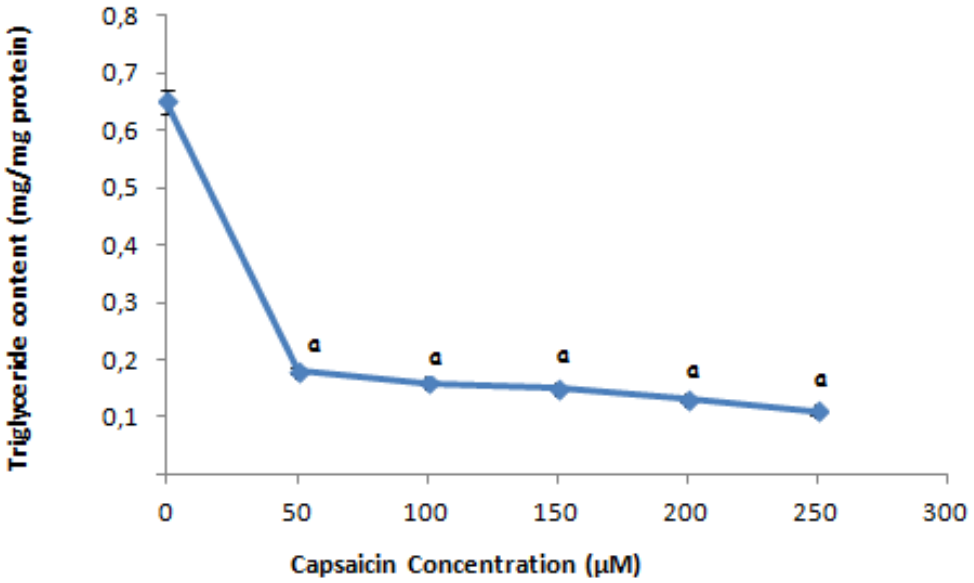

Fig. 5. The effect of capsaicin on the inhibition of intracellular triglyceride content. Values are means ± SE, n = 3.
[a] The differences were statistically significant comparing with the control group ($p<0.05$)

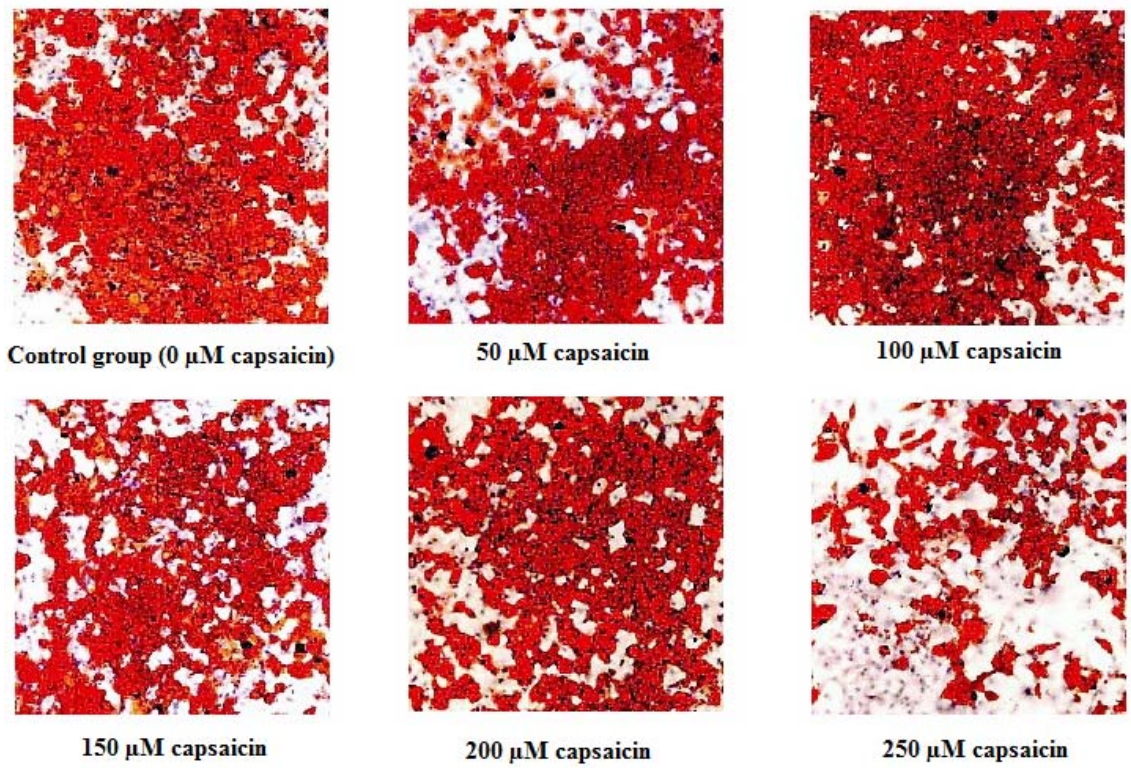

Fig. 6. Oil Red O staining showed the morphological changes of 3T3-L1 cell line with different concentrations of capsaicin. The cells were photographed at magnification x100.

Changes in expression of transcription factor genes in differentiated 3T3-L1 adipocytes treated with capsaicin were analyzed by using gene-specific primers and real-time RT-PCR. Genes analyzed include PPARγ, C/EBPα and SREBP-lc. The results were normalized to expression of β-actin and are expressed as relative mRNA level compared with the average expression in cells incubated without capsaicin (=1.00). Comparing to control group, PPARγ mRNA level was 0.52±0.04, 0.44±0.03, 0.42±0.06, 0.36±0.05 and 0.28±0.06 for the 0, 50, 100, 150, 200 and 250 µM capsaicin treatments, respectively ($p<0.05$) (Figure 7). C/EBPα mRNA level was 0.75±0.04, 0.69±0.08, 0.65±0.06, 0.63±0.11 and 0.61±0.09 comparing to control group, respectively ($p>0.05$) (Figure 8). Comparing to control group, SREBP-lc mRNA level was 0.81±0.09, 0.76±0.09, 0.73±0.08, 0.71±0.06 and 0.68±0.07 for the 0, 50, 100, 150, 200 and 250 µM capsaicin treatments, respectively ($p>0.05$) (Figure 9).

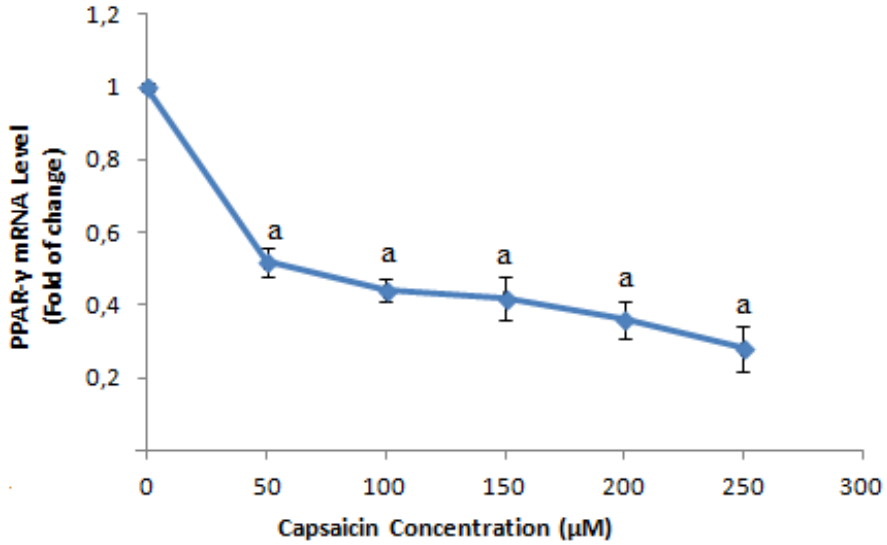

Fig. 7. The effect of capsaicin on PPARγ mRNA level. The results were normalized to expression of β-actin and are expressed as relative mRNA level compared with the average expression in cells incubated without capsaicin (=1). Values are means ± SE, n = 3. [a]The differences were statistically significant comparing with the control group ($p<0.05$)

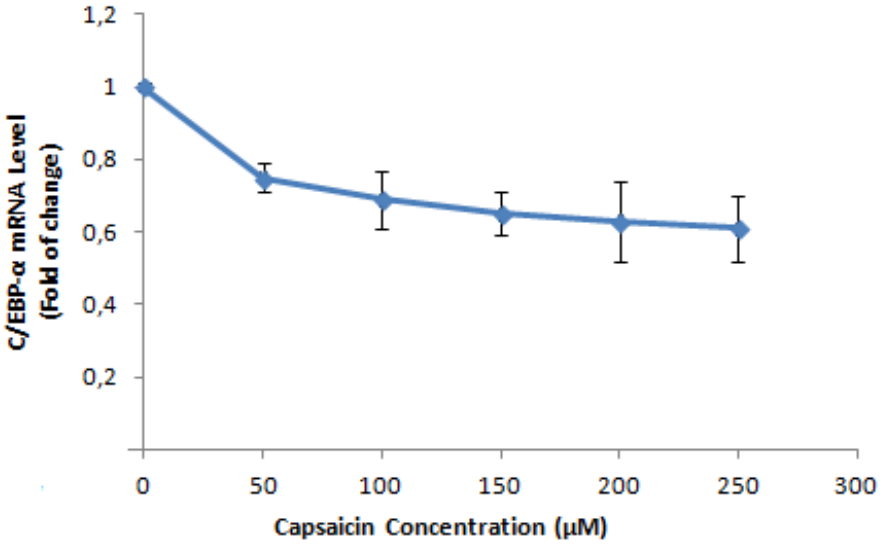

Fig. 8. The effect of capsaicin on C/EBPα mRNA level. The results were normalized to expression of β-actin and are expressed as relative mRNA level compared with the average expression in cells incubated without capsaicin (=1). Values are means ± SE, n = 3. There were no significant differences between the groups ($p>0.05$).

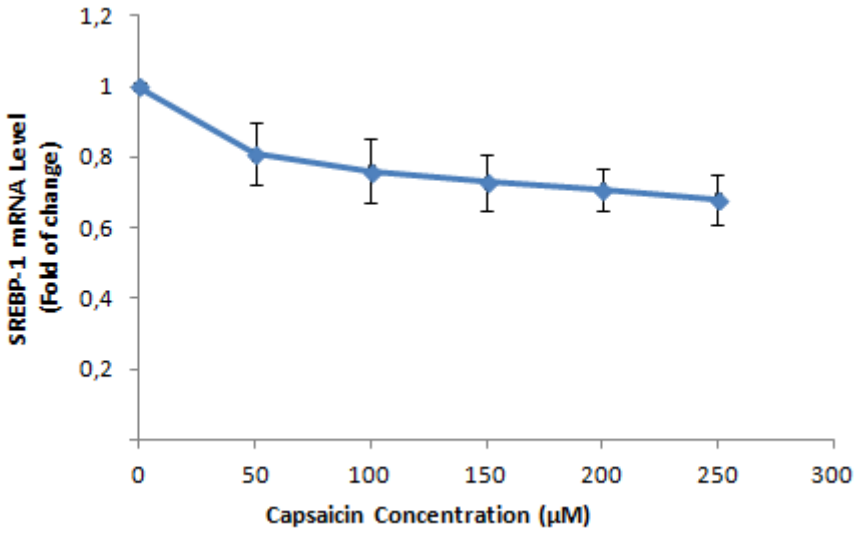

Fig. 9. The effect of capsaicin on SREBP-1c mRNA level. The results were normalized to expression of β-actin and are expressed as relative mRNA level compared with the average expression in cells incubated without capsaicin (=1). Values are means ± SE, n = 3. There were no significant differences between the groups ($p>0.05$).

4. Discussion and Conclusion

Obesity is a multifactorial disease with many contributing factors that are interconnected such as the genetic, environmental, and abnormal regulation of fat metabolism. Because obesity is an exceedingly complex group of diseases, it can even be characterized as a syndrome (1,2). Adipose tissue consists of adipocytes, which store triacylglycerol as a fuel for the body. Excess adipose tissue leads to insulin resistance, thereby increasing the risk of type 2 diabetes mellitus and cardiovascular disease. Adipocyte differentiation plays a crucial role in obesity (3,4). Wang and Jones (35) proposed that decreased preadipocyte proliferation and adipocyte lipogenesis are mechanisms of antiobesity. Adipose tissue remodeling is an ongoing physiological process that occurs throughout adult life.

3T3-L1 preadipocyte cell line is a useful model in adipocyte biology and can demonstrate most biological and biochemical reactions *in vitro*. This cell line has been well characterized in its ability to undergo complete differentiation into mature adipocytes. The process of adipogenesis involves the alternation of cell morphology, growth arrest and clonal expansion and lipid storage (5).

A variety of naturally occurring flavonoids have been found to possess beneficial effects on health, and these compounds have drawn attention because of their relative safeness and accumulated evidence of anti-obesity and antidiabetic effects in animals and humans (36,37). Capsaicin is one of the most known flavonoid that plays a critical role on adipocyte metabolism according to several laboratory studies (8). It has been reported that capsaicin has potent antiobesity effects, shown by a decrease in body weight and fat mass *in vivo* and the suppression of adipogenesis *in vitro* (38). The anti-proliferatory and lipolytic effects of capsaicin have been attributed to their ability to modulate various signaling pathways, specially, the control of cell proliferation and survival (39). However, the precise target of their anti-proliferatory effect has remained unresolved (40). Here, transcription factors can be a possible main target of capsaicin for anti-obesity activity.

The purpose of this study was to investigate the effects of capsaicin on fat metabolism in 3T3-L1 pre-adipocytes in vitro. Specifically this study examined the impacts of capsaicin treatment on lipolysis by observing its effect on lipolytic marker and adipogenesis by observing its effect on transcription factors.

In the cell viability analysis by tripan blue staining, treatment of 3T3-L1 preadipocytes with capsaicin increased the induction of cell apoptosis in a dose-dependent manner (Figure 1). Decreasing of cell viability via MTT assay and increasing of LDH release clearly indicated that capsaicin caused the inhibition of cell population growth of 3T3-L1 preadipocytes (Figure 2 and 3). Our data showed that the treatment of 3T3-L1 preadipocytes with capsaicin increased the apoptotic cell population in a dose-dependent manner. According to current study, reduction of cell viability started on 50 µM capsaicin treatment and slowed down at the increasing

concentrations. Hwang et al (6) and Hsu and Yen (9) showed that treatment with high concentrations of capsaicin from 50 to 250 μM decreased the cell population. Hsu and Yen (9) indicated that IC_{50} value of capsaicin in 3T3-L1 adipocytes was 45 μM. Han et al (41) has reported that concentrations of capsaicin of 50-500 μM decreased cell viability by 20-65% during the 72 h incubation.

GPDH is a cytosolic enzyme that has a critical role on lipid biosynthesis by providing glycerol backbone for triglyceride synthesis in adipocytes. In this regard, the GPDH activity has been used as a specific marker to quantify the degree of differentiation. Also, it may reflect the rate of lipogenesis, because in adipocytes the increase in triglyceride storage could be sustained only in the presence of sufficient supply of glycerol 3-phosphate. This implies the triglyceride reduction effect of capsaicin may be partly due to the inhibition of differentiation in the later period of time (30). Our data indicated that the exposure of 3T3-L1 adipocytes to capsaicin caused a significant decrease in GPDH activity and the content of intracellular triglycerides (Figure 4 and 5). The results support the hypothesis that capsaicin activates the hydrolysis of lipid stored in adipocytes and the excretion of glycerol from the cells, and hence reduces the lipid content of the cells. Similarly, Ahn et al (42) reported that addition of Kochujang extract (Korean fermented red pepper paste) stimulated the release of glycerol from differentiated 3T3-L1 adipocytes into the medium. There are several possible mechanisms that could account for the capsaicin induced reduction in intracellular triglyceride content in 3T3-L1 adipocytes including enhanced fatty acid oxidation, lowered levels of fatty acid uptake, increased rates of lipolysis and reduction in triglyceride synthesis or any combination of these mechanisms.

Oil Red O staining showed that 3T3-L1 cell count and lipid contents dramatically decreased after 250 μM capsaicin treatment as compared with control group. This result demonstrated that capsaicin efficiently blocks the adipocyte differentiation and adipogenesis in 3T3-L1 preadipocytes.

Although there is evidence supporting the reduced adipocytic lipogenesis is one of the mechanisms of antiobesity, better understanding of endogenous mechanisms involved has increased interest at the molecular level. At the molecular level, adipogenesis is driven by a complex transcription cascade involving the sequential activation of PPARγ, C/EBPα and SREBP-lc. C/EBPα is rapidly and transiently expressed after the addition of differentiation hormonal mixture and act synergistically to induce the expression of PPARγ, C/EBPα and SREBP-lc, the master adipogenic transcription factors (14). It is clear that PPARγ, C/EBPα and SREBP-lc play a pivotal role in terminal differentiation together by activating the transcription of genes involved in adipocyte phenotypes (24). Adipose differentiation is up-regulated by PPARγ, C/EBPα and SREBP-lc families. In order to elucidate whether capsaicin plays an inhibitory role in adipogenesis, we investigated the effect of capsaicin on the PPARγ, C/EBPα, and SREBP-lc (20).

In this study, it was revealed that exposing 3T3-L1 preadipocytes and differentiating postconfluent preadipocytes to different doses of capsaicin decreased PPARγ, C/EBPα and SREBP-1c mRNA levels as compared with their controls without treatment in dose dependent manner. This finding again confirms the active inhibitory role of capsaicin in down-regulating the PPARγ, C/EBPα and SREBP-1c in the progression of adipogenesis. In this experiment, our data showed that down-regulation of PPARγ, C/EBPα and SREBP-lc expression in capsaicin treated cells was observed similar to the reduction of triglyceride content and GPDH activity.

Feng et al (39) reported that yellow capsicum extract reduces weight gain in 3T3-L1 cell line by influencing adipocyte differentiation through suppressing. C/EBPα and SREBP-1c have similar levels of expression in the whole cell extracts from 3T3-L1 cells with capsaicin. They showed that capsaicin treatment in those doses did not exert the suppression effect dramatically. In our study, consistent with observations by others that inhibiting 3T3-L1 cell line by capsaicin was through forceful inhibition of PPARγ-induced transcriptional activity. But this inhibition was not statistically significant in C/EBPα and SREBP-lc mRNA levels after the capsaicin treatment. The reason of this condition can be the regulation of SREBP-1c activity is mediated by the proteolytic cleavage of SREBP-1c precursor and the transportation of its active form into the nucleus, while the transcriptional activity of PPARγ depends on the presence of its ligand and/or protein phosphorylation and dephosphorylation (17,21,23).

In conclusion, the results of this study clearly showed that capsaicin exerts antiproliferative activity in 3T3-L1 preadipocytes. A pronounced inhibitory effect of capsaicin on adipogenesis was observed particularly at 250 μM concentration. Capsaicin inhibits proliferation of preadipocytes

and prevents its differentiation into mature adipocytes, thus reducing adipogenesis. These data provide a novel mechanism through which capsaicin can provide anti-obesity benefits in obesity. The potential anti-obesity property of capsaicin can partially be attributed to its suppressive effects in adipogenesis through the down-regulation of three important transcription factors; PPARγ, C/EBPα and SREBP-1c. This study demonstrated that capsaicin treatment inhibited the adipogenesis through the down-regulation of transcription factors, especially PPARγ. Alternative mechanisms may involve cell cycle arrest and the induction of apoptosis. Since capsaicin is the main component found in hot pepper, the consumption of hot pepper may contribute to the maintenance of body weight and prevent the development of obesity.

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