Cinnamon Extract Changes the Expression of miRNAs26-b, 29a, 223 and320 in Insulin Resistant Adipocytes

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Abstract
Objective: Insulin resistance (IR) is the major cause in Type 2 diabetes mellitus (T2DM). Expression of some miRNAs can be changed in response to a drug treatment for IR, and used as the biomarker in IR. This study set out to determine the effect of cinnamon extract (cinnamaldehyde) on some miRNAs expression in IR adipocytes.

Materials and Methods: In this In-vitro study the 3T3L1 cells were expanded in Dulbecco’s modified Eagle’s medium (DMEM), differentiated into adipocytes phenotype and insulin resistant with high glucose medium, then the cells were treated with cinnamaldehyde. To determine of the miRNAs profiling in 3T3L1 adipocytes, insulin-resistant adipocytes and treated insulin-resistant adipocytes quantitative real-time PCR method was performed.

Results: IR adipocytes exhibited a significantly increase in miRs 29a, 223 and 320 expression, and decrease in miR26-b expression in compare to the normal adipocytes (P-value<0.001 and P-value= 0.024 respectively). However in response to cinnamaldehyde in IR adipocytes, expression of miRs 29a, 223 and 320 were down-regulated while expression of miR26-b was up-regulated nearied it to the normal level (P-value= 0.003 and P-value= 0.002 respectively).

Conclusion: IR changes expression of intended miRs, so that cinnamaldehyde treatment helps to improve and normalize the changes. Cinnamon as the herbal product can be helpful for IR particular in adipose tissue.

Keywords: Insulin resistance, Diabetes, miRNAs, Cinnamon, Cinnamaldehyde, Adipocytes

Introduction

Type 2 diabetes mellitus(T2DM) as a common metabolic disease in the world is caused with low response of cells to normal amount of insulin as insulin resistance(IR) (1,2). Insulin sensitivity in cells and down-stream signaling related to insulin response were studied by several studies as important factors of pathologies in T2DM (3-6). The majority of individuals with T2DM are IR. Importantly, IR peoples are at risk for serious complications, including heart disease, some of cancer, polycystic ovarian syndrome,
Cinnamon & miRNAs 26-b, 29a, 223 and 320 and non-alcoholic fatty liver (7,8). Timely identification in IR can help to control of diabetes and its complications. MiRNAs (small non-coding single stranded RNAs) are considered as biomarkers for multiple diseases including cancer, cardiovascular, diabetes and obesity in initiation and progression of diseases (9-11). Studies report variable expression of serum miRNAs in IR persons and T2DM compared to insulin sensitive controls. Some studies have shown relationship between some miRNAs and its effects with diabetes and some molecular signaling so that expression profiles of miRNAs were changed and led to some complications as insulin resistance (12-17). However herbal products are used for anti-diabetic purposes (6,18,19). There was a number of in vitro and in vivo studies showing that cinnamon improves both IR and glucose metabolism. Cinnamon has multiple ingredients include polyphenoles, cinnamic acid, cinnamyl alcohol and cinnamaldehyde. Cinnamaldehyde is an effective compound in blood glucose with some known roles as autophosphorylation of insulin receptor, glycogen synthase activation and decrease of lipid level. However, the detailed mechanism of this anti-diabetic activity has not yet been clarified and is still controversial (20-22). This study set out to evaluate the effect of cinnamon extract on some miRs as 26-b, 29a, 320 and 223 in IR 3T3L1 compared with insulin-sensitive adipocytes as controls.

Materials and Methods
Cell line of mouse 3T3-L1 was prepared from Iranian Biological Resource Center (IBRC C10152), Dulbecco’s modified Eagle’s medium (DMEM) (include high or low glucose) from InoClon (InoClon.com) and fetal bovine serum (FBS) from Gibco (Bioidea company). 6-NBDG (6-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-6-deoxyglucose was purchased from Invitrogen by Life Technologies. Penicillin-streptomycin, trypsin-EDTA solution, isobutyl methylxanthine (IBMX), insulin, dexamethasone, oil red O, cinnamaldehyde (W228613), rosiglitazone were obtained from Sigma (Sigma Aldrich.com). RNU6 extraction kit, cDNA kit, real time kit and miRs primers were prepared from Qiagen (40724 Hilden, Germany).

3T3-L1 cell culturing and adipose phenotype differentiation
Cultivation of cells were done with high glucose DMEM in 6-plates and supplemented with, 10% of fetal bovine serum (FBS), 10 mM HEPES, 2 mM of glutamine and 1% solution of penicillin-streptomycin. The cells were cultured in a humidified environment with 95% air containing of 5% CO2 and 37 °C (23,24). For insulin-resistance studies and after 70-80 % of confluence of the cells, differentiation of the cells to adipocyte accompanied with especial medium including 10% FBS, 0.5 mM isobutyl methyl xanthine (IBMX), 0.25 µM dexamethasone, 10 µg/ml of insulin and 2 µM of Rosiglitazone. 48 hours later, the cells treated with differentiating medium with only 10µg/ml insulin for 2 days. Then high glucose DMEM replaced and changed fresh medium every 48 hours. Typically the cells converted to adipocyte phenotype and make lipid droplets after a few days. After 9 days, the morphology of the cells became as adipocytes that can be observed with staining of Oil Red O under Olympus microscope (25-28).

Staining of cells and confirmation of adipocyte differentiation
After culturing of 3T3L1 cells for 14 days, the cells were washed with phosphate buffered saline (PBS) for twice and fixed by 4% formalin diluted with PBS for 1 hour at environment temperature. Then the cells by 60% iso-propanol were washed and dried in environment. We used stock solution of 0.5g Oil Red O in 100ml iso-propanol which working solution was diluted in proportional 60% of stain and 40% distilled water. The cells stained for 2 hours and washed with...
distilled water and after drying observed by microscope (29,30).

**3T3L1 adipocytes conversion to insulin resistance**
To make IR, the cells cultured in 6-plates treated with 5.5mM glucose DMEM for 48 h. Then adipocytes treated with 25mM glucose (high glucose) and 1µM insulin (high insulin) for 24 h. Evaluation of IR adipocytes was determined by glucose uptake (31-34).

**Glucose uptake assay**
3T3-L1 adipocytes were in two groups: I. Insulin sensitive adipocytes (Non-IR) that were treated to 100mg/dl glucose with and without insulin (10ng/ml) II. IR adipocytes were treated with 100mg/dl glucose with and without insulin (10ng/ml) for 20 h. The adipocytes were starved with DMEM without serum and low glucose for 3 h. Then cells washed with PBS for three times and incubated with in low glucose DMEM containing 10ng/ml of insulin for 20 h. Controls were Non-IR cells without insulin. Residual glucose in culture medium was determined using photometric method with commercial glucose oxidase (GO) kit and glucose uptake was measured (35,36).

**Drug treatment**
The insulin resistant adipocytes were treated with cinnamaldehyde (100µg/ml concentration added in DMEM with 10%FBS) in 6-plates for 2 h at 37°C. Then, after collection of the cells and washing with PBS, RNA according to the protocol of kit was extracted.

**Quantitative real-time PCR (Q-PCR)**
Extraction of RNA according to kit protocol (Qiagen) was done by TRIZol. The purity and quantity of RNAs was estimated by nanodrop and absorbance at 260 and 280nm. Then cDNA made using of RT Kit (Qiagen). The miRNAs sequences were taken by miRBase site. Expression of miRNAs was determined by SYBR green. The level of miRNAs expression was calculated with the cycle threshold (Ct), that normalized with U6 snRNA (as internal control) and calculating of $2^{\Delta\Delta Ct}$. The samples were measured for triplicate.

**Statistical analysis**
SPSS software (version 16) was used. Data are shown as the mean ± SD. For statistical comparisons in two groups Student’s t-test was used, whereas for differences in multiple groups, ANOVA was used. $P$-value< 0.05 was significant limits.

**Ethical considerations**
Our study was confirmed by Ethics Committee in Shahid Sadoughi University of Medical Sciences, Iran (IR.SSU.MEDICINE.REC. 1395.23).

**Results**
Glucose uptake and confirmation of IR adipocytes is showed in Figure 1. Our result showed, glucose uptake following insulin stimulation in IR adipocytes was significantly less than Non-IR adipocytes ($P$-value= 0.008). With 0mM of insulin, there was no significant difference in glucose transport in IR adipocytes and Non-IR adipocytes ($P$-value= 0.225). As shown the figure 2 in IR adipocytes, expression of miRs29a, 223 and 320 had up-regulated (350, 40 and 82 times fold respectively) ($P$-value< 0.001) while miR26-b had down-regulated approximately 5 times fold ($P$-value= 0.024). Cinnamaldehyde treatment caused different expressions of miRNAs in IR 3T3-L1 adipocytes. MiR26-b expression was up-regulated (3.5 fold change) ($P$-value= 0.002) while miRs29a, 223 and 320 expression were down-regulated (0.03, 0.03 and 0.06 fold change respectively) with cinnamaldehyde ($P$-value= 0.003 and $p$-value= 0.004).

**Discussion**
In our study miR26-b was down regulated and miRs 29a, 223 and 320 were up regulated in
insulin resistant adipocytes compare to normal adipocytes.
Also cinnamon extract led to changes in the miRNAs expression in IR adipocytes. According to our results miR26-b expression had increased and returned to near normal level and conversely miRs 29a, 223 and 320 expression had decreased. Low response to normal insulin secretion is an important aspect in T2DM. In this case...
signaling of down-stream in insulin are almost inefficient (3). Some anti-diabetic drugs can improve this condition in different manner and help to blood glucose homeostasis. Leonardiniet alshowed that thiazolidinediones (TZDs) play a role in sensitization of insulin by activating of peroxisome proliferator-activated receptor (PPARγ) (37),and Inzucchi et al in a study showed that metformin, a biguanide and as anti-diabetic drugs, can be useful to insulin sensitivity and control of blood glucose in T2DM (4).

Cinnamon, as herbal product has anti-diabetic effects. Nikzamir et al revealed that cinnamaldehyde in treated cells a significant increase in glucose transporter 4 (GLUT4) gene expression and its up regulation( a glycoprotein that leads to glucose balance and consume glucose in adipose tissue and muscles) (22).

Hardie also showed that cinnamaldehyde increases the levels of peroxisome proliferator-activated receptor γ (PPARγ) and activates adenosine monophosphat-activated protein kinase (AMPK) that induces insulin sensitivity (38). Despite all the studies, molecular and cellular mechanism in cells especially adipocytes is not recognized.

MicroRNAs, small non-coding RNAs, play a role in various aspects in biological reactions including expression and regulation of gene, differentiation of cells and molecular metabolism. Many studies had shown different expression of miRNAs in persons with T2DM or insulin resistance compared to healthy groups with insulin sensitivity. The findings showed that hyperglycemia changed miRNAs expression so that led to up or down regulation of them (14,39-42).

Some studies showed that hyperglycemia enhanced expression of some miRNAs as miR320 (40-50 fold) or miR29-a (1.5 to 2 fold) (33,43,44). However agonists of PPARγ and the other insulin sensitizers, including metformin and TZDs are able to reduce expression of some miRNAs as miR320 in IR persons responders to TZD therapy (14,23,28,44).

Conclusions
The present study showed that cinnamon extract changed miRNAs expression related inIR similar to other studies. Changes in miRNAs can help to improve in insulin sensitivity following herbal medicine therapy. More studies on animal or human model are suggested.

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Conflict of Interest
There is no conflict of interest about the publication of this article.

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