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Original article

The role of probiotic content of kefir in the adipogenic differentiation process of 3T3-L1 cells

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Summary. *Objective:* Kefir is traditional fermented milk that is reported to have various health benefits such as preventing obesity. The aim of this study was to investigate the effect of kefir on lipid accumulation and cell differentiation in adipocytes. *Materials and Methods:* Kefir was prepared from milk and kefir powder fermentation. Then, kefir supernatant and pellet fractions were incubated for 24 h and 48 h with mature 3T3-L1 adipocytes. Cytotoxic effect of kefir on the cells was measured by MTT assay. Lipid accumulation was detected by Oil-red O staining. Anti-adipogenic activities of kefir fractions were investigated distribution of ANGPTL-4, leptin and PPAR- γ in mature 3T3-L1 adipocytes using indirect immunoperoxidase technique. *Results:* In the MTT assay, 0.1 mg/dl dilutions of kefir supernatant and pellet were found to be effective on adipocytes growth and differentiation after both 24 h and 48 h incubations. Lipid accumulation was reduced by both kefir supernatant and pellet in 3T3-L1 adipocytes for 24 h. As a result of immunohistochemical staining, ANGPTL-4 immunoreactivity was significantly increased in 3T3-L1 cells after treated with kefir fractions. Additionally, leptin immunoreactivity was also decreased significantly in the mature adipocytes after treated with kefir pellet. There were no differences in expression of PPAR- γ in all groups. *Conclusion:* Kefir fractions have prevented lipid accumulation by preserving the cell structure on 3T3-L1 adipocytes. The increased level of ANGPTL4 expression may be mediated inhibition of lipid accumulation. Moreover, kefir supernatant may play important role in preventing weight gain due to reduced leptin level.

Keywords: Kefir, Obesity, Leptin, ANGPTL-4, PPAR-γ

Introduction

Obesity is a chronic disease and defined as the accumulation of fat in the human body beyond the amount (1). It is known to increase the risk factor of chronic and serious diseases, such as cardiovascular disease, diabetes mellitus type 2, hypertension, dyslipidemia and cancer (2). Obesity is resulted from factors, including genetics and environmental factors such as gut microbiota.

The gut microbiota plays a strong role in the control of obesity through a more efficient way for better energy yield from consumed foods and by modulating dietary or the host-derived compounds (3). Gut microbiota affects immune cells via microbial products including metabolites such as short-chain fatty acids (SCFAs) which can affect adipogenesis. Gut microbiota and obesity contributing mechanism is still unclear. However, it has been suggested that extra calories, increased lipoprotein lipase (LPL) activity, lipogenesis, increased intestinal permeability could contribute to gut microbiota related obesity (1, 4-7). Probiotics affect these mechanisms by increasing the short fatty acids (8). SCFAs induce satiety, decrease

liver lipogenesis, increase levels of PYY, GLP-1, leptin and reduce lipid accumulation in adipose tissue. Thus, the increase in the amount of short-chain fatty acids makes a strong contribution to weight control (9).

Diet and food components are important factors in regulating the composition and the function of gut microbiota. Kefir is one of the factors that have regulatory effect on gut microbiota (10). It is obtained from kefir grains which are rich in probiotic content. At the end of the fermentation of kefir grains, lactic acid, bioactive peptides, exopolycsaccharides, antibiotic, small amounts of alcohol and various bacteriocins are produced. According to Codex Alimentarius, kefir contains 2.7% protein, 0.6% lactic acid and less than 10% fat. The total number of microorganisms is at least 10^7 CFU/mL and the number of yeasts is minimum 10^4 CFU/mL. It also has beneficial effects on lactose digestion, blood pressure, antioxidant activity, cancer prevention, cholesterol and glucose metabolism (11). In addition of these effects, kefir is one of the potential dietary solutions to body weight control due to its probiotic properties (12).

Fasting induced adipose factor (FIAF) is known as ANGPTL-4 and it has got a protective mechanism against diet-induced obesity. ANGPTL-4 shows this protective effect of obesity by inhibiting lipoprotein lipase (LPL) (1, 7, 13). It is secreted by liver, intestine and adipose tissue and main regulator of adipocyte. As a result of increased activity of LPL, the amount of fatty acids and fat storage increases, thus increasing the risk of developing obesity (14,15). The probiotics increase the amount of ANGPTL-4 with positive effects on microbiota and inhibit the LPL activity and show a protective effect against obesity (13,16).

Differentiated 3T3-L1 adipocytes are widely used *in vitro* model in the study of adipogenesis. Differentiation of the cells is dependent on culture conditions including supplements, induction time and number of passage. Treatment of 3T3-L1 preadipocyte cell line with pro-differentiation agents convert cells into mature adipocytes (17). In many in vitro studies related with adipogenic cells are preferred 3T3-L1 cells (18-20).

The role of probiotics in obesity and related diseases has been explained by reducing fat storage and reduces the inflammatory cytokines. However, the cellular mechanisms of kefir on adipocyte differentiation and lipid accumulation have remained unclear. The present study is to determine the effect of kefir on fat accumulation and ANGPTL4, leptin and PPAR- γ expression in 3T3-L1 adipocytes.

Materials and Methods

Sample preparation and reagents

Kefir powder was supplied by Danisco (CHOOZ-IT™ Kefir DGLYO 1000 I, 1284649). Kefir composition consists of lactic acid bacteries such as Lactococcus lactis subsp., Leuconoctoc sp., Lactobacillus sp., Streptococcus thermophilus, kefir grains microflora and kefir grains. Kefir powder was added into pasteurized milk (500 mL pasteurized milk, 2.5 mg kefir powder). The fermentation was performed for 16 hours at room temperature. Grains were filtered out from the fermented medium. Then kefir was centrifuged at 4500 rpm for 30 minutes at room temperature. After centrifugation, kefir supernatant and pellet fractions were separated from each other. Fractions were then freeze dried and powered. Dried kefir fractions were diluted in phosphate-buffered saline (PBS, Frederick, MD217104, USA) and stored at 4°C until used.

Cell culture and adipocyte differentiation

3T3-L1 cells were supplied from ATCC (CL-173 Rockville,MD, USA) and cultured in preadipocyte medium (ZenBio, PM-1-L1). Culture medium was changed every two days. After the cells reached to 90-100 % confluency, they were subcultured using 0.25% trypsin-EDTA solution (Biochrom, L2143). 3T3-L1 preadipocytes were cultured with differentiation medium (ZenBio, #cat DM-2-L1) to stimulate mature adipocyte differentiation for 21 days and medium was changed every two days. The cells were fixed on culture days of 3, 7, 14 and 21. After 21 days, the 3T3-L1 differentiation medium was replaced with 3T3-L1 adipocyte maintenance medium (ZenBio, #cat AM-1-L1).

Oil Red O staining

Oil Red O staining method was used to stain lipid droplets in the 3T3-L1 cells. Stok 0.5% Oil red O (Chem-cma SC, 203749A) solution was dissolved in isopropanol (2-propariol) (Sigma Aldrich, 19516). The mixture was filtered through filter paper, diluted with

distilled water in a 3:2 dilution and stored at 4°C for 24 h. Fixed cells were incubated with Oil red O solution for 15 min at room temperature and then, they were washed with distilled water. Cells were visualized by a light microscope and photographed (Olympus BX40, Tokyo, Japan).

Cell viabilty

The cytotoxicity effect of kefir fractions were measured using a MTT assay (3 - (4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide) (Biotium, #30006). MTT is based on colorimetric measurement of reduction of 3 - (4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide which is reduced by living cells to yield purple formazan product. Stock kefir supernatant and pellet fractions were prepared in PBS and final dilution was 100 mg/ml. After then, and the solution was diluted in culture medium with five different concentrations (0.02, 0.05, 0.1, 0.2, 0.5 and 1 mg/mL). 3T3-L1 adipocytes were collected, suspended in medium and seeded in 96-well culture dishes at a density of 5×10^4 cells in each well with 100 µl medium. Negative control wells had culture medium neither contained any cells nor kefir grains, and positive control wells had only cells with culture medium without kefir grains and incubations were performed for 24 and 48 h. After incubation time, 10 µl MTT solution was added into each well and incubated for 4 h at 37 °C in 5% CO2, after that, 200 µl DMSO was added into each well to dissolve the formazan salts. The absorbance was measured at 540 nm with spectrophotometer (Versa Max, Molecular Device, Sunnyvale, USA). All experiments were performed in triplicate for each extract.

Immunohistochemistry

Distributions of leptin, ANGPTL-4 and PPAR γ in 3T3-L1 adipocytes were analyzed using indirect immunoperoxidase technique. 3T3-L1 adipocytes were fixed with 4% of paraformaldehyde for 30 minutes after 24 h incubation with kefir grains and they were then washed with PBS for 2 times, each for 30 min. Cells were permeabilized with 0.1 % Triton-X-100 (Sigma Aldrich, 9002931) for 15 minutes on ice, they were washed again with PBS. For inhibiting endogenous peroxidase enzyme activity, they were incubated with 3% of H_2O_2 (Sigma Aldrich, D30926) for 10 min at room temperature. At the end of this procedure, cells were washed again with PBS (3x3 min) and blocking serum (Histostatin- Plus Kit HRP, 859043, Thermo Fischer) was added and allowed to incubate for 1 hour at room temperature. The blocking serum was discarded after incubation time and anti-leptin (1:50) (Prointech, 17436-1-AP), anti-ANGPTL-4 (1:300) (Prointech, 18374-1-AP) and anti PPAR γ (1:50) (Prointech, 15540-1-AP) primary antibodies were added and incubated in a humid environment for overnight at 4°C. The cells were then washed 3x3 min with PBS and biotinylated secondary antibody (Histostatin- Plus Kit HRP, 859043, Thermo Fischer) were added and incubated for 30 min. At the end of this period, cells were washed again with PBS and streptavidin peroxidase complex (Histostatin-Plus Kit HRP, 859043, Thermo Fischer) was added and incubated for 30 min. After washing with PBS, immunoreactivity was observed using 3,3' diaminobenzidine (DAB) chromogen (ScyTek Laboratories, ACK125) for 5 minutes. DAB solution was then washed with distilled water and they were stained with Mayer's hematoxylin (Merck Millipore, 109249) to counterstain. They were covered with mounting medium and examined under light microscope (Olympus BX40, Tokyo, Japan). Immunocytochemical staining was evaluated by H-SCORE analysis method.

Statistical analysis

All data was described as mean ± standard deviation (SD). Statistical differences were determined using by SPSS packet programmed (SPSS-21). The differences between the groups were analyzed with Kruskal-Wallis and Mann Whitney U test. A p value of <0.05 was considered as statistically significant.

Results

Cell viability and cytotoxicity

3T3-L1 adipocytes were treated with different concentrations of (0.02, 0.05, 0.1, 0.2, 0.5 and 1 mg/mL) kefir supernatant and pellet for 24 and 48 h. The cell viability was determined as described above by MTT assay. 0.2, 0.5 and 1 mg/mL concentrations of kefir supernatant and pellet resulted in a dramatic decrease of 3T3-L1 adipocytes proliferation (Figure 1

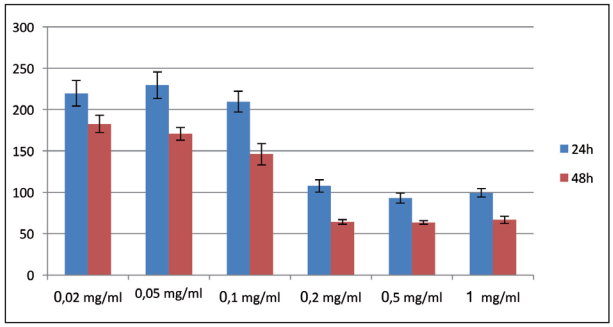

Figure 1. Effect of kefir supernatant on cell viability of 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with different concentrations of kefir supernatant for 24 or 48 h. Viability was quantitated by the MTT assay.

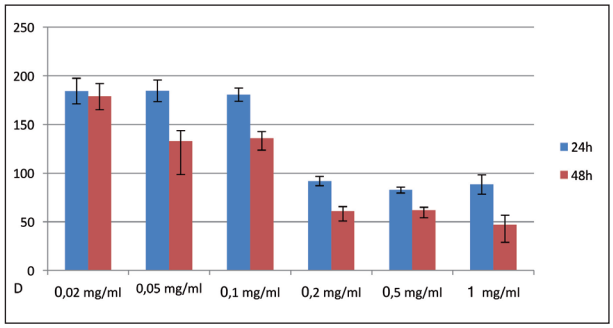

Figure 2. Effect of kefir pellet on cell viability of 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with different concentrations of kefir pellet for 24 or 48 h. Viability was quantitated by the MTT assay.

and 2). Based on results, kefir supernatant and pellet at 0.1 mg/ml for 24 h incubation were used in all subsequent experiments.

Oil red O staining

3T3-L1 preadipocyte cells differentiation protocol was applied and then the cells were fixed on days 3, 7, 14 and 21. Lipid droplets were observed on the 3rd day of 3T3-L1 cells and the accumulation of lipid was increased according to incubation time (Figure 3). On day 14 (Figure 3, G) and 21 (Figure 3, H), >95% of the cells contained lipid droplets and there was similar oil red positive lipid droplets. Therefore, the mature adipocytes evaluation in the study was used only for 14 days during culture time.

3T3-L1 adipocytes were incubated with kefir supernatant and pellet (0.1 mg/ml) for 24 h. Kefir supernatant and pellet treated 3T3-L1 adipocytes showed less intracellular lipid accumulation decreased versus control cells. Especially, lipid droplets was smaller in the 3T3-L1 cells when compared to control group (Figure 4).

Immunohistochemical evaluation

The H-SCOREs of ANGPTL-4, leptin, PPAR-γ intensities in 3T3-L1 adipocytes treated with kefir supernatant and pellet for 24 h are given in Table 1. ANGPTL-4 immunreactivity was strong in 3T3-L1 adi-

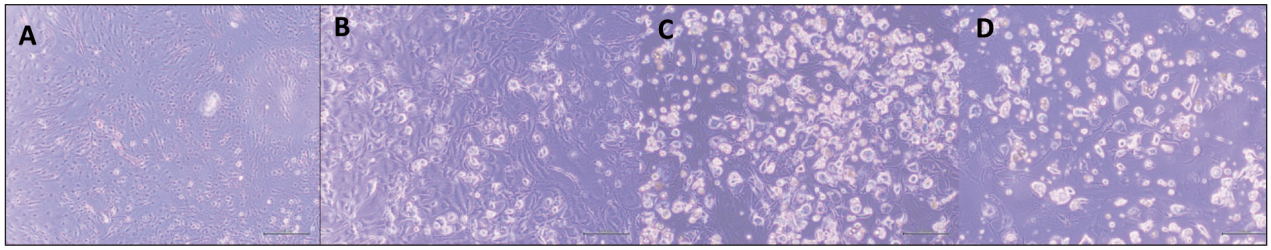

Figure 3. Lipid accumulation in 3T3-L1 adipocytes in culture (A-D) and Oil Red O staining (E-H) without kefir grains treatment. Day 3 (A, E), day 7 (B, F), day 14 (C, G), day 21 (D, H) Scala bars = 50 μm.

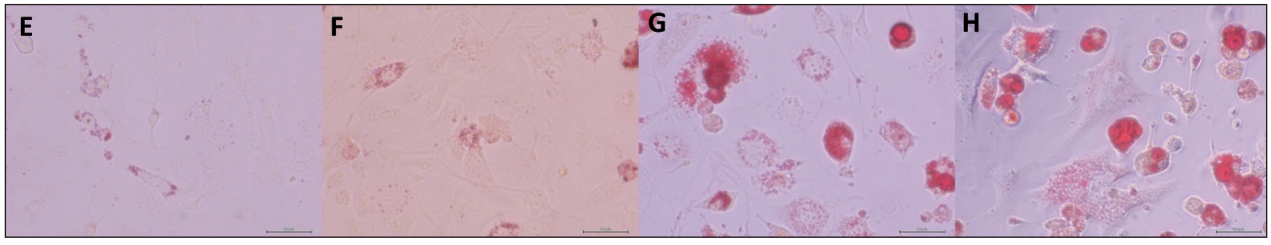

Figure 4. Oil Red O staining of 3T3-L1 adipocytes with kefir supernatant (A), pellet (B) and without kefir grains (control, C). Scala bars = 50 μm

pocytes treated with kefir supernatant and moderate in kefir pellet treated 3T3-L1 adipocytes. The immunoreactivity of ANGPTL-4 was significantly higher in 3T3-L1 adipocytes treated with both kefir supernatant and pellet compared to control group (Figure 5 A-C, Table 1). While ANGPTL-4 immunoreactivity was higher in 3T3-L1 cells after incubation with kefir supernatant than kefir pellet group, however, this increased immunoreactivity was not significant (Figure 5 A-C, Table 1). Immunolabeling of leptin was mild in both kefir supernatant (Figure 6B) and kefir pellet (Figure 6C) treated 3T3-L1 cells. Leptin expression was dramatically decreased in the kefir pellet group, this immunoreactivity was statistically significant when compared to kefir supernatant group (Table 1).

Intensity for PPAR-γ was mild and modarate in 3T3-L1 adipocytes that treated with kefir supernatant (Figure 7B) and kefir pellet (Figure 7C), respectively. The PPAR-γ immunoreactivity was also moderate in control group (Figure 7A). Statistical analysis showed that PPAR-γ H-SCORE results were not significant when compared with control group (Table 1).

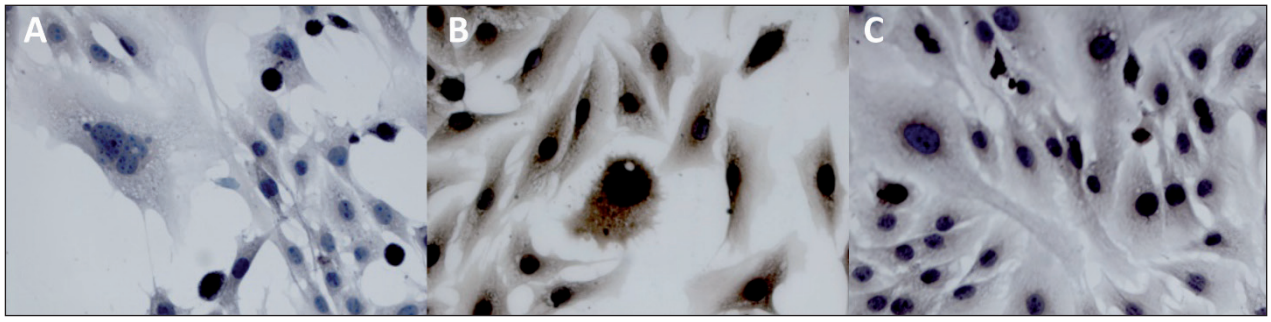

Figure 5. Immunoreactivity of ANGPTL-4 in 3T3-L1 adipocytes from control (A), kefir supernatant (B) and kefir pelet (C) groups. Scala Bars = 50 μm

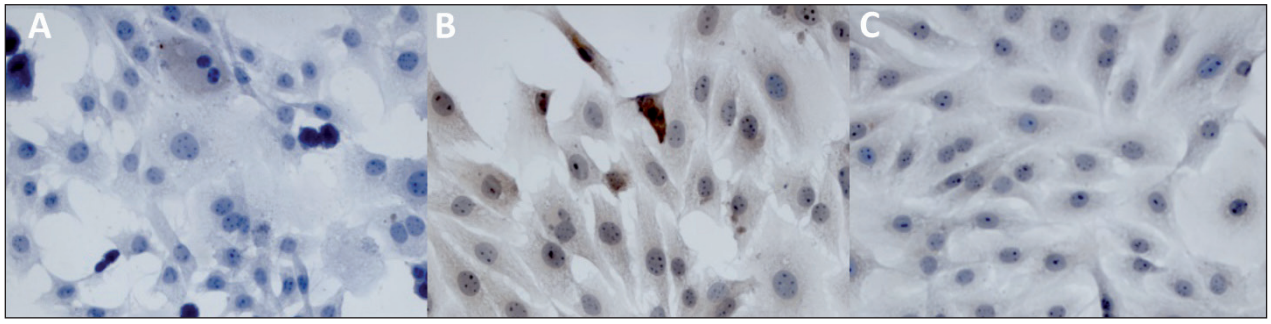

Figure 6. Immunoreactivity of leptin 3T3-L1 adipocytes from control (A), kefir supernatant (B) and kefir pelet (C) groups, Scala Bars = 50 μm

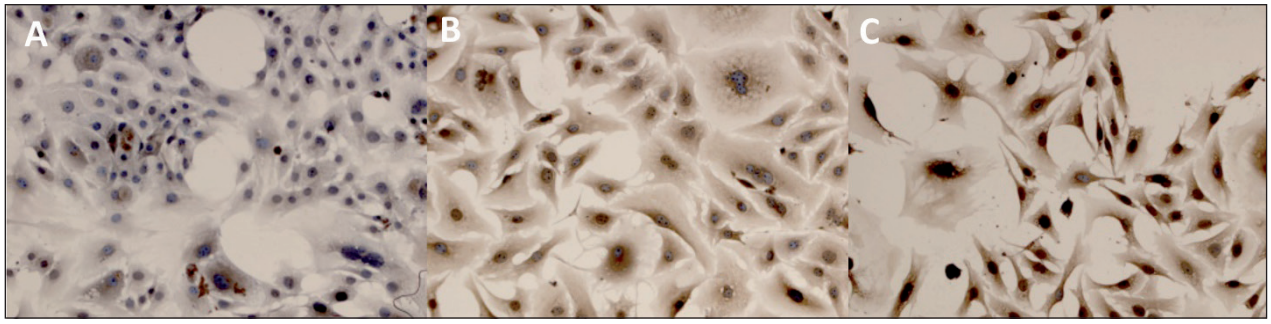

Figure 7. Immunoreactivity of PPAR-γ 3T3-L1 adipocytes from control (A), kefir supernatant (B) and kefir pelet (C) groups Scala Bars = 50 μm

Table 1. The H-SCORE datas for ANGPTL-4, leptin and PPAR- γ in control and treated with either kefir supernatant or kefir pellet of 3T3-L1 adipocytes

	ANGPTL-4	LEPTIN	PPAR- γ
Control group	265,46 ± 25,54	211,37 ± 11,39	239,06 ± 22,68
Kefir supernatant	374,42 ± 27,47[a,]	210,63 ± 6,15	229,90 ± 16,13
Kefir pellet	317,06 ± 45,05[b]	203,73 ± 3,83[c]	243,13 ± 22,98

Data is expressed as means ± SD and were compared by Mann-Whitney U test.
[a] The data was significant when compared with control group (p = 0.009).
[b] The data was significant when compared with control group (p = 0.047).
[c] The data was significant when compared with kefir supernatant treated cells group (p = 0.014).

Discussion

Kefir is a traditional fermented milk product with natural probiotic content. It has numerous beneficial health effects such as hypocholesterolaemic effect, anti-inflammatory effect, anti-oxidant activity, weight gain control etc. (11, 21). Our results showed that both kefir supernatant and pellet fractions inhibited lipid accumulation in 3T3-L1 adipocytes. Also, ANGPTL-4 expression is significantly increased in 3T3-L1 mature adipocytes after treatment with both kefir supernatant and pellet fractions. In addition, leptin immunoreactivity was significantly decreased after treated with kefir pellet. PPAR-γ expression was not affected after kefir supernatant and pellet treatment.

3T3-L1 cells are preferred for better understanding of lipid metabolism and adipogenesis (18-20). In this study, 3T3-L1 cells were used to determine the effect of different grains of kefir on adipogenesis via histochemically and immunohistochemically. Because of the differentiation of 3T3-L1 cells dependent on culture time, to obtain mature adipocyte, the time manner of the preadipocyte differentiation was firstly investigated. The mature adipocytes were analyzed with lipid accumulation after Oil Red O staining. The lipid accumulation was similar in adipocytes on day 14th and 21th day culture period, therefore, throughout the study 14 day culture time was used. Secondly, the molecular mechanism of kefir was investigated expressions of ANGPTL-4, leptin and PPAR- γ in all groups

ANGPTL-4 is a member of angiopoietins that the protein is synthesized mainly from liver, intestine and adipose tissue which is also known as fasting-induced adipose factor (FIAF) (22). It is defined as the target gene of peroxisome proliferator- activated receptor protein- α (PPAR-α) and PPAR- γ (23,24). It regulates lipoprotein lipase (LPL) activity (25,26), stimulates intracellular adipocyte lipolysis (27), modulate glucose and lipid metabolism (22). Our results showed that, ANGPTL-4 immunoreactivities were significantly increased in 3T3-L1 cells after kefir treatment groups for 24 h. According to these results, kefir may provide weight control by inhibiting LPL and reducing fat accumulation in adipocytes. In addition, kefir supernatant increased ANGPTL-4 expression more than kefir pellet through probiotic content. The intestinal microbiota is improved with probiotic uptake and ANGPTL-4 expression is supported. Consequently, kefir supernatant is more effective in weight control than pellet. Both grains of kefir on ANGPTL-4 activity were first revealed by this study.

Leptin is primarily secreted from white fat tissue (WAT) (28) and controls food intake and energy expenditure (29). It prevents weight gain by increased lipolysis (29), decreased lipogenesis (30), activating POMC neurons and inhibiting NPY/AGRP neurons (31). In our results, there was no significant difference in kefir pellet and supernatant fractions compared to the control group. In addition, kefir supernatant has similar expression levels compared to the control group but more than the pellet. This effect is demonstrated by its probiotic content. Thus, it has an effective weight gain by maintained adipogenic cell characteristic and preventing cell differentiation after kefir incubation. However, leptin expression was significantly lower in the kefir pellet group than kefir supernatant group. Reduced leptin expression with kefir consumption has a positive effect on leptin resistance in obese individuals. Rosa et al. (12) was investigated the effect of kefir on serum leptin levels and they found that it significantly increased leptin levels. In another study, conducted on 21 volunteer individuals, changes in leptin levels of individuals consuming kefir were examined. Researchers

found a significant increase in leptin levels in the group consuming kefir (32).

Ligand activating transcription factors are one of the subclasses of the nuclear hormone receptor superfamily. Peroxisome proliferator-activated receptors (PPARs) are the subfamily of this group. The PPAR subfamily has got 3 isotopes; PPAR-α, PPAR-β/δ, PPAR-γ (33). These three forms are found in different tissues, have different ligand specificity and have different physiological roles (34). PPAR- γ is predominantly secreted in adipose tissue (35). It plays an important role in adipogenesis by increasing the regulation of lipids into adipose tissue, liver and muscle; modulation of adipocytokine release; improving insulin resistance (36). Also, it is the key regulator in adipocyte differentiation and effective in lipid metabolism through endogenous and synthetic ligands (37). Finally, it inhibits inflammation by regulating leptin and adiponectin levels (38). PPAR- γ plays a role in the management of obesity through these effects. In our study, PPAR- γ immunoreactivity was similar in kefir pellet treated 3T3-L1 cells when compared control group. The kefir supernatant group showed a slight decrease in PPAR- γ expression through probiotic content. According to these results, PPAR- γ expression did not change during adipogenesis with or without kefir grains. Ho et al. (39) suggested that PPAR- γ expression was significantly reduced after incubation with kefir. In another study on mice, was also PPAR- γ expression decreased with kefir treatment (40). The PPAR- γ expression may depend on kefir contents, in our study we have used kefir grains which differ from each other therefore, PPAR- γ expression might not be effective in this study, or different expressions were not evaluated with immunohistochemically, the mRNA levels could be analyzed.

As a result, 3T3-L1 cells incubated with kefir fractions and reduction in the amount of stained lipid was detected Kefir supernatant reduced the lipid accumulation in lipid droplets. The lipid accumulation inhibitory effect was more observed in the kefir pellet group than kefir supernatant. Decreased lipid accumulation after kefir grains, may be associated with the effect of enhancing ANGPTL-4 expression. So, this increase inhibits LPL activity and prevents fat accumulation. In addition, other mechanisms during adipogenesis could be also investigated in future studies.

Conclusion

In conclusion, the effects of kefir on lipid accumulation and cell differentiation were investigated. For this purpose, accumulation of lipid was evaluated with Oil red O staining and the expressions of ANGPTL-4, leptin and PPAR- γ were examined immunohistochemically. As a result, kefir has prevented fat accumulation via inhibiting of molecular pathways of lipid accumulation. Therefore, kefir may prevent weight gain and adipogenesis, thus, this study will guide us to explain the role of kefir in obesity.

Acknowledgements

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Disclosure statement

The authors declare no conflict of interest.

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