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Effect of Garlic Oil on Fatty Acid Accumulation and Glycerol-3-Phosphate Dehydrogenase Activity in Differentiating Adipocytes

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ABSTRACT : Garlic oil (GAR, Allium sativum L.) has been studied as a feed additive to improve animal production performance and decrease methane emission in ruminants. The present study was designed to determine the possible effect of GAR on fatty acid composition and accumulation in animal fat tissue using a cell model. 3T3-L1 preadipocytes at 2×10^4 mL^{-1} were seeded to 24-well plates and allowed to proliferate to reach confluence. The cells were then treated with media containing 0, 2.5, 5, 10, 20 and 40 μg mL^{-1} of GAR during the differentiation period for 8 days. Media containing dexamethasone, methyl-isobutylxanthine and insulin was applied during the first 2 days of the early differentiation period. On day 8 sub-sets of the wells were stained with oil red-O and the remaining cells were harvested for determination of glycerol-3-phosphate dehydrogenase [EC 1.1.1.8] (GPDH) activity (n = 6) and cellular fatty acid concentration (n = 6). It was found that supplementation of GAR increased ($p<0.05$) the ratio of monounsaturated fatty acids/saturated fatty acids in the adipocytes and showed inhibitory effect ($p<0.05$) on the post-confluent proliferation. With relative low dosage, GAR (5-20 μg mL^{-1}) increased ($p<0.05$) the GPDH activity without affecting the cellular fatty acid concentration, while a high dosage (40 μg mL^{-1}) inhibited ($p<0.05$) fatty acid accumulation and decreased GPDH activity. Supplementation of GAR had an effect on cell post-confluent proliferation, differentiation and fatty acid accumulation. However, the effect may be diverse and depends on the dose applied. (**Key Words :** 3T3-L1 Adipocytes, Fatty Acids, Garlic Oil, Glycerol-3-phosphate Dehydrogenase)

INTRODUCTION

Garlic oil or garlic (GAR, *Allium sativum* L.) has been tested in animal model and human for their functional effects such as prevention of cardiovascular disease, antioxidation and antidiabetic effect (Ackemann et al., 2001; Brace, 2002; Thomson and Ali, 2003; Liu et al., 2005, 2006; Lim et al., 2006; Sarkar et al., 2006; Yalçın et al., 2007). It also has been tested as natural feed additive for dairy cow and beef cattle to improve performance of production and was found that GAR supplementation could increase dietary energy efficiency and decrease methane emission (Busquet et al., 2005; Chaves et al., 2008; Yang et al., 2008). However, little information was available on effect of garlic oil on development of animal fat tissue and its possible functionality on cell metabolism of adipocytes.

Fatty acid composition and accumulation, especially those of the unsaturated fatty acids in animal products such as meat and milk, are very important factors on product quality. 3T3-L1 Cell lines have been used as model of adipocyte to study fatty acid accumulation, differentiation of preadipocytes and glucose utilization (Green and Kehinde, 1974). With additional stimulators such as a mixture of dexamethasone, methyl-isobutylxanthine and insulin (DMI) the cells are able to start differentiation from preadipocytes to adipocytes (Ntambi and Kim, 2000). As result of lipogenesis and differentiation in the early differentiating adipocytes, the accumulated fatty acids in the cells can be measured and used for comparison, which can be the combined result of both lipogenesis and lipolysis in the mature adipocytes. Glycerol-3-phosphate dehydrogenase [EC 1.1.1.8] (GPDH) is a key enzyme for triglyceride biosynthesis (Wise and Green, 1979) and has been used as differentiation marker that reflects the fatty acids synthesis rate and the incorporation of glucose to the cellular lipids in adipocytes.

With hypothesis that supplementation of GAR may affect fatty acid accumulation and composition of adipocytes and differentiation of preadipocyte to adipocytes,

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the present study was conducted to determine the possible effects of GAR in 3T3-L1 cells during the overall differentiation period. Concentration of cellular individual fatty acid, the ratio of monounsaturated fatty acids (MUFA)/saturated fatty acids (SFA), GPDH activity and profiles of cells stained with or without oil red-O were determined and compared among the cells treated with or without GAR.

MATERIALS AND METHODS

Experimental treatments and cell culture

3T3-L1 cell line was obtained from ATCC™ (VA, USA). The cells with passages of 5 were used in this study. The cells were seeded to 24-well plates at a density of 2×10^4 cells mL^{-1} in Dulbecco's modified Eagle's medium (DMEM, Sigma, MO, USA) containing 10% fetal bovine serum (FBS, Sigma, MO, USA) in a humidified 5% CO2 atmosphere at 37°C for proliferation until reach confluence (He et al., 2006a). The confluent cells were treated with media containing 0, 2.5, 5, 10, 20, or 40 μg mL^{-1} of GAR during the overall differentiation period (Day 1-8). GAR (Allium sativum, contains 26% Allyl mercaptan, 18% Allyl trisulphide and 1.5% allicin) was obtained from AXISS France SAS (Bellegarde-sur-Valserine Cedex, France). The pure ethanol was used as carrier for the GAR and added to media in 0.1%. The media during the early differentiation period (Day 1-2) were supplemented with 0.1 μmol L^{-1} dexamethasone (Sigma, MO, USA), 1 mmol L^{-1} methyl-isobutylxanthine (Sigma, MO, USA) and 0.1 μmol L^{-1} insulin (Bovine insulin, Sigma, MO, USA) for starting the differentiation of the preadipocytes. The cell cultures were photographed by using a microscope (Olympus CKX41, Olympus, Japan) with a digital camera (Moticam 2300, Motic China Group Co., Ltd., China). On Day 8 at the end of experiment the cells were harvested for determination of GPDH activity, cellular fatty acids concentration, and remained four sub sets of the wells for Oil Red-O staining.

Harvesting cells

At the end of culture experiment, the cells in total 12 sub sets of wells were harvested for further extractions and analysis of GPDH activity (n = 6) and cellular fatty acids (n = 6). The remains were stained with Oil-Red O and Mayer's hematoxylin (He et al., 2009). To harvest the cells for counting the number and analysis of cellular fatty acids, the cells were washed with warm phosphate buffered saline (PBS, pH 7.08, calcium and magnesium free) and dissociated by incubation in PBS solution containing 0.25% trypsin and 100 mg L^{-1} EDTA in 37°C for 5 min. The cells were counted using a haemocytometer. For analysis of GPDH activity, the cells were washed with cold PBS. Then 0.5 ml Tris-EDTA (pH 7.5) was added to each well. The cells were collected mechanically and crushed under an ultrasonic reactor. After centrifugation at 12,800×g at 4°C for 5 min the supernatant was taken for analysis of GPDH activity. All the harvested cells and extracts were stored under -80°C for further analysis.

GPDH [1.1.1.8] activity and protein analysis

The GPDH activity was measured by method of Wise and Green (1979) with modifications (He et al., 2009). The measurement was done in 98-well micro plate. The following aliquots were added to the well in sequences: 50 μL solution (pH 7.5) that was formed by 0.5 mol L^{-1} triethanolamine, 10 mmol L^{-1} EDTA and 10 mmol L^{-1} 2-mercaptoethanol (TEA solution); 100 μL 5 mmol L^{-1} dihydroxyacetone phosphate (DHAP); 200 μL 0.5 mmol L^{-1} β-nicotinamide adenine dinucleotide with reduced form (NADH) and 50 μL of extract. The disappearance of NADH at 25°C was measured by a spectrophotometric method at 340 nm with microplate spectrophotometer. The activity of GPDH was expressed as units per min = [NADH (100 nmole)×change of OD340]/[1.25×time (min)×protein (mg)]. Protein content was measured with Total Protein Kit (Micro Lowry, Onishi & Barr Modification, Sigma-Aldrich Inc., MO, USA) based on method of Lowry et al. (1951).

Extraction of cellular lipids

The cellular lipids were extracted by using a procedure reported previously (He et al., 2006b). The harvested cells that had been stored under -80°C freezer were then transferred and washed into a test tube with 4 mL of isopropanol. Then 4 mL of hexane was added and crushed under ultrasonic reactor. After crushing the cells, 4 mL water was added to the mixture and the tubes were centrifuged at 2,000×g at 5°C for 5 min. Then the upper layer was transferred to a new tube. After the hexane was evaporated under nitrogen in a 38°C water bath, the residue was stored at -80°C for further analysis.

Methylation and determination of fatty acids

A combined base/acid methylation method (Kramer et al., 1997) with modifications was used (He et al., 2009). Nonadecanoic acid (C19:0) methyl ester (100 μL, 5.96 mg mL^{-1} hexane Nu-Chek Prep, Inc., MN, USA) was used as an internal standard added to the tubes containing lipids. Then 2 mL of sodium methoxide (0.5 mmol L^{-1} in methanol) was added to each tube and flushed with nitrogen and mixed completely. They were placed in a 50°C water bath for 10 min. After that 1 mL boron triflouride (14% in methanol) was added and reheated in a 50°C water bath for another 10 min. After cooling down 5 mL water and 2 mL hexane were

added to the tube and mixed completely. They were allowed to stand for 10 min and the upper layer (hexane) was taken into a GC vial flushed with nitrogen for fatty acid determination by gas chromatography. Fatty acid methyl esters were quantified by a gas chromatograph (Hewlett Packard GC System 5890; Mississauga, ON) equipped with a flame ionization detector and SP-2560 fused silica capillary column (100 m with 0.2 mm film thickness; Supelco Inc., Oakville, ON). Samples were loaded on to the column via 5 μL splitless injections. The initial oven temperature (120°C) was held for 15 min and then increased at 5°C min^{-1} to 160°C, and held for 15 min. Next the temperature was increased at 4°C min^{-1} to 240°C and held for 30 min. Inlet and detector temperatures were maintained at 220°C, and 275°C, respectively. Helium carrier gas flow rate through the column was 1.7 mL min^{-1}. Hydrogen flow to the detector was 34 mL min^{-1}, air flow was 320 mL min^{-1} and helium make-up gas flow rate was 29 mL min^{-1}. Peaks in the chromatograms were identified and quantified using pure methyl ester standards (Sigma-Aldrich Inc., Oakville, ON) and reported as μg fatty acid per 10^5 cells. The recovery rates and correction factors were calculated based on the internal standard C19:0 fatty acid.

Statistical analysis

Analysis of variance (ANOVA) was conducted using SAS-PROC MIXED (SAS, 1997) to determine the effect of GAR at various dosages on cell number, cellular GPDH activity, fatty acids concentration and the ratio of saturated fatty acids to monounsaturated fatty acids. The significant was set as $p \leq 0.05$.

RESULTS

Adipocyte profile and viability

Images of the live cells on Day 8 before harvested at the end of experiment are shown in Figure 1. Most of the cells in each group are differentiated to adipocytes showing accumulated oil drops which was confirmed by oil red-O stained (unpublished). GAR treatments at dosages of 20 and 40 μg mL^{-1} (Figure 1E, F) resulted in a lesser proportion of lipids accumulated adipocytes compared to the control and those received GAR treatments with dosages of 2.5, 5 and

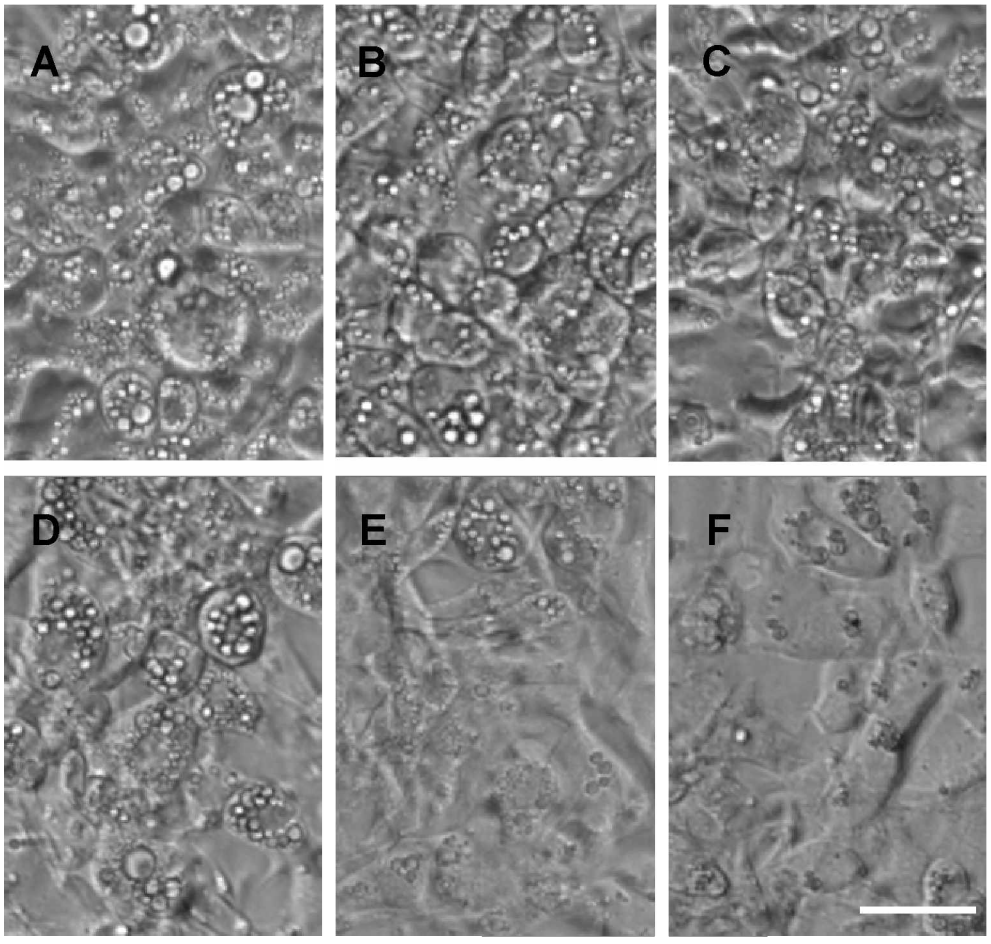

Figure 1. Photos of 3T3-L1 adipocytes on Day 8 of post-confluence after treated with garlic oil (GAR) 0, 2.5, 5, 10, 20, 40 μg mL^{-1} (photos of A, B, C, D, E and F respectively) during the experimental period. Bar = 50 μm.

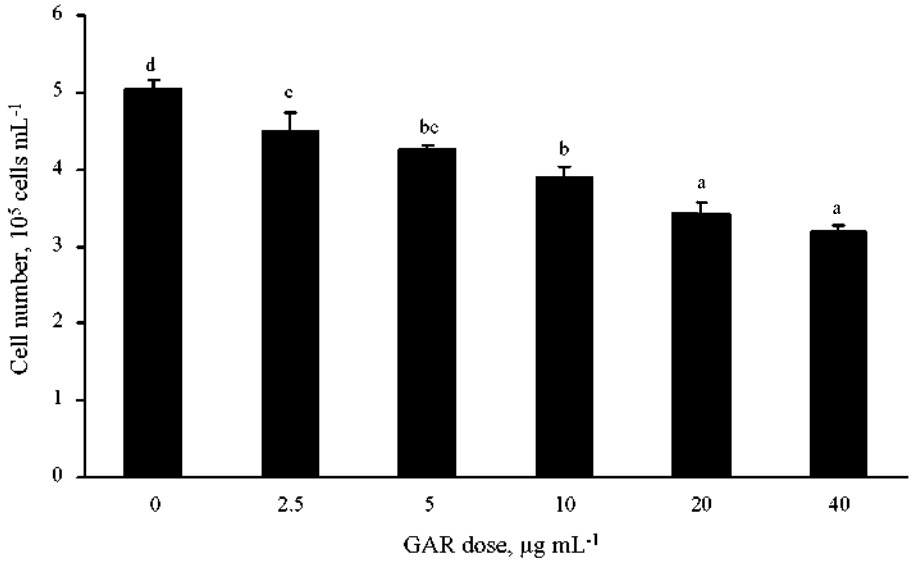

Figure 2. Comparison on number of cells harvested on Day 8 of post-confluence after treated with or without garlic oil (GAR) during the experimental period. a-d: means (±SE, n = 6) without the same letter differ significantly ($p<0.05$).

10 μg mL^{-1} (Figure 1A-D).

The average density of the cells harvested on Day 8 of post-confluence were ranged from 3.2×10^5 to 5×10^5 cells mL^{-1}, which were 16 to 25 folds of that at the beginning (2×10^4 cells mL^{-1}). The GAR treatment resulted in a significant decrease in the final cell number and showing its inhibitory effect on post-confluence proliferation of adipocytes in a dose-dependent manner (Figure 2).

Cellular GPDH activity

Supplementation of GAR at dosages up to 10 μg mL^{-1} to the media increased cellular GPDH activity in a dose-dependent manner (Figure 3). Compared to the control supplementation of 5, 10 and 20 μg mL^{-1} GAR to the media resulted in higher ($p<0.05$) cellular GPDH activity in the adipocytes that were harvested at the end of the experiment. However, a relatively high dosage of 40 μg mL^{-1} GAR did not significantly change the cellular GPDH activity compared that in the control.

Ratios of MUFA to SFA

Ratio of MUFA to SFA was increased with GAR supplementation and in a dose-dependent manner (Figure 4). The GAR supplementation with 5, 10, 20 and 40 μg mL^{-1}

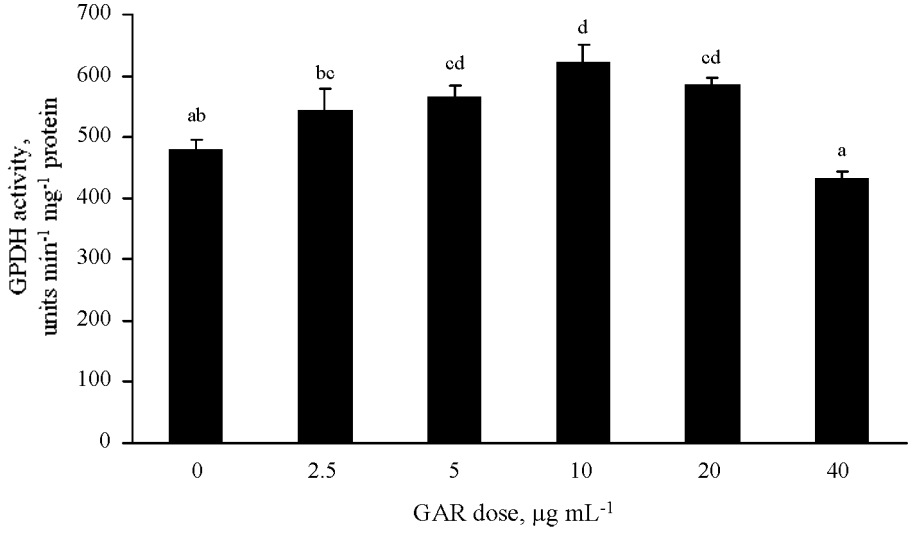

Figure 3. Comparison on activity of glycerol-3-phosphate dehydrogenase [EC1.1.1.8.] (GPDH) in the 3T3-L1 adipocytes after treated with or without garlic oil (GAR) during the experimental period. a-d: means (±SE, n = 6) without the same letter differ significantly ($p<0.05$).

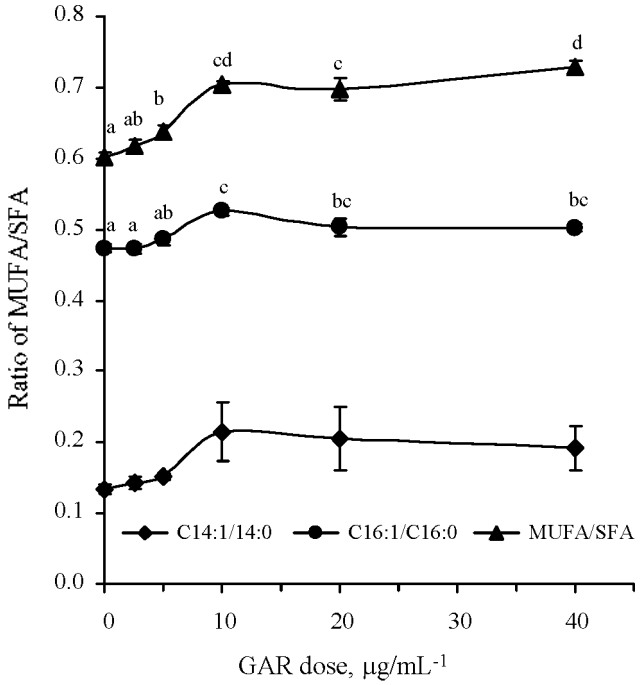

Figure 4. Comparison on ratios of monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA) in the 3T3-L1 adipocytes after treated with or without garlic oil (GAR) during the experimental period. a-d: means (±SE, n = 6) without the same letter differ significantly ($p<0.05$).

resulted in a higher ($p<0.05$) ratio of MUFA/SFA. Ratio of C16:1/C16:0 increased in a dose-dependent manner with GAR supplementation increase from 0 to 2.5, 5 and 10 μg mL^{-1} and became stable with further increment in GAR dosage. No significant difference was found on ratio of C14:1/C14:0 among the treatments although the pattern of changes in the ratio with increment of GAR dosage was similar to that of C16:1/C16:0.

Cellular fatty acids concentration

A comparison of cellular concentration of fatty acids including palmitic acid (C16:0) and palmitoleic acid (C16:1) are shown in Table 1. The C16:0 and C16:1 are among the main components which represent about 50% and 20% of cellular fatty acids, respectively. The supplementation with 2.5, 5 or 10 μg mL^{-1} GAR had no significant effect on cellular fatty acids. However, additional 40 μg mL^{-1} GAR in the media resulted in the lowest ($p<0.05$) MUFA and SFA in the cells. The supplementation of 20 μg mL^{-1} GAR also decreased ($p<0.05$) cellular SFA but not the MUFA neither total fatty acids. The supplementation of 10, 20 and 40 μg mL^{-1} GAR to the media decreased the amount of cellular myristic acid (C14:0), myristoleic acid (C14:1), C16:0 and C16:1 in a dose-dependent manner. The amount of stearic acid (C18:0) or oleic acid (C18:1) was generally greater in treatments with 5 μg mL^{-1} or higher GAR than that in the control.

DISCUSSION

GAR has been tested as an alternative feed additive of antibiotic to improve animal production performance and decrease methane emission in ruminant based on its antimicrobial activities. It is of interesting to clarify effect of GAR on development and composition of fat tissue which are related to the product quality. Through using 3T3 cell line it is possible to obtain many useful information such as effects of GAR supplementation on morphology, lipogenesis related enzyme activity and fatty acid concentration and composition in adipocytes during differentiation and fatty acid accumulation periods (He et al., 2006a, 2006b, 2009).

The profile of fatty acids, especially ratio of monounsaturated fatty acids/saturated fatty acids is very important information, which may reflect the influence of biological active material on cell membrane fluidity (Field et al., 1997). The accumulation of cellular fatty acids including SFA and MUFA are correlated to GPDH activity in adipocytes during differentiation period (He et al., 2006b). The present study found that supplementation of

Table 1. Concentration of cellular fatty acids in the 3T3-L1 after treated with or without garlic oil (GAR) during the experimental period

FA (μg 10^5 cells^{-1})	GAR Treatment (μg mL^{-1})						SEM	p-value
	0	2.5	5	10	20	40		
C12:0	0.07	0.07	0.08	0.07	0.08	0.08	0.01	0.68
C14:0	0.79^{b}	0.80^{b}	0.76^{b}	0.57^{a}	0.50^{a}	0.43^{a}	0.05	<0.001
C14:1	0.11	0.11	0.12	0.11	0.09	0.08	0.01	0.38
C16:0	5.44^{cd}	5.62^{d}	5.54^{cd}	4.93^{bc}	4.37^{b}	3.38^{a}	0.20	<0.001
C16:1	2.59^{c}	2.66^{c}	2.69^{c}	2.58^{c}	2.19^{b}	1.70^{a}	0.10	<0.001
C18:0	0.62^{a}	0.68^{ab}	0.72^{b}	0.71^{ab}	0.77^{b}	0.72^{b}	0.03	0.05
C18:1 cis-9	1.18^{a}	1.35^{ab}	1.40^{b}	1.42^{b}	1.38^{b}	1.26^{ab}	0.06	0.05
C18:1 cis-11	0.30	0.32	0.32	0.32	0.32	0.31	0.01	0.83
SFA	6.92^{cd}	7.16^{d}	7.10^{cd}	6.28^{bc}	5.72^{b}	4.62^{a}	0.27	<0.001
MUFA	4.17^{b}	4.45^{b}	4.53^{b}	4.42^{b}	3.97^{b}	3.35^{a}	0.17	<0.001
Total FA	11.59^{bc}	12.13^{c}	12.18^{c}	11.24^{bc}	10.20^{b}	8.42^{a}	0.45	<0.001

[a-c] Means (n = 6) without the same letter differ significantly ($p<0.05$).

GAR up to 10 μg mL^{-1} increased the cellular MUFA in a dose dependent manner (Figure 4). The supplementation of GAR with relative high dose (20-40 μg mL^{-1}) did not increase or even resulted in a less accumulation of fatty acids (Table 1), however, the ratios of MUFA/SFA, C14:1/C14:0 and C16:1/C16:0 were still in a high level. The improvement effect of GAR was independent to the changes of total fatty acid concentration and GPDH activity. It is suggested that the anti-oxidation components in GAR may attribute the most to this (Yamasaki et al., 1994).

GAR may have diverse effects on differentiation and cellular fatty acid accumulation when supplemented to the media at various doses. In the present study it was found that although supplementation with GAR in dosages of 5, 10 and 20 μg mL^{-1} improved GPDH activity (Figure 3), it brought no significant elevation in fatty acids accumulation. In turn, supplementation of GAR at relative high dosages of 20 and 40 μg mL^{-1} resulted in less fatty acid accumulation. In a previous study (He et al., 2007) a combined effect of supplementation with GAR and CLA t10, c12 on adipocyte differentiation was investigated. It was found that supplementation of 10 μg mL^{-1} GAR alone increase the cellular fatty acid accumulation, while the same dosage of CLA t10, c12 and the combination of the CLA and GAR significantly decreased that and resulted in a very low ratio of MUFA/SFA. It is suggested that the overall effect of GAR on cellular fatty acids accumulation was affected by the dosage applied. The lower doses of GAR did not affect or even increase the cellular fatty acid accumulation through stimulating the GPDH activity, while the very high doses of GAR may decrease it.

The major metabolite compounds in garlic oil which have been tested for the possible functionalities are allyl mercaptan, allyl trisulfide and allicin (Amagase et al., 2001; Wu et al., 2002). The allicin in GAR (approximately 1.5%) is unstable and can be decomposed and metabolized to allyl disulfide and allyl mercaptan in liver according to Egen-Schwind et al. (1992). It was found that supplementation of allyl mercaptan depressed the cell viability but had no effect on fatty acid incorporation into cellular lipids in Hep-G2 cells (Xu and Simon Cho, 1999). The present study found that supplementation with GAR decreased viability of cells harvested at the end of experiment in a dose dependent manner (Figure 2). This inhibition on viability may be caused by the allyl mercaptan component from the GAR. A study by Zelikoff et al. (1986) found that garlic oil and diallyl trisulfide could shorten the cell-doubling time and stimulate proliferation of NIH 3T3 cells while a recent study found many flavonoids and phenolic acids inhibited proliferation of 3T3-L1 cells (Hsu and Yen, 2007). The antioxidation effect of these components may inhibit the cell growth of the cell lines including those cancer cells used in these studies. Allyl trisulfide is the main sulfur compound in garlic oil that may have the biological activities such as antibiotic and anticancer found in animal and human studies. The function of garlic oil such as antidiabetic and antiobesity is still less well understood. Intake of garlic oil was found lowered glucose level in normal male but not female subjects (Zhang et al., 2001). Liu et al. (2006) reported that treatment with GAR improved glucose tolerance in rats with diabetes. However, a treatment with diallyl disulfide alone did not show the same effect, which suggest it may attribute to other compounds in the GAR or their combination with diallyl disulfide. The organosulfur compounds could also be metabolized to allyl mercaptan after being absorbed into blood (Lawson and Wang, 1993).

In conclusion, the present study found that supplementation of GAR increased ratio of MUFA/SFA in adipocytes and showed certain inhibitory effect on the post -confluent proliferation of the cells, which may be attributed by the anti-oxidation components in GAR. The diverse effects of GAR on differentiation and lipid accumulation of adipocytes were found when various dosages applied. With a relative low dosage GAR increased the GPDH activity without significantly affecting the cellular fatty acid concentration. It could turn to inhibit fatty acid accumulation and down the GPDH activity when a high dosage (40 μg mL^{-1}) was applied.

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