Adipose tissue miRNA level variation through conjugated linoleic acid supplementation in diet-induced obese rats

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Background. Conjugated linoleic acid (CLA), which is an octadecadienoic acid isomer, is believed to play different positive physiological roles, such as lowering body fat. Due to some reported side effects of CLA, like lipodystrophy and impaired glucose metabolism, it is important to establish its safety by understanding detailed molecular mechanisms. One of these mechanisms may be the role of this dietary agent in modifying the function and activity of microRNAs (miRNAs).

Objectives. The aim of the study was to investigate how adipocyte miR-27a and miR-143 expression may be influenced by CLA in obese rats.

Material and methods. In this study, 24 male Wistar rats were randomly divided into normal-fat diet (NFD) and high-fat diet (HFD) groups. After 8 weeks, the rats were weighed and half of the diet-induced obese rats were randomly selected to receive 500 mg CLA per 1 kg body weight for 4 weeks. At the end of this period, epididymal fat was isolated to investigate the expression level of miRNAs by real-time polymerase chain reaction (RT-PCR).

Results. After 12 weeks, the obese rats in the HFD group, compared with rats in the NFD group, demonstrated a significant decrease in the expression of miR-27a (p < 0.05) and a significant increase in the expression of miR-143 (p < 0.05). In the group which had received CLA for a 4-week period, these events were reversed. Moreover, the rats in this group gained less weight than other rats in HFD groups, although the difference was not statistically significant.

Conclusions. In conclusion, this study demonstrated that CLA, as an anti-obesity agent, may minimize abnormal changes in miRNA expression in obesity. This suggests a new pathway for weight loss; however, further studies are needed.

Key words: obesity, microRNA, high-fat diet, conjugated linoleic acid

The growing global prevalence of obesity, which is recognized as a disease by the American Medical Association, has had damaging effects on various aspects of life and has even increased mortality and morbidity rates as a result of cardiovascular diseases, cancers and metabolic syndrome.[1,2] On the one hand, various approaches such as increasing physical activity, diet therapy, surgery, and medication have demonstrated only partial success. On the other hand, several anti-obesity drugs have been withdrawn from the market because of their proven adverse effects.[3] Hence, an increasing demand for safe and effective anti-obesity drugs has arisen.

In this regard, it is necessary to consider regulatory mechanisms in order to find effective agents for obesity control. Recently, microRNAs (miRNAs) as epigenetic regulators in obesity have attracted considerable attention from researchers. The miRNAs are short-length, noncoding RNAs which, by pairing with the 3'-untranslated region (UTR) of their target mRNAs, control gene expression.

Besides the important roles of miRNAs in different processes such as proliferation, differentiation, apoptosis, growth, and development, it seems that several miRNAs are deregulated in animals and humans with obesity. However, there is insufficient evidence regarding the role of these small molecules in the metabolism of adipose tissue.[4,5] Among the numerous miRNAs existing in adipose tissue, a few of them are differentially expressed in obesity or demonstrate regional variance in their expression.[6]

Recently, some studies have shown that miRNA expression in adipose tissue may possibly affect the pathogenesis of diseases related to high-fat diets. Some miRNAs can be modified by epigenetic mechanisms when the diet composition is changed.[7–9] Thus, understanding the mechanisms by which miRNAs exert their potential roles in adipose tissue metabolism may possibly lead to the discovery of new paths in the development and treatment of obesity.[10] However, very few studies have addressed this issue and have considered the effect of different kinds of diet or dietary agents on miRNA variations.[11,12]

Therefore, a hypothesis can be put forward that investigating the use of pharmacological agents such as fat burners in obesity management can elucidate the probable mechanisms for their anti-obesity action.[13] One agent that is considered an anti-obesity drug is conjugated linoleic acid (CLA), which contains a linoleic acid isomer (C18:2, n-6).[14] A large proportion of the obese population is interested in CLA supplements, claiming body fat-lowering effects. Conjugated linoleic acid is considered a "natural" compound without dangerous side effects.[15] However, this fatty acid inhibits the entry of glucose into the adipocytes, and may lead to hyperinsulinemia and increases in inflammatory markers. Consequently, there are doubts regarding the exact mechanisms of action of CLA in adipocytes that result in the reduction of body fat, and regarding the safety of using this compound as a dietary supplement.[14]

From among numerous adipocyte miRNAs, miR-143 and miR-27, which are up- and downregulated (respectively) during adipocyte differentiation, were selected for further investigation.[16–18]

The expression of miR-143 was reported to be increased in mice fed a high-fat diet, which in turn caused hyperinsulinemia and impaired glucose tolerance. On the other hand, in mice with reduced miR-143 level or activity, undesirable blood glucose changes were modified, and consequently, this microRNA was regarded as a potential candidate to be targeted in type 2 diabetes as well as obesity treatment.[7] Furthermore, a high-fat diet was found to lead to reduced expression of miR-27a in concordance with amplified peroxisome proliferator-activated receptor γ (PPARγ) activity, which is a well-known transcription factor in adipocyte differentiation.[8,18] Therefore, recommendation of dietary agents that induce miR-27a or inhibit miR-143 seems to be logical to decelerate adipogenesis. Consequently, the aim of this study was to determine the variations in expression of these 2 miRNAs in adipose depots in the epididymis of rats fed a high-fat diet before and after CLA supplementation.

Material and methods

Animals and diet

Twenty-four 8-week-old male albino Wistar rats were obtained from the Animal Laboratory Unit of Ahvaz Jundishapur University of Medical Sciences (Iran). The rats, which weighed 150–200 g, were housed in groups of 2 per cage and maintained on a 12-hour light/dark cycle at 22°C with a relative humidity at 50 ±5%, and were fed a standard chow diet and tap water ad libitum. After a 1-week acclimation period, they were randomly allocated into 2 different diet groups: a normal-fat diet (NFD) for the entire experimental procedure (a semi-purified NFD), (n = 8) as the control group; and a high-fat diet (HFD) for the entire experimental procedure (a semi-purified HFD to induce obesity) (n = 16).

The basic diet in this study was a semi-purified form of the American Institute of Nutrition (AIN)-93M diet. The proportional compositions of the 2 diets are presented in Table 1.[19] Fresh portions were prepared twice a week and stored at 4°C. After the 8th week, the HFD group was randomly subdivided into 2 categories (n = 8 in each): a control group, which received HFD + 1 mL of corn oil per day for 4 weeks by oral gavage; and a CLA group, which received HFD + 500 mg per kg body weight of CLA (Tonalin® CLA; Natural Factors, Coquitlam, Canada, composed of a 75% mixture of c9, t11 and t10, c12 isomers in a 50:50 ratio) dissolved in 0.5 mL of corn oil (1 mL total) per day for 4 weeks by oral gavage.

During the course of the experiment, body weights were recorded weekly to verify the obesogenic effects of the HFD. At the 12th week, the animals were fasted for 10 h before

Table 1. Modified* American Institute of Nutrition diet composition

Ingredients in 1 kg of diet	Normal-fat diet [g]	High-fat diet [g]
Corn starch	465.6	237
Casein (>85% protein)	140	140
Dextrinized corn starch	155	78
Sucrose	100	50
Soybean oil	40	185
Margarine	0	185
Fiber	50	50
Mineral mix (AIN-93M-MX)	35	35
Vitamin mix (AIN-93-VX)	10	10
L-Cystine	1.8	3
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.008	0.01
Total energy [kcal]	3600	5350

*AIN-93M Purified Diet for Laboratory Rodents was modified.[19] Fiber was replaced by wheat bran in the formulation.

being sacrificed and then white adipose tissue (WAT) from epididymal fat was isolated and directly submerged in RNA later solution (RNA Stabilization Reagent; Qiagen, Hilden, Germany) to stabilize and keep cellular RNA in situ, and was kept at −80°C until analysis.

All the procedures carried out in this study involving animals were approved by the ethical committee for experimental animal care at Ahvaz Jundishapur University of Medical Sciences (NRC9204).

MicroRNA extraction from adipose tissue

To extract total miRNA from WAT taken from NFD- or HFD-fed rats, approx. 100 mg of tissue was homogenized in 1–2 mL of Qiazol (Qiagen) using a digital homogenizer (WiseTis Homogenizer HG-15D, Grafstal, Germany). Thereafter, miRNA isolation was carried out utilizing the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of extracted miRNA was measured using the NanoDrop Spectrophotometer 2000c (Thermo Fisher Scientific, Inc., Waltham, USA) and its integrity was confirmed by 2% agarose gel electrophoresis. The RNA samples were directly frozen and stored at −80°C.

MicroRNA quantification was initiated by reverse transcription of the RNA samples and then amplification by Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA) and specific primers. Reverse transcription and real-time polymerase chain reaction (RT-PCR) were carried out using a miScript Reverse Transcription Kit (Qiagen), miScript SYBR Green PCR Kit (Qiagen) and miScript Primer Assay (Qiagen) in accordance with the manufacturer's instructions. All the primers were manufactured by Qiagen (Hs_RNU6-2_11 Cat. No. MS00033740, RN_miR-143_1 Cat. No. MS00000420 and RN_miR-27a_1 Cat. No. MS00000147) and their sequences were not revealed.

MiR-27a and miR-143 levels were normalized to RNA U6. Real-time PCR was performed on a StepOne Real Time PCR System (Applied Biosystems). The RT-PCR thermal cycling included 15 min incubation at 95°C, followed by 40 cycles of a 3-stage temperature profile of 94°C for 15 s, 55°C for 30 s and finally 70°C for 30 s. All the samples were run in triplicate and the fold changes in the miRNA level were calculated by the comparative cycle threshold (Ct) method $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct$ miRNA − Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples − ΔCt untreated controls.

Statistical analysis

The statistical analysis was performed using the Statistical Package for Social Sciences v. 18.0 (SPSS Inc., Chicago, USA) The results were expressed as means ± standard error of the mean (SEM). The normality and homogeneity of variances were tested with the Kolmogorov-Smirnov test. Student's t-test and a 1-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test were employed to compare the mean differences between the groups. A p-value <0.05 was considered statistically significant.

Results

Effects of high-fat diet and normal-fat diet on weight gain

Body weights at the beginning were not different between the 2 groups (p-value >0.05). At the 8th week, the HFD-fed rats gained significantly more weight than the controls and developed the obese phenotype (Table 2).

Effects of conjugated linoleic acid on weight gain

Four-week treatment with CLA reduced the animals' weight gain throughout the treatment period; however, this effect was not statistically significant (Table 3).

Table 2. Effects of NFD and HFD on rats' weight gain over 8 weeks

Group	n	Baseline weight [g]	p-value	8-week weight [g]	p-value
NFD	8	169.66 ±8.91	0.67[a]	246.38 ±9.48	0.009[b]
HFD	16	173.00 ±4.02		296.43 ±5.36	

Values are expressed as means ± standard error of the mean (SEM). NFD – normal-fat diet; HFD – high-fat diet; [a] insignificant weight difference between NFD and HFD at baseline; [b] significant weight difference between NFD and HFD at the 8th week.

Table 3. Effect of CLA on weight gain rate over 4 weeks in obese rats

Group	n	8-week weight [g]	12-week weight [g]	Weight gain [g]	p-value
NFD	8	246.38 ±6.48	264.62 ±8.03	18 (7.3%)	
HFD	8	300.50 ±13.16	336.43 ±15.10	35.9 (11.9%)	0.08[a]
CLA	8	288.38 ±7.60	312.68 ±9.60	24 (8.3%)	

Values are expressed as means ± standard error of the mean (SEM). NFD – normal-fat diet; HFD – high-fat diet; CLA – conjugated linoleic acid; [a] significant difference between CLA and control HFD in weight gain over 4 weeks.

Effects of high-fat diet on adipose tissue miR-143 and miR-27a expression levels

Parallel to the weight gain in HFD-fed rats, the expression levels of the miR-27a and miR-143 were altered in the adipose tissue of the obese rats compared to the NFD-fed rats. The HFD reduced miR-27a expression to 0.021-fold and increased miR-143 expression to 2.3-fold compared to the NFD-fed rats (Fig. 1).

Effects of conjugated linoleic acid on adipose tissue miR-143 and miR-27a expression levels

After 4 weeks, RT-PCR results showed that overexpression of miR-143 and downregulation of miR-27a in the adipose tissue induced by the HFD were weakened in the rats in the CLA group compared to the obese rats fed only a HFD. An increase in miR-27a expression to 2.26-fold was accompanied by a decrease in miR-143 to 0.42-fold following daily doses of 500 mg of CLA/kg of body weight (Fig. 1).
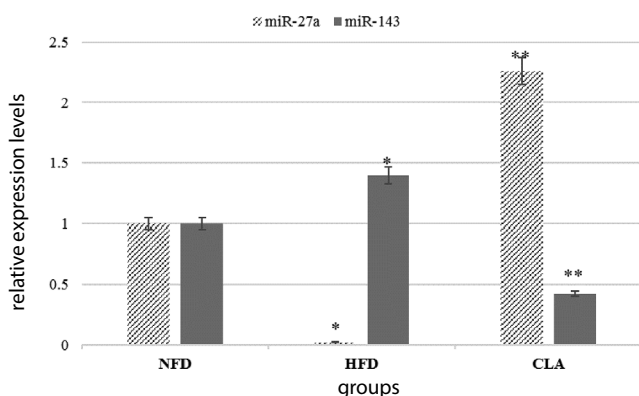

Fig. 1. Relative expression levels of miR-143 and miR-27a in the adipose tissue of rats fed a NFD or HFD in comparison with the CLA group. MicroRNA levels were normalized to U6 small nuclear RNA. The means ± standard error of the mean (SEM) of 8 rats in each group is shown.

* values with significant changes relative to the NFD group (* $p < 0.05$);
** values with significant changes relative to the HFD group (** $p < 0.05$);
NFD – normal-fat diet; HFD – high-fat diet; CLA – conjugated linoleic acid.

Discussion

Although CLA supplementation (in both animals and humans) has been found to bring about changes in body weight and some other beneficial effects in previous studies, the current results are very conflicting.

One of the main results of this study was the deceleration of the weight-gain trend in the CLA group in comparison with the HFD group. As shown in Table 3, the weight-gain rate in the HFD+CLA group was close to that of the NFD group (8.3% vs 7.3%); however, the final weight difference between the HFD group and the HFD+CLA group was not statistically significant. It seems that CLA does not diminish food intake in rodents (we did not measure food intake) and its effects are independent of the amount of fat in the diet, while some protein sources may change the effects of CLA on adipocyte fat content and the production of adipocytokine.[15] Despite the reduction in the fat content of CLA-fed animals reported in various studies, there was not much difference between the total body weights of the treated animals and the controls. As suggested by a few investigators, this happens mainly because of hepatomegaly and splenomegaly, which have been attributed to CLA supplementation.[20]

Furthermore, the weight changes in this study were judged only by body weight, while fat mass is a more sensitive index for evaluating obesity in animals. Woods et al. reported that rats fed a HFD for 10 weeks demonstrated a 10% increase in body weight. However, a 35–40% increase in body fat was detected compared with animals fed a NFD.[21] Another study has verified that a decrease in adipose mass was evident as early as after 1 week of feeding the animals CLA.[22]

It should be noted that regional differences in responsiveness to CLA have been reported. To determine cell proliferation response to CLA supplementation, it seems logical to expose animals to CLA during fetal growth, when most pre-adipocyte hyperplasia occurs.[22]

On the other hand, a few adverse effects of CLA have been reported, mainly due to the 10-trans, 12-cis isomer. For example, increased production of eicosanoids, pro-carcinogenic effects and negative alterations in glucose and lipoprotein metabolism have been attributed to CLA.[14,23] Its pro-oxidant effect enhances lipid hydroperoxide and lessens total antioxidant defenses, which leads to adverse effects on the lipid profile.[24] Thus, understanding the mechanism of action of CLA is vital for establishing an anti-obesity drug such as CLA.

In addition to determining the real impact of CLA on adiposity, it is necessary to ascertain the extent of the positive alterations obtained. Furthermore, it will be necessary to consider reciprocal interactions between dietary CLA and hormones such as leptin and insulin.[25]

As in most studies, a mixture of 2 major isomers (9-cis, 11-trans and 10-trans, 12-cis in equal proportions) was utilized in this study. In addition, a synthetic NFD was utilized to adjust the confounding effects of the control diet. In most other studies, a chow diet is used, although its basic composition is totally different from the experimental HFD and this is a noteworthy point.[26] Therefore, in this research, find the AIN-93 M diet was used as the basic diet and was formulated with different fat contents.

Since CLA has been reported to diminish fat deposition in animals, Parra et al. carried out a study to determine whether or not CLA administration affects the expression of miRNAs in the adipose tissue of mice. They found that miR-143 was downregulated in animals fed a standard-fat diet and treated with CLA,[27] which is in line with our results.

The association between abnormal miRNA expression and anomalies in adipogenesis alongside obesity may be a reason to target these molecules in obesity management. On the other hand, because the targets of miR-143 and miR-27a have not been identified, the association between the alterations in miRNAs brought about by CLA administration and fat accumulation needs further investigation.[27]

Supplementation with CLA in obese rats led to significant modifications in miR-27a and miR-143 expression in adipose tissue, suggesting probable pathways through which CLA mediates its biological effects in weight changes.

Esau et al. studied miRNA expression arrays in pre-adipocytes and demonstrated that miR-143 normally induces adipocyte differentiation and is overexpressed in obesity.[28] We obtained similar results. As in the present study, Takanabe et al. reported a 3.3-fold greater expression of adipose tissue miR-143 in obese mice; this was associated with over-expressions of PPARγ and activator-protein 2 (AP2), which are adipocyte differentiation markers.[17] Transcriptional factors (TF) such as C/EBP (α, β, δ), fatty acid synthase and fatty acid binding proteins genes can control adipocyte differentiation.[29] Similarly, *miR-143*, which mainly acts through *ERK5* (extracellular signal-regulated kinase 5) and *MAPK7*, can modulate the aforementioned TFs and control adipogenesis.[6,28,30]

In contrast, miR-27a in mature adipocytes of the obese mice was downregulated in comparison with lean mice.[18] A diet inducing non-alcoholic fatty liver disease (NAFLD) (high fat or high carbohydrate content) has been shown to decrease the expression of miR-27, -122, and -451, while upregulating miR-429, -200a and -200b expression in rat livers, which is consistent with our results in the HFD group.[7]

Investigating the protein and mRNA levels of key molecules and the expression levels of some genes related to adipogenesis or lipolysis, such as PPARγ, CEBPs and FAS, could be important and interesting. Since every single miRNA is able to control hundreds of target mRNAs, exploration of miRNA profile alteration is suggested; it was not possible for the researchers in the present study.

Conclusions

Complementary studies regarding the modification roles played by dietary agents or supplements in miRNA expression and other related genes are needed, not only to confirm the potential of miRNAs as novel prognostic metabolic biomarkers, but also to find new and more specific goals in obesity management, as well as the treatment of other metabolic disorders. However, we must bear in mind that studies evaluating the effects of nutrients on miRNA levels have mainly focused on the oncologic aspects and there are limited studies in line with ours.[12]

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