International Journal of Nutrition Sciences

Journal Home Page: ijns.sums.ac.ir

ORIGINAL ARTICLE

Protective Effect of Alginate Extraction of Brown Algae on MicroRNAs in Type 1 Diabetes of Rats

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ARTICLE INFO

Keywords:
MicroRNAs
Diabetes
α-L-guluronic acid
β-D-mannuronic acid
Rat

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Received: August 19, 2024
Revised: November 12, 2024
Accepted: November 20, 2024

ABSTRACT

Background: MicroRNAs expressions that are known as Post-transcriptional repressors of gene expression in animals and plants have an important role in diabetic consequences and pathogenesis. This research aimed to investigate the expression of miR-34a, miR-126, and miR-125a-5p levels for diagnosis of diabetes, and the effect of α-L-guluronic acid and β-D-mannuronic acid supplementation on expression patterns of these MicroRNAs in diabetic rats.

Methods: Total MicroRNAs levels were measured by Quantitative reverse transcription polymerase chain reaction (qRT-PCR) method. Body weight, fasting insulin level, food intake and Vitamin D level in four groups were also assessed after 4 weeks.

Results: The analyses illustrate marked declines in expression of miR-126 (p=0.009) and miR-125a-5p (p=0.019) levels in the control diabetic group when compared to the control healthy group. Expression levels of miR-126 (p=0.026) and miR-125a-5p (p=0.041) were upregulated considerably in diabetic rats after treatment with β-D-mannuronic acid and α-L-guluronic acid, respectively.

Conclusion: These results suggest that use of miR-126 and 125a-5p is a sensible criterion for diagnosing diabetes. β-D-mannuronic acid is also considered a new therapeutic agent to correct disturbances in the MicroRNAs profile of diabetic rats.

Please cite this article as: Sharifzadeh M, Esmaeili-Bandboni A, Emami MR, Mirshafiey A, Javanbakht MH. Protective Effect of Alginate Extraction of Brown Algae on MicroRNAs in Type 1 Diabetes of Rats. Int J Nutr Sci. 2024;9(4):318-324. doi: 10.30476/ijns.2024.102136.1314.

Introduction

Diabetes Mellitus as a metabolic disease is determined by a progressive hyperglycemia as a result of insulin resistance in different tissues such as adipose tissue, liver and muscle and lead to insufficient production of insulin and due to β-cell dysfunction. Patients with history of type 2 diabetes may suffer from cardiovascular diseases (CVDs) as the most cause of death in these patients (1-3). Oxidized low-density lipoproteins (Ox-LDL) play a crucial role in initiation and progression of atherosclerosis. In diabetic patients, the serum

ox-LDL level increases with the length of diabetes, even though the patients' LDL-cholesterol level is maintained in the normal range (4). Ox-LDL-stimulated monocytes release inflammatory cytokines such as interleukin-6 (IL-6), IL-2, tumor necrosis factor-alfa (TNF-α), transforming growth factor-beta (TGF-β) and Mir-125a-5p that can interfere with uptake of ox-LDL by monocytes (5). In diabetic subjects, Mir125a-5p is upregulated by Ox-LDL and inflammatory cytokine as a protecting factor and can interfere with their function. It is worthy to mention that one of the leading causes of mortality in diabetic patients as atherosclerosis is exacerbated by Ox-LDL (5, 6).

Alginate is commonly extracted from brown algae. Its chain is made of homopolymeric regions of β-D-mannuronic acid and α-L-guluronic acid (7). It was shown that Na-alginate (at 600 mg/kg) significantly increased the weight of diabetic rats in comparison to the diabetic control, and decreased pre-prandial and postprandial glucose levels (8). α-L-guluronic acid was demonstrated to have immunomodulatory effects by decreasing the expression of Toll-like receptor 2 (TLR2) and Nuclear factor kappa B (NF-kB) genes in common variable immunodeficiency (CVID) patients (9). The β-D-mannuronic acid as an extract of algae drug plays a practical role in the augmentation of inflammatory diseases (10). In addition, β-D-mannuronic acid with low molecular weight has no reported toxicity when compared with other anti-inflammatory drugs (11).

MicroRNAs are short RNAs (about 19-24 nucleotides) that repress post-transcriptionally gene expression in eukaryotes. There are evidences suggesting that MicroRNAs are involved in extreme pathological processes (12, 13). Also, a previous study revealed that MicroRNAs are visible in the serum and plasma of mice, rats, humans and other animals. The levels of MicroRNAs in serum due to their reproducibility are stable among individuals of the same species (14). It was found that the expression of MicroRNAs profiles change in diabetes too (15). MiR-34a could induce apoptosis by inducing G0/G1 arrest in mouse pancreatic beta cells. In addition, higher expression of miR-34a may be related to the pathogenesis of Type 1 diabetes (T1DM). The serum level of miR-34a increases in diabetes, and in this regard MiRNA can be considered as a potential biomarker for diagnosis of T2DM (16). In addition, the expression of MiR-34a gene increases in atherosclerosis plaques of human (17, 18). Moreover, the other MiRNA which is involved in diabetes as a protective agent is named MiR-125a-5p. Upregulation of miR-125a-5p was observed in regulatory T (Treg) cells extracted from pancreatic draining lymph nodes (PLN) of T1D patients. Tumor necrosis factor receptor-2 (TNFR2) and C-C Chemokine Receptor type-2 (CCR2) were identified as miR-125a-5p target genes. Increase in expression of miR-125a-5p in Treg cells of diabetic patients can result in a decrease in CCR2 expression, and thus restrict their migration and operation in the pancreas (19). Also, expression of miR-126 was illustrated to decrease in T1DM (20). Furthermore, the miR-126 level acts as an essential factor in the efferocytosis process of apoptotic myocytes by macrophages in hyperglycemia conditions such as diabetes (21, 22).

Due to high prevalence of diabetes and its complications all over the world, as well as the lack of adequate studies on the effects of α-L-guluronic acid and β-D-mannuronic acid on serum levels of MicroRNAs, the present study was conducted in the animal model of type I diabetes using Sprague-Dawley rats to determine the effectiveness of α-L-guluronic acid and β-D-mannuronic acid on expression of three MicroRNAs of miR-34a, miR-126, miR-125a-5p.

Materials and Methods

Twenty-four young adult Sprague–Dawley rats (180-260 g) were used for this experimental study. Before the start of the experiments, the rats were housed under recommended temperature of 20-22°C and humidity of 55-65% for two days. A standard diet known as chow diet was started in a 12:12-hour light-dark cycle for optimal adaptation of animals. Protocol of animal care was approved by the Animal Care and Research Committee of Tehran University of Medical Sciences with registration number of 9411468009. The experimental model of type I DM (insulin-dependent DM) was induced by a single intraperitoneal injection of 60 mg/kg of streptozotocin (STZ, Sigma, St. Louis, MO) in 1 mM of phosphate buffer. Forty eight hours after injecting STZ, DM was confirmed with the animal's fasting blood sugar measurement. A glucometer was utilized to measure fasting serum glucose, which was considered an index of DM development and rats were considered to have DM if their glucose level was above 200 mg/dL. The rats became hyperglycemic two days after the injection and continued to have high blood sugar levels until they were sacrificed. At the end of study after 12 hours fasting, all rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg) as described before, and were then sacrificed under chloroform anesthedia (23).

The rats were divided into four equal groups of healthy control rats (Group I), STZ-induced diabetic control rats (Group II), diabetic rats treated with

α-L-guluronic acid (Group III) (Sigma R2625, USA), and diabetic rats treated with β-D-mannuronic acid (Group IV) (Sigma R2625, USA). Each group consisted of six animals. Group I received 0.5 mL normal saline, while group II was given identically the vehicle flaxen oil. Group III received 25 mg/kg of α-L-guluronic acid, and Group IV was administered with the same amount of β-D-mannuronic acid. In both intervention groups, the injections were intraperitoneally for four weeks. During the first three days of treatment, injections were given once per day, while other injections were every other day.

A peripheral blood specimen was drawn from each rat (6 mL/rat), and ethylene diamine tetra-acetic acid (EDTA) was used as an anticoagulant. The plasma was separated by centrifuging the blood samples at 1200 ×g for 10 minutes at 4°C. The plasma was stored in RNase-free tubes (1.5 mL) at -80°C for further analysis. At the end of the study which lasted four weeks, the rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and finally under chloroform anesthesia were euthanized. The hybrid-R miRNA isolation kit (GeneAll, South Korea) was utilized to extract microRNAs from rats' plasma. The procedure was carried out according to the manufacturer's recommendations. The concentration and purity of the extracted miRNAs were subsequently measured using the Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometer.

The first strand of cDNA was synthesized from miRNA templates using a stem loop cDNA synthesis primer, as described by Kramer in 2011 (24). Forward primers for each miRNA (miR-34a, miR-126, miR-125a-5p and miRNA-16 as a house keeping gene) were designed according to the Kramer and Busk methods (Bioneer Co., Daejeon, Korea) (24, 25). The sequences of all these forward primers were presented in Table 1.

First strands of cDNA were synthetized from total miRNA (5 µL) using the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara Bio, Siga, Japan), according to the manufacturer's protocol. Subsequently, the expression of MicroRNAs in treated and control groups were detected by real-time quantitative PCR using miScript SYBRGreen PCR kit (Takara Bio, Siga, Japan) and Applied Biosystem step one plus equipment. In each reaction, 2 µL of cDNA, 10 µL of SYBR Green I Master mix, 1 µL of specific primers, 1 µL of upstream universal primer, 5.5 µL of H_2O, and 0.5 µL of Rox were utilized. The real-time PCR was carried out in triplicate, and miR-16 was used as an endogenous reference for normalization. Relative changes in gene expression were calculated using 2-ΔΔCt, defined as △△Ct=(Ct MicroRNA - Ct miRNA-16) of the treatment group minus (Ct MicroRNA - Ct miRNA-16) mean of the control group (26). To measure glucose levels, the glucose oxidase protocol of a glucose kit (Biochemistry, Tehran, Iran) was applied. For the measurement of insulin levels, an ELISA kit (DiaMetra, Perugia, Italy) was used.

To analyze the data, the Kolmogorov-Smirnov test was used to test for normal distribution of the gene expression and final food intake data. Due to the non-normal distribution, non-parametric methods such as the Kruskal-Wallis test and one-way ANOVA were employed between-group analysis of the variables. On the other hand, fasting blood glucose and fasting insulin levels, and weight were normally distributed, and thus, the one-way ANOVA test with LSD post hoc was utilized to compare groups. To compare the mean concentration of different microRNAs in the control and intervention groups, the Kruskal-Wallis test was used. A minimum level of significance of 0.05 was applied to indicate statistical significance.

Results

Table 2 displays the body and biochemical characteristics of healthy and diabetic rats both at the baseline and after the respective interventions. For the diabetic rats treated with α-L-guluronic acid, it was found that the body weight increased significantly when compared to the diabetic control group. However, blood glucose level did not decrease in the control diabetic rats following treatment. In addition, there was no significant difference in the initial weight (p=0.879) or initial food intake (p=0.633) between the studied groups.

Table 1: Sequences of primers.

Primer name	Sequences
Stem loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACNNNNNN
Revers primer	CCAGTGCAGGGTCCGAGGTA
F-miR-34a	GGGGTGGCAGTGTCTTAGCT
F-miR-29b	GGGGGGTAGCACCATTTGAAATC
F-miR-125a-5p	GGGGTCCCTGAGACCCTTTAAC
F-miR-126a	GGGGGTCGTACCGTGAGTAAT
F-miR-16	GGGGGGTAGCAGCACGTAAATA

RT: Reverse Transcription, F: Forward primer.

Table 2: Body and biochemical characteristics in healthy and diabetic control groups at the baseline and after the interventions with α-L-guluronic acid and ß-d-mannuronic acid.

Variable	Group				*P* value
	α-L-guluronic acid (n=6)	Diabetic rats ß-D-mannuronic acid (n=4)	DC(n=6)	HC(n=5)	Between groups
Weight before intervention (g)	232±37	244±45	231±45	223±18	0.879
Final weight (g)	337±105[a]	272±61	208±41	303±32	0.031
Food intake before intervention (g)	21±1	23±1	24±5	23±3	0.633
Final food intake (g)	26±1	25±1	23±3	30±3	0.005
Fasting blood sugar before (mg/dL)	261±86[b]	264±47[b]	300±86[b]	96±14	0.001
Fasting blood sugar final (mg/dL)	241±145	330±115[c]	379±130[c]	92±14	0.007
Final fasting insulin (µIU/mL)	5±1[a]	6/095[a]	4	5[a]	0.81

DC: Diabetic control, HC: Healthy control. a: The mean values are statistically significantly higher than the diabetic control group (p<0.05). b: The mean values are statistically significantly higher than the healthy control group (p<0.05). c: The mean of the values is statistically significantly higher than the healthy control group (p<0.05).

However, a significant difference was observed in relation to the final weight (p=0.031) and regarding the final food intake (p=0.005). The analysis showed differences in the expression levels of miR-126 and miR-125a-5p in the sera between groups (healthy, diabetic control groups, and two diabetic groups treated with weather α-L-guluronic acid or β-D-mannuronic acid). Mir-34a expression level rose slightly in the diabetic group when compared to the healthy group (p=0.268). Furthermore, after treatment with α-L-guluronic acid and ß-D-mannuronic acid supplementation in diabetic rats, the expression of miR-34a decreased slightly in both treatment groups. MiR-126 expression declined remarkably in the diabetic groups when compared to the healthy control group (p=0.009). After treatment with α-L-guluronic acid, and ß-D-mannuronic acid supplementation, the expression of mir-126 was upregulated. This change was significant in ß-D-mannuronic acid group (p=0.026), while in the α-L-guluronic acid group was not significant (p=0.177, Figure 1a). Also, the expression of miR-125a-5p decreased dramatically in the diabetic groups when compared to the healthy control group (p=0.019). This change was reversed after treatment with α-L-guluronic acid and D-mannuronic supplementation. Fold change caused by α-L-guluronic acid was significant (p=0.041). On the other hand, ß-D-mannuronic acid treatment was not efficient (p=0.124, Figure 1b).

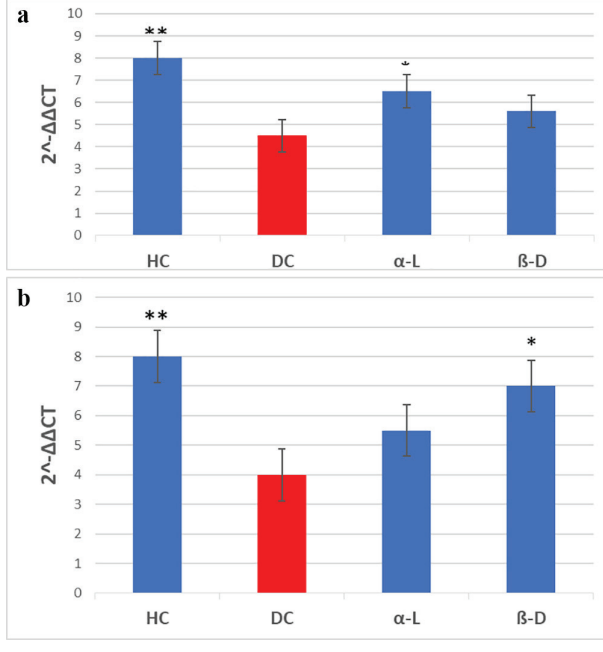

Figure 1: Circulating levels of miR-126(1a) and miR-125a-5p(1b) in sera of healthy control (HC), diabetic control (DC), α-L (α-L-guluronic acid.) and β-D (β-D-mannuronic acid) groups. qRT-PCR as the extraction technique was performed for miRNAs of plasma samples in four groups. *, **, *** depict $p\leq0.05$, $p\leq0.01$, $p\leq0.001$, respectively.

Discussion

Present work illustrated that plasma level of miR-126 and miR-125a-5p which known as a protective agents declined in control diabetic groups and ß-D-mannuronic acid and α-L-guluronic acid were able to rescue the expression of miR-126 and miR-125a-5p. Epigenetic and genetic changes were found to affect diabetes and progression of insulin resistant (27, 28). The involvement of MicroRNAs in cellular activities including proliferation, differentiation, and metabolic integration and also some diseases like diabetes was previously described (29). The major finding of our study was that miR-34a was upregulated and miR-125a-5p and miR-126 declined remarkably in the diabetic rats when compared to the healthy rats. Moreover, after treatment with single supplement (ß-D-mannuronic acid), mir-126 in diabetes rats inclined remarkably. In addition, after treatment with another selective supplement (α-L-guluronic acid), mir-125a-5p increased remarkably in diabetic rats. According to the report of Jahromi *et al.*, β-D-mannuronic acid is a novel immunosuppressive drug and able to modify signaling by toll-like receptors

(TLR) suppressing the nuclear factor-κB (NF-κB) and miR-146a as a new therapeutic approach (30).

A previous study revealed that miR-34a may be related to the pathogenesis of T2DM (17). Liu *et al.* illustrated that miR-34a can induce apoptosis by inducing G0/G1 arrest in mouse pancreatic beta cells (17). Also, Kong *et al.* have shown that in patients with a history of diabetes, the serum miR-34a level was significantly upregulated in comparison to non-diabetics patients (31). Serum miR-126 has been plummeting in impaired glucose tolerance (IGT), impaired fasting glucose (IFG) subjects and T2DM patients when compared to healthy controls (22). It has significantly increased in IGT-IFG and T2DM patients after diet therapy, insulin intake, and exercise for duration of six months. Furthermore, miR-126 is highly enriched in endothelial cells and contributes to the repair and maintenance of angiogenesis, and vascular integrity (32, 33).

Our results were in agreement with the findings of a previous study which showed that miR-126 in plasma of control diabetic group plunged when compared to control healthy group group, and could be considered as a biomarker for starting the vicious complications of diabetes like CVDs (34). Also, after treatment with β-D-mannuronic acid, the expression of miR-126 recovered significantly in comparison to the diabetic control group that had not received the treatment. In a previous study, individuals with a long history of diabetes and hyperglycemia were included that may reduce the expression of mir126 and thus expose the patients to CVDs (33), while our study was designed over one-month period. In addition, mir126 which is considered as a protective factor declined showing that mir-126 not only in long term but also in short term is affected by diabetes.

Mir-125a-5p can regulate the expression of genes in the mitogen-activated protein kinase (MAPK) signaling pathway that is important in T2DM pathogenesis. Ciccacci *et al.* and Herrera *et al.* showed that the expression of this miRNA declined in human and rat with T2DM when compared to the healthy individuals, respectively (35, 36). Our results confirmed the findings of these studies and revealed that miR-125a-5p could significantly drop in diabetic control group when compared to control healthy group. Also, after treatment with α-L-guluronic acid, the expression of miR-125a-5p rose significantly in comparison to the diabetic control group that did not receive the treatment.

As reported before, alginate could decrease fasting plasma glucose level in diabetic rats (8). Our study support a previous study which depicted that supplementation with α-L-guluronic acid and β-D-mannuronic acid can decrease fasting blood glucose level; but this effect was not significant (37). Present study revealed the same findings that the body weight of diabetic rats increased after treatment with α-L-guluronic acid. In addition, the fasting insulin level inclined in the two treated groups, while more efficacy was seen with ß-D-mannuronic acid therapy (37). However, foregoing study illustrated that α-L-guluronic acid led to a marked increase in final food intake of Sprague Dawley rats; while our study did not demonstrate the same results.

Diabetes has a complex etiology, which involves, genetics, diet, and environmental factors that affect the diagnosis and treatment. Currently, physician and health staff are using traditional methods using levels of HbA1c and glucose; however, these biomarkers are not early diagnostic factors for diabetes mellitus (38, 39). Hence, the discovery of the new therapeutic agents and biomarkers is necessary. Furthermore, it was shown that alginate as a dietary fiber can decrease post-prandial glucose level (40). Our findings showed that α-L-guluronic acid and β-D-mannuronic acid as a component of alginate could have beneficial effects on diabetes. It is also reasonable to measure mRNAs and inflammatory cytokines but due to budget restriction we had limitations to evaluate all these markers.

Conclusion

Mir125a-5p and mir126 were shown as biomarkers for diagnosis of hyperglycemia and also diabetes. Due to destructive consequences that can occur by diabetes, therapeutic agents that can reverse the miRNAs values such as α-L-guluronic acid and β-D-mannuronic acid were demonstrated as new supplementary medicines in diabetes which may need further researches too.

Acknowledgment

The authors would like to thank all the members that have participated in the research.

Authors' Contribution

M.S: Conceptualization, Investigation, Writing Original Draft Preparation, and Data Analysis. A.E and MR.E: Supervision, Methodology, Review & Editing. A.M and MH.J: Visualization, and Data Curation, Project Administration and Resources.

Conflict of Interest

None declared.

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