Ginger oil-mediated down-regulation of adipocyte specific genes inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes

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ABSTRACT

Dietary ginger is known to supress body weight gain and body fat accumulation. We investigated the effects of ginger oil on adipogenesis, proliferation, apoptosis and lipolysis of 3T3-L1 adipocytes. After treatment with ginger oil, adipocytes were stained with Oil Red O to visualize lipid droplets and then measured for lipid content. Mature adipocytes were treated with ginger oil (100 and 200 µg/ml), and the expression of adipocyte-specific genes were investigated using real-time RT-PCR. Ginger oil inhibited adipogenesis and adipocyte proliferation as evidenced by dose-dependent increase in apoptosis. Interestingly, the ginger oil effectively suppressed the expression of PPARγ (peroxisome proliferator-activated gamma), C/EBPα (CCAAT/enhancer binding protein-alpha) and SREBP1c (sterol regulatory element binding protein-1c) and subsequently suppressed the expression of AKT1 (thymoma viral proto-oncogene 1) gene, which inhibits proliferation and differentiation and induces apoptosis in adipocytes. It could also prevent obesity by inducing lipolysis via upregulation of expression of LIPE (gene for hormone sensitive lipase) and TNFα (tumor necrosis factor alpha) in mature adipocyte. In conclusion, ginger oil may alter fat mass by directly inhibiting adipogenesis and inducing apoptosis in adipocytes and therefore could have applications in the treatment of obesity.

Keywords: Anti-obesity, adipogenesis, apoptosis, adipocyte-specific genes, ginger oil.

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INTRODUCTION

The incidence of obesity is rising at an alarming rate and has become a major public health concern with unexpected costs. Obesity facilitates the development of metabolic disorders such as diabetes, hypertension, and cancer. The main causes for obesity are including regulation of genes, metabolism, physical activity, diet and life style changes.

Adipogenesis is a complex process accompanied by coordinated changes in morphology, hormone sensitivity, lipid metabolism and expression of several adipocyte-specific genes like peroxisome proliferator-activated gamma (PPARγ), CCAAT/enhancer binding protein-alpha (C/EBPα), and sterol regulatory element binding protein-1c (SREBP1c) (Rayalam et al., 2008). These transcription factors in turn coordinate the expression of genes involved in the lipolytic process, mainly lipoprotein lipase (LPL) and hormone-sensitive lipase (LIPE) (Jeon et al., 2004). Indeed, many studies have shown that

down-regulation of PPARγ, C/EBPα, and SREBP1c inhibit 3T3-L1 adipogenesis (Rayalam et al., 2008; Jeon et al., 2004; Noh et al., 2013) and that these genes act as molecular targets to prevent obesity.

The number of adipocytes increases as a result of increased proliferation and differentiation; decrease in adipose tissue mass arises from the loss of lipids, either through lipolysis and/or the death of fat cells through apoptosis (Yang et al., 2006). Induction of apoptosis in adipocytes, in turn, could also reduce the number and growth of adipocytes. Previously, it was reported that apoptosis in adipocytes reduced the risk of obesity and prevent the development of obesity-associated diseases, in particular, cancer (van Kruijsdijk et al., 2009). The thymoma viral proto-oncogene 1 (AKT1) gene is an important regulator of cell proliferation and differentiation. Activation of this gene enhances production of the enzyme, AKT1 kinase, which alters many signalling pathways. AKT1 kinase helps to regulate cell proliferation and apoptosis (Wu et al., 2013) and also plays an essential role in adipocyte differentiation (Tzeng and Liu, 2013). Studies have reported that knock-down of the AKT1 pathways inhibited proliferation and induced apoptosis in mature adipocytes (Wu et al., 2013; Tzeng and Liu, 2013). Tumour necrosis factor-alpha (TNFα) is a multi-functional cytokine regulating a variety of cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism. TNFα is known to suppress many transcriptional factors, including PPARγ, C/EBPα, and glucose transporter 4 (GLUT4) through distinct molecular pathways which lead to metabolic dysregulation (Chae and Kwak, 2003; Kim et al., 2005). TNFα also directly alters the lipid metabolism in adipocytes through inhibiton of lipogenesis and stimulation of lipolysis (Cawthorn and Sethi, 2008; Park and Kim, 2011).

Ginger is the rhizome of the plant, *Zingiber officinale* Rocoe (Zingiberaceae family). In Ayurvedic medicine, ginger is used as a treatment for rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation and diabetes (Lantz et al., 2007). Studies have revealed that dietary ginger has biological effects, such as anti-inflammatory (Lantz et al., 2007), antioxidant (Ippoushi et al., 2003), antithrombotic (Thomson et al., 2002), anticancer (Surh, 2002), antihyperlipidemic (Matsuda et al., 2009), anti-obesity (Westerterp-Plantenga et al., 2006), antibacterial (Mesomo et al., 2013), and antifungal (Ficker et al., 2003) activities. The active pungent compound in ginger, 6-gingerol, also has been shown to prevent obesity by ameliorating hyperlipidemia (Kadnur and Goyal, 2005), decreasing serum cholesterol (Fuhrman et al., 2000) and triglycerides (Bhandari et al., 2005). However, there are limited studies on the biological effects of an essential oil from ginger, commonly called as ginger oil. The aim of this study was to investigate the effect of ginger oil on adipogenesis, lipolysis, proliferation and apoptosis of adipocytes and the regulation of adipocyte specific genes to ameliorate obesity and obesity-associated risk factors.

MATERIALS AND METHODS

Plant preparation and extraction of essential oil

Chinese ginger (*Zingiber officinale* Roscoe), was purchased from the local market. The fresh-ginger was chopped into small pieces and ground into paste using a kitchen blender. Essential oil was obtained from the fresh ginger paste (100 g) by hydrodistillation for 4 h in a Clevenger apparatus. The oil was dried over anhydrous sodium sulphate and stored at -20°C until used.

Cell culture

Mouse pre-adipocyte cells, 3T3-L1 (ATCC, Manssas, VA), were grown and maintained in a humidified incubator containing 5% CO_2 at 37°C. DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin-Streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA) was used as the culture medium. Cells were subcultured after reaching 80% confluence.

Differentiation of 3T3-L1 cells (Adipogenesis assay)

Differentiation of 3T3-L1 preadipocytes to mature adipocytes were carried out according to the modified method of Manaharan et al. (2013). The 3T3-L1 preadipocytes were maintained in 10% DMEM in an atmosphere of 5% CO_2 at 37°C. To induce differentiation, the postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with 10% DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, St. Louis, MO, USA) and 0.25 µM dexamethasone in six-well plates. On day 3, the medium was replaced with 10% DMEM containing 10 µM of insulin. Medium was replaced with fresh medium (10% DMEM with 10 µM of insulin) every two days until day 14.

Effect of ginger oil on 3T3-L1 adipogenesis

To evaluate the effect of ginger oil on adipogenesis, the ginger oil (20 µl) was added at various concentrations (50 to 800 µg/ml) in the differentiation medium (from day 3 until day 14). After day 14, the lipid droplets in mature adipocytes were stained and visualized through Oil Red O staining.

Oil-Red-O staining

Differentiated 3T3-L1 cells were stained using the Oil-Red-O, as described by Manaharan et al. (2013). The cells were washed once with phosphate buffer saline (PBS) and fixed with 10% formalin for 30 min. The fixed cells were washed three times with PBS. A volume of 1 ml of Oil-Red-O in isopropanol (60% Oil-Red-O) was added to each well and cells were incubated at room temperature for 1 h. The plates were rinsed three or four times with pure water and examined under an Inverted Research Microscope (Nikon , ECLIPSE TI-S) and the images were captured.

Quantification of lipid content in 3T3-L1 adipocytes

The Oil-Red-O stained adipocytes were washed with PBS and allowed to air dry. Lipid content were extracted using 100% isopropanol and the absorbance was measured using a

Table 1. List of genes used in real time RT-PCR.

Gene symbol	Gene name	Assay ID	Accession number
18S	Eukaryotic 18S rRNA	Mm03928990_g1	NR_003278.3
PPARγ	Peroxisome proliferator activated receptor gamma	Mm01184322_m1	NM_001127330.1
C/EBPα	CCAAT/enhancer binding protein (C/EBP), alpha	Mm00514283_s1	NM_007678.3
SREBP1c	Sterol regulatory element binding transcription factor 1	Mm00550338_m1	NM_011480.3
LIPE	Lipase, hormone sensitive	Mm00495359_m1	NM_001039507.2
AKT1	Thymoma viral proto-oncogene 1	Mm01331626_m1	NM_001165894.1
TNFα	Tumor necrosis factor alpha	Mm00443258_m1	NM_001278601.1

The targeted-genes selected for this study and their corresponding assay ID along with accession number are available on Applied Biosystems website (www.appliedbiosystems.com). Assay ID refers to the "Applied Biosystems Gene Expression Assays" inventoried kit with proprietary primer and Taqman® probe mix. Assay ID with "Mm" prefix denotes " Mus musculus". All gene expression assays used were probed with FAM/MGB dye. Eukaryotic 18S rRNA was used as the endogenous control.

spectrophotometer (Bio-Rad, MA, USA) at the wavelength of 520 nm.

Cell proliferation assay (MTT)

The effect of the ginger oil on proliferation of 3T3-L1 adipocytes was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT, Merck, Frankfurter, Darmstadt, Germany) assay as described by Yu et al. (2011). The 3T3-L1 cells were seeded (5×10^3 cells/well) in 96-well plate and were differentiated as described earlier. After day 14, the adipocytes were treated with ginger oil and resveratrol (positive control) at various concentration (50 to 800 µg/ml) for 24 h. The culture medium was removed and 20 µl of MTT (5 mg/ml in DMSO) were added to each well, followed by incubation for 4 h. The formation of formazan crystal was visualized under light microscope. The formazan crystals was dissolved by adding 100 µl DMSO to each well. The absorbance was measured on a Bio-rad microplate reader (Bio-Rad, Hercules, CA, USA) at the wavelength of 570 nm. The effect of ginger oil on poliferation of differentiated 3T3-L1 cell was assessed as cell viability as percentage of control, where vehicle-treated control cells (0.1% DMSO) were taken as 100% viable.

Apoptosis assay

The effect of ginger oil on apoptosis was assayed using ApoStrand™ ELISA Apoptosis Detection Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) as per the manufacturer's instructions.

Lipolysis assay

Lipolytic activity was determined by measuring glycerol released from hydrolyzed triglycerols into culture medium (Chai et al., 2011). The extent of lipolysis induced by ginger oil was assayed by using the Lipolysis assay kit for 3T3-L1 adipocytes: glycerol detection (Zen-Bio, Durham, NC, USA) as per the manufacturer's instructions.

Regulation of gene expression (RT-PCR)

Total RNA was extracted from mature 3T3-L1 adipocytes using Rneasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The integrity of the RNA extracted from the sample was verified and quantified using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA). First-strand cDNA was synthesized after the total RNA was extracted by using the High Capacity RNA to cDNA Kits (Applied Biosystems, California, USA). All the primers and probes for Taqman® real-time PCR were prepared by Applied Biosystems as depicted in Table 1. Gene expression levels were analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) using the StepOnePlus real-time PCR system. The cycle conditions were: 95.0°C for 20 min, followed by 40 cycles of 95.0°C for 1 min, and 60.0°C for 20 min. The relative expression levels of targeted genes (PPARγ, C/EBPα, SREBP1c, LIPE, AKT1 and TNFα) were evaluated using the standard curve ($\Delta\Delta C_T$) method. Standard curves for targeted genes were obtained by amplification of serially-diluted mixtures cDNA samples (4-fold dilutions), with five dilution points, each in triplicate. The mRNA expression levels of targeted genes were normalized by using 18S rRNA as an endogenous control.

Statistical analysis

All experiments were performed at least, in triplicate. Analysis at every time point from each experiment was carried out in triplicate. Means, standard errors, standard deviations, one way ANOVA and Student's paired t-test were calculated from replicates within the experiments and analyses were done using SPSS version 16. Statistical significance was accepted at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Effect of ginger oil on cell proliferation and apoptosis of 3T3-L1 adipocytes

Increased proliferation of adipocytes subsequently increases production of cytokines and inflammatory cytokines leading to adipocyte dysfunction. Obesity associated with adipocyte dysfunction is believed to play a crucial role in the development of cancer, insulin resistance, type 2 diabetes mellitus and obesity-related cardiovascular diseases (van Kruijsdijk et al., 2009). Adipocyte dysfunction results in altered levels of adipocytokine, which directly affects cell proliferation, apoptosis, invasive growth, and angiogenesis (Gonzalez-
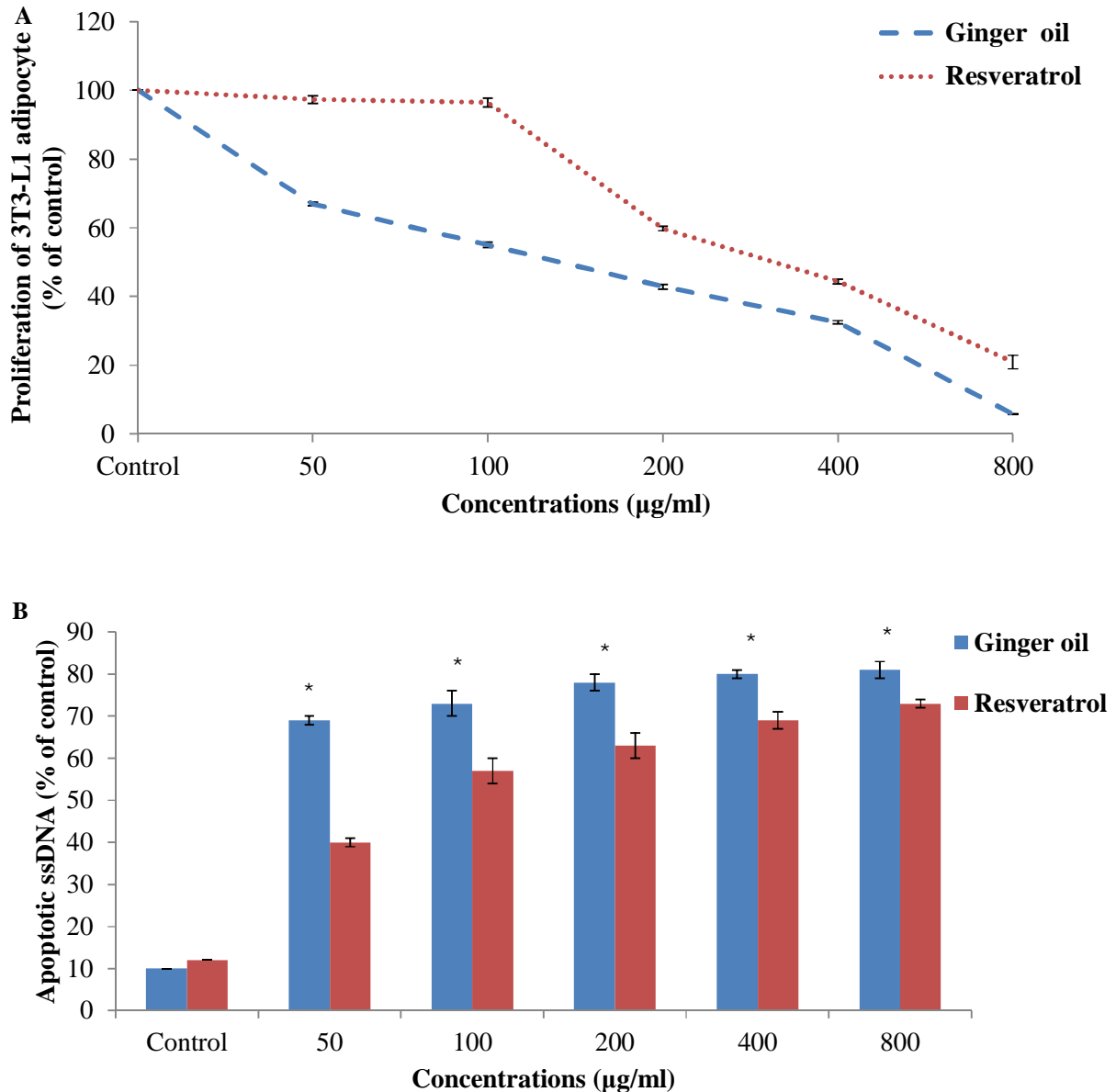
Figure 1. (A) The effect of ginger oil on proliferation of 3T3-L1 adipocytes. Cell proliferation after 24 h of treatment with ginger oil at indicated concentrations (50-800 µg/ml) was determined by the MTT colorimetric assay. Data are presented as means ± SD, n=3. Student's paired *t*-test shows significant value,*p < 0.05 vs. resveratrol. (B) The effect of ginger oil on induction of apoptosis in 3T3-L1 adipocytes. Apoptosis was quantified by detecting the ssDNA at the wavelength of 405 nm using an ELISA plate reader. Data are expressed as means ± SD, n=3. Student's paired *t*-test shows significant value, *p < 0.05 vs. control.

Castejon and Rodriguez-Casado, 2011). Recent interest has focused on decreasing proliferation and increasing apoptosis of adipocytes, which in turn can control adipocyte dysfunction and obesity-related carcinogenesis (Rayalam et al., 2007). Therefore, in this study we evaluated the anti-proliferative and pro-apoptotic effects of ginger oil in mature 3T3-L1 adipocytes.

As shown in Figure 1A, the ginger oil effectively inhibited proliferation of 3T3-L1 adipocyte dose-dependently with an IC$_{50}$ value of 120 ± 1.8 µg/ml. The

ginger oil significantly (*p < 0.05) inhibited proliferation of mature adipocytes far better than resveratrol in all the concentrations tested (Figure 1A). Resveratrol, a naturally occuring phytochemical found in red wine and grape juice, has been shown to inhibit proliferation of 3T3-L1 pre-adipocytes (Hsu and Yen, 2006) and mature adipocytes, as well as to induce apoptosis and cell cycle arrest in various cell lines (Rayalam et al., 2007). Ginger oil, at the lowest concentration tested (50 µg/ml), inhibited 3T3-L1 adipocyte proliferation by 34 ± 1.2%, whereas

lower concentrations of resveratrol showed no significant effect on inhibition of cell proliferation.

To determine whether the inhibition of cell proliferation was due to its apoptotic mechanism, the extent of cellular apoptosis was quantified by the ApoStrand ELISA apoptosis detection kit. ApoStrand ELISA only detects single stranded DNA (ssDNA), which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis (Frankfurt and Krishan, 2001). As shown in Figure 1B, the ginger oil induced apoptosis dose-dependently as compared to the control. The results showed that the ginger oil at 50, 100, 200, 400 and 800 µg/ml significantly (*p < 0.05) induced apoptosis by 69 ± 2.5%, 73 ± 1.7%, 78 ± 1.2%, 80 ± 2.0%, and 81 ± 1.4%, respectively (Figure 1B). Ginger oil exhibited far higher induction of apoptosis, from a concentration as low as 50 µg/ml, than the positive control, resveratrol (Figure 1B). These results are in agreement with the earlier ones, suggesting that decreased proliferation of 3T3-L1 adipocytes are due to the induction of apoptosis. However, further studies are needed to investigate the molecular mechanism leading to apoptosis by ginger oil in matured 3T3-L1 adipocytes.

Effect of ginger oil on 3T3-L1 adipogenesis

Adipogenesis involves differentiation of pre-adipocytes into mature adipocytes. This is accompanied by an increase in the expression of adipocyte-specific genes including adipocyte fatty acid binding protein and lipid-metabolising enzymes. Activation of PPARγ (peroxisome proliferator activated receptor gamma) by its ligands is a key process for adipocyte differentiation. Adipogenesis and fat accumulation are associated with the occurance and development of obesity.

We investigated the effect of ginger oil on the differentiation of 3T3-L1 pre-adipocytes. Through Oil Red O staining, a dose-dependent decrease in the lipid droplets was observed (Figure 2B) in the 3T3-L1 cells treated with ginger oil compared to the control (0.1% DMSO) (Figure 2A). Ginger oil was found to greatly decrease lipid droplets in mature adipocytes from concentrations as low as 50 µg/ml (Figure 2B) and was far better than 300 µg/ml of resveratrol (Figure 2A). Reveratrol is well established as an inhibitor of adipogenesis and it suppresses the expression of PPARγ, C/EBPα, and SREBP1c (Rayalam et al., 2008). Recently, Tzeng and Liu (2013) reported that down-regulation of PPARγ, C/EBPα (CCAAT/enhancer binding protein-alpha) and AKT1 kinase (serine-threonine protein kinase 1) in adipocytes contribute to the inhibition of adipogenesis. These molecules have also been known to be involved in the cell cycle, cell proliferation and differentiation, migration and apoptosis. It can be postulated that ginger oil has antiadipogenic property and could down-regulate the expression of PPARγ, C/EBP,

SREBP1c and AKT1. However, this can only be further confirmed via gene expression studies on adipocytes.

We also quantified the lipid content in mature 3T3-L1 adipocytes treated with ginger oil at various concentrations (50 to 800 µg/ml). The lipid accumulated in the mature adipocytes were extracted using 100% isopropanol (Manaharan et al., 2013). As shown in Figure 3, ginger oil significantly (**p < 0.001) decreased lipid content in mature adipocytes in a dose-dependent manner as compared to the control. An approximately 85% decrease in the lipid content of mature adipocytes was observed compared to the control at the highest concentration of ginger oil (800 µg/ml, Figure 3). These results correspond to the Oil Red O staining observation (Figure 2B). Decrease in the lipid content is not only due to the decreased adipogenesis but also due to the effect of ginger oil on cell proliferation, indicating that ginger oil could be a good inhibitor of adipogenesis and a far better one than the natural adipogenesis inhibitor, resveratrol.

Effect of ginger oil on 3T3-L1 lipolysis

In addition to antiadipogenic effects, we also investigated the effects of ginger oil on lipolysis. Lipolysis is an important metabolic pathways that regulates adipose tissue, weight and obesity. Lipolysis plays a central role in the regulation of energy balance by hydrolizing triglycerides (TG) into glycerol and free fatty acids (FFA). This process releases FFA into the circulation where they may be either re-esterified in the adipocyte or be transported to other tissues and exert effects throughout the body. Elevated adipocyte lipolysis has been observed in obese diabetic individuals. Hormone sensitive lipase (gene, LIPEor HSL) is the enzyme responsible for regulating lipolysis in mature adipocytes by catalyzing the hydrolysis of TG, resulting in the release of glycerol and FFA [31]. Activation of adenylate cyclase (AC) in the lipolytic pathway, increases cyclic AMP (cAMP) levels which in turn activate LIPE to regulate lipolysis in mature adipocytes (Robidoux et al., 2004).

Our results show that treatment with the ginger oil for 24 h significantly (**p < 0.001) stimulates lipolysis as evidence by increased glycerol content in the culture medium (Figure 4A). A dose-dependent induction of lipolysis was observed as compared to the control (Figure 4A). A previous study reported that insulin inhibits lipolysis through AMP kinase-dependent enhancement of perilipin expression as well as the prevention of HSL phosphorylation (Brasaemle, 2007). Similar findings were obtained in our study where the insulin showed anti-lipolytic activity compared to the control and the ginger oil (Figure 4A). As shown in our result, the lipid droplets inside the mature 3T3-L1 adipocytes were surrounded by perilipin coating, and the coating was obviously thickened by the insulin treatment (Figure 4C) during differentiation. Perilipin, one of the major lipid droplet-coating proteins, is
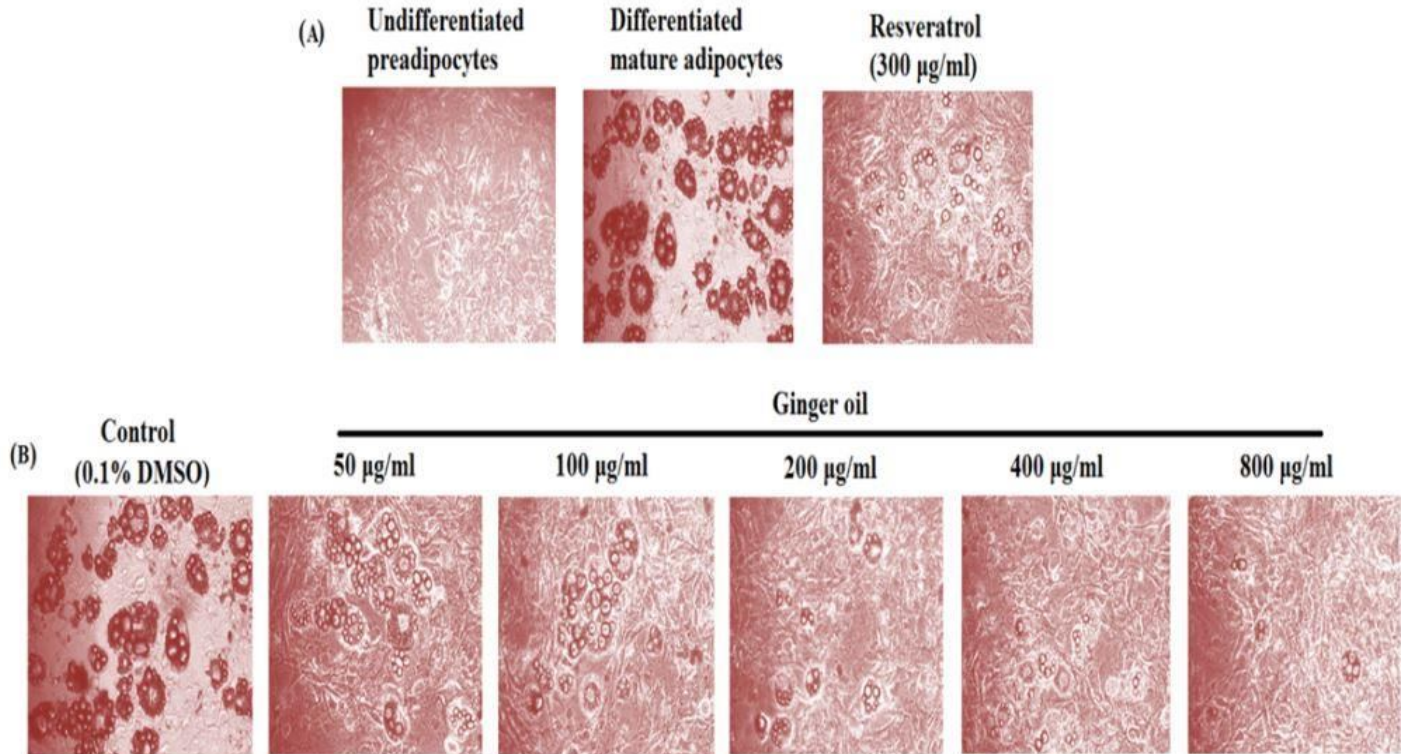
Figure 2. Oil Red O staining of mature 3T3-L1 adipocytes after day 14. (A) Images of undifferentiated pre-adipocytes (day 0), differentiated mature adipocytes (after day 14), and resveratrol (300 μg/ml)- treated mature adipocytes. (B) The effect of ginger oil (50 to 800 μg/ml) on differentiation and lipid accumulation in 3T3-L1 adipocytes. Cultures treated with vehicle (0.1% DMSO) served as control. Images captured using Inverted fluorescence microscope (Nikon, ECLIPSE TI-S) at 100X magnification.
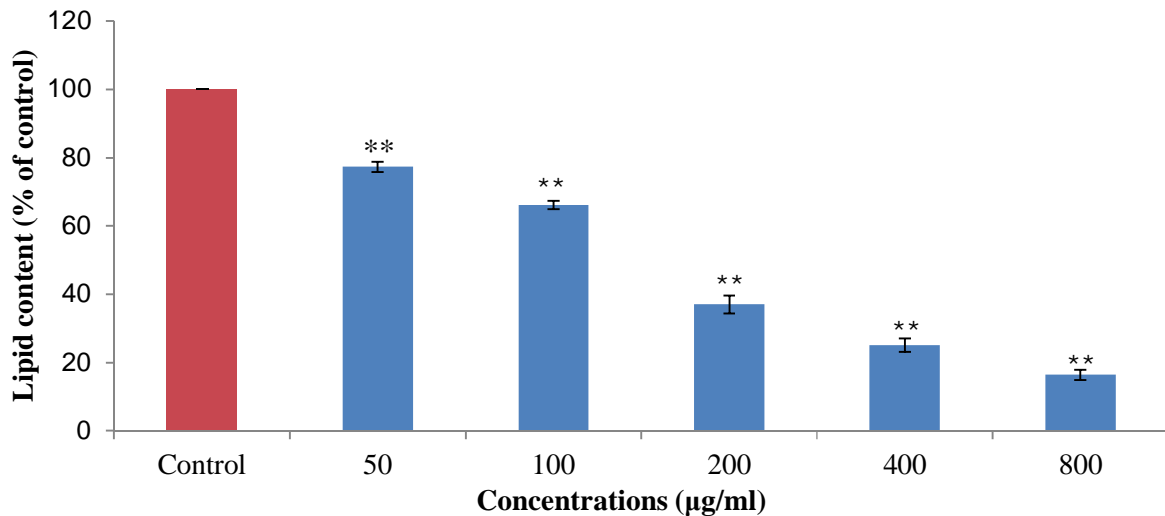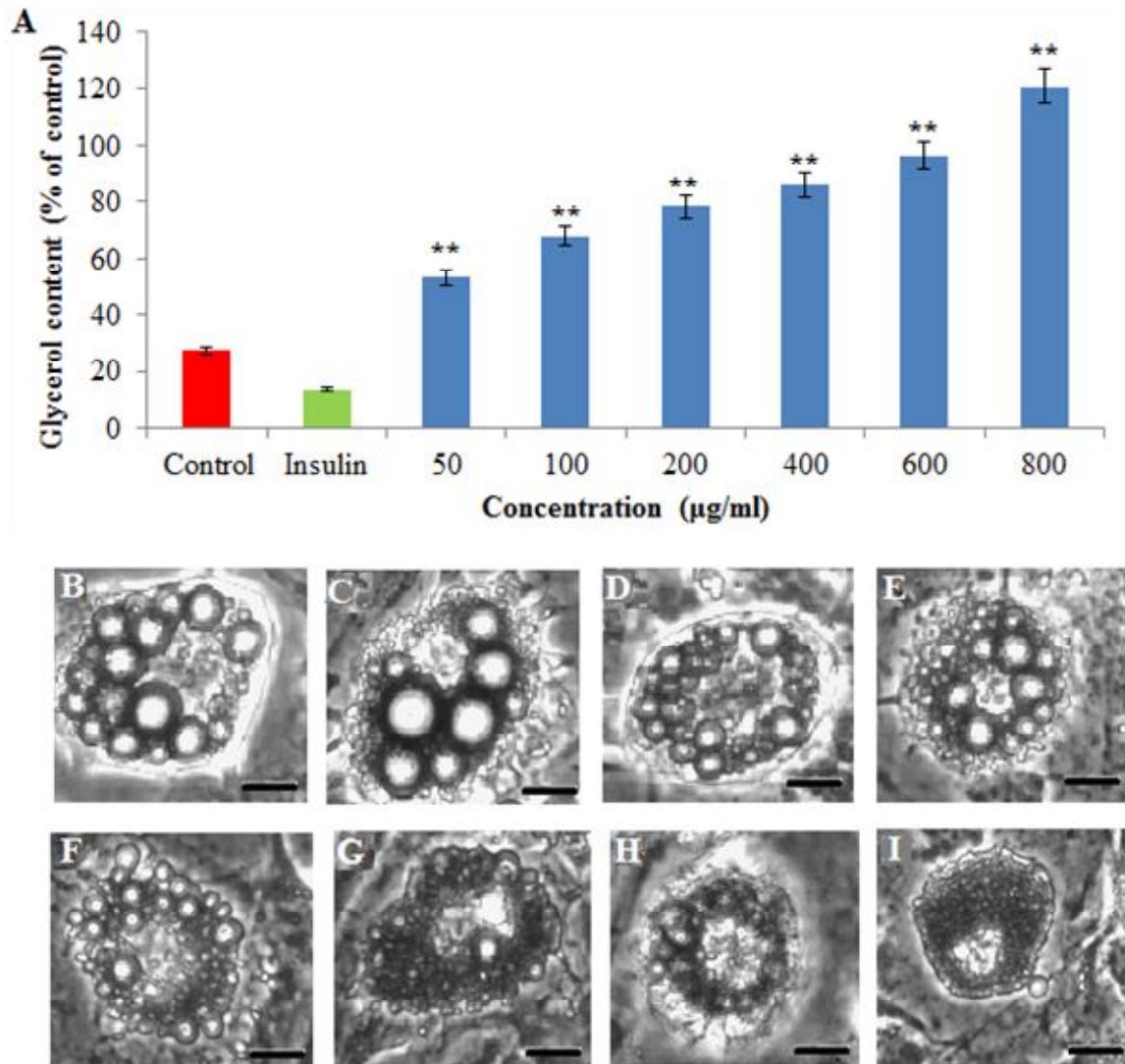

Figure 3. Effect of ginger oil (50 to 800 μg/ml) on lipid content of mature 3T3-L1 adipocytes. Lipid content in adipocytes (fat droplets) was extracted after staining with Oil-Red-O using 100% isopropanol and the absorbance was measured at 520 nm. Data are expressed as means ± SD, n=3. One-way Anova shows significant values, **$p < 0.001$ vs. control.

known to stabilize lipid droplets and retard lipolysis in adipocytes (Brasaemle, 2007). Thus, it is likely that

insulin suppresses lipolysis by increasing the expression of perilipin. Scientific studies have shown that

Figure 4. (A) Effect of ginger oil (50 to 800 µg/ml) on glycerol content of mature 3T3-L1 adipocytes. Glycerol content in culture medium after 24 h treatment with ginger oil was quantified using the Lipolysis assay kit and the absorbance was measured at 540 nm. Data are presented as means ± SD, n = 3. One-way Anova showed significant values, **p< 0.001 vs. control. Representative optical images of matured 3T3-L1 adipocytes with scale bars = 10 µm. (B) Control adipocytes filled with large lipid droplets, (C) Insulin (10 µM) treated adipocytes with dark, thick perilipin coated lipid droplets, (D-I) Fragmentation of lipid droplets and reduction of adipocyte sizes after treatment with ginger oil; 50, 100, 200, 400, 600 and 800 µg/ml, respectively.

fragmentation of lipid droplets could also enhance lipolysis by increasing the surface area of lipid droplets for LIPE binding activity (Brasaemle et al., 2009; Than et al., 2012). Lipolytic agents, such as epinephrine, the ß-adrenergic agonist, stimulate lipid droplet fragmentation by activating the cAMP/PKA pathway, which in turn, leads to phosphorylation of perilipin and HSL (Than et al., 2012). As shown in Figure 4D-4I, the ginger oil indeed caused a dose-dependent fragmentation of lipid droplets, reduced the thickness of their perilipin coating and also reduced the sizes of matured adipocytes compared to the control (Figure 4B). The exact molecular mechanism regulating lipolysis in adipocytes remain unclear. Thus,

further studies were carried out to investigate the regulation of lipolytic genes in mature adipocytes after treatment with ginger oil. We found that the ginger oil, at concentrations of 100 and 200 µg/ml, significantly (**p < 0.001) up-regulated the expression of LIPE (Figure 5) in the matured adipocytes. It clearly shows that increased LIPE expression increased the phosphorylation of perilipin and stimulated fragmentation of lipid droplets and led to lipolysis (Figure 4). Many studies reported that LIPE overexpression is an important factor in the prevention of obesity by modulating lipolysis and controlling the catabolism of cellular fat stores in adipocytes (Lass et al., 2011).

Figure 5. Effect of ginger oil on expression of PPARγ, C/EBPα, SREBP1c, LIPE, AKT1 and TNFα in mature 3T3-L1 adipocytes. Eukaryotic 18S rRNA was used as the endogenous control. Data are expressed as means ± SD, n=3. One-way ANOVA show significant values, *p<0.05, **p< 0.001 vs. endogenous control.

A recent study by Lasa et al. (2012), reported that resveratrol enhances lipolysis in adipocytes through the adenosine monophosphate activated protein kinase (AMPK) signalling pathway. Studies with adipocytes have suggested that activation of AMPK inhibits HSL phosphorylation (Watt et al., 2004) but activates the adipose triglyceride lipase (AGTL) activity in the cytosol and also on the lipid droplets (Yin et al., 2003). AGTL activation is stimulated by an activator protein called CGI-58 which depends on phosphorylation of perilipin and leads to lipolysis in adipocytes (Lasa et al., 2012). Our results revealed that LIPE expression was down-regulated following treatment with resveratrol (Figure 5). It clearly shows that resveratrol inhibits HSL phosphorylation. Further studies are required to confirm the ability of resveratrol to regulate AGTL expression in adipocytes.

We also observed that ginger, oil at 100 and 200 µg/ml, significantly (**p < 0.001) up-regulated the expression of TNFα far better than resveratrol (Figure 5). TNFα stimulates lipolysis via a glucose-dependent mechanism that likely involves transcription factors, mainly JNK, ERK1/2 and NFκB, resulting in up-regulation of cAMP and down-regulation of perilipins [40]. In addition to its anti-lypolytic action, TNFα is also known to inhibit adipogenesis by preventing the induction of PPARγ and C/EBPα expression. Our results showed that ginger oil inhibits adipocyte differentiation (Figure 2B) and decreases the lipid content (Figure 3) and this could be due to the expression of TNFα.

Overall, the ginger oil stimulates lipolysis in matured adipocytes by up-regulating the expression of LIPE and TNFα. Thus, the ginger oil may serve as a novel therapeutic target for prevention of obesity and obesity-associated adipocyte dysregulation.

Effect of ginger oil on the expression of adipocyte-specific genes in mature 3T3-L1 adipocytes

Identification of a potential molecular target and its possible pathway may promote prevention of obesity and obesity-related metabolic diseases. In this study, we evaluated the regulation of adipogenic-specific genes in mature 3T3-L1 adipocytes for better understanding of its molecular mechanism. The expression level of genes was quantified using real time RT-PCR system. The list of genes investigated and the probes and primers used are shown in Table 1. Our results showed that ginger oil, at concentrations of 100 and 200 µg/ml, significantly (*p < 0.05, **p < 0.001) reduced the expression of PPARγ, C/EBPα, SREBP1c and AKT1 as compared to the control (Figure 5). Resveratrol is known as an PPARγ, C/EBPα and SREBP1c inhibitor (Rayalam et al., 2008) and was used as a positive control in this experiment. Interestingly, ginger oil at a concentration of 200 µg/ml was found to be supressing the expression of PPARγ, C/EBPα, SREBP1c, AKT1 far better than resveratrol (Figure 5).

The members of the C/EBP family of transcription factors, C/EBPα and PPARγ, play crutial roles during adipogenesis and activate the expression of genes associated with adipocyte maturation. Our results suggest that ginger oil inhibited 3T3-L1 adipogenesis by

down-regulating adipogenic transcription factors, in particular PPARγ and C/EBPα. Several studies have demonstrated that PPARγ and C/EBPα co-regulate each other's expression (Noh et al., 2013). PPARγ is the most important gene during adipogenesis, while, C/EBPα might play more of an accessory role for PPARγ by inducing and maintaining PPARγ expression (Noh et al., 2013).

As expected from previous findings, the ginger oil showed a higher fold change in PPARγ expression than C/EBPα at concentrations of 100 and 200 μg/ml (Figure 5). The ginger oil-mediated down-regulation of C/EBPα is attributed to the higher fold change in PPARγ expression. Activation of PPARγ induces the expression of genes controlling adipocyte fatty acid metabolism, including SREBP1c (Rayalam et al., 2008). In this study, the inactivation of PPARγ by ginger oil, in turn, suppress SREBP1c expression in 3T3-L1 adipocytes. A recent study showed that inhibition of adipogenesis is associated with the suppression of genes in C/EBPα and SREBP1c dependent pathways (Lee et al., 2013). The SREBP1c molecule regulates lipogenic genes that encode enzymes involved in triglyceride synthesis and desaturation of fatty acids with a subsequent series of activations promoting the expression of target genes in a SREBP1c dependent pathway. The inhibition of SREBP1c gene expression in adipocytes has been accompanied by a decrease in gene expression of fatty acid synthase (FAS) and other target genes in the SREBP1c dependent pathway (Lee et al., 2013). Down-regulation of SREBP1c by ginger oil might reduce fatty acid synthesis and may result in the inhibition of lipid accumulation in adipocytes.

In addition, phosphorylation of AKT1 promotes the differentiation of pre-adipocyte 3T3-L1 cells into mature adipocytes and enhance adipocyte proliferation. Lack of AKT1 phosphorylation was found to inhibit differentiation of 3T3-L1 cells and induced apoptosis in mature adipocytes (Tzeng and Liu, 2013). Ginger oil exhibited a dose-dependent decrease in AKT1 expression as compared to the control (Figure 5). Interestingly, ginger oil at a concentration of 100 μg/ml was able to be inhibits AKT1 expression far better than resveratrol at 300 μg/ml (Figure 5). Resveratrol is well studied and known to induce apoptosis in mature adipocytes via supressing adipocyte-specific genes (Rayalam et al., 2008). Our results show that ginger oil is a more potent inhibitor of AKT1 expression and inducer of apoptosis compared to resveratrol. Knockdown of AKT1 pathway might be a potential therapeutic target to prevent obesity by modulating proliferation and differentiation of adipocytes. We postulate that ginger oil may serve as a potential therapeutic agent against obesity as shown by it inhibiting adipogenesis and inducing apoptosis in 3T3-L1 adipocytes by down-regulating the expression of adipocyte-specific genes in particular, PPARγ, C/EBPα, SREBP1c and AKT1.

CONCLUSION

In conclusion, ginger oil was able to inhibit adipogenesis, retard proliferation, induce apoptosis and stimulate lipolysis in mature 3T3-L1 adipocytes. Ginger oil was also clearly far better than resveratrol in inducing apoptosis and inhibiting 3T3-L1 cell proliferation at all the concentrations tested. To the best of our knowledge, this is the first time that ginger oil has been reported to have anti-adipogenic, anti-proliferative, pro-apoptotic and as well lipolytic effects on 3T3-L1 adipocytes. The pungent active compounds in ginger oil might contribute to these properties. Ginger oil was found to also down regulate the expression of adipocyte specific genes, PPARγ C/EBPα, and SREBP1c. In addition to that, ginger oil also inhibited the expression of the AKT1 gene, which has been shown to play crucial role in regulating proliferation, differentiation and controlling apoptosis of adipocytes. Suppression of these molecular targets could contribute to the anti-adipogenic, anti-proliferative and pro-apoptotic properties of the ginger oil. Apart from that, up-regulation of LIPE and TNFα genes by ginger oil plays a key role in lipolysis of adipocytes. Therefore, these findings provide a more detailed evaluation of the anti-obesity potential of ginger oil and could be useful in the development of new therapeutic strategies for obesity and obesity-related diseases.

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Conflict of interest

The authors have declared no conflict of interest.

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