MicroRNA-146a and Metabolic Disorder

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Abstract
MicroRNA-146a is one of the inflammation related microRNAs that constitutes an imperative part in the immune system. The present study was designed to evaluate whether microRNA-146a and its effect on the expression of target proteins (TRAF6 and IRAK1) are involved in metabolic disorders such as type II diabetes. In the current study, we investigated the expression levels of microRNA-146a in kidneys of twenty diabetic rats. The results showed a four-fold increase in microRNA-146a level in the kidneys of diabetic rats which was associated with a significant reduction in the expression of IRAK1 with no changes in TRAF6 expression levels. On the contrary, significant increase in NF-κB, TNFα, and IL6 and IL1β concentrations in kidney tissues of diabetic rats was observed. In conclusion, MicroRNA-146a can be used as a marker in case of diabetes.

Keywords: NF-κB, microRNA-146a, Diabetic Nephropathy, Diabetes type II-IL6, TNFα-IL1β

Introduction
MicroRNAs belong to a class of small non-coding regulatory RNA containing about 22 nucleotides in length [1]. They act through binding to the 3’UTR (untranslated region) of target mRNA leading to transcriptional repression or degradation of the target mRNA at the post-transcriptional level. The expression of MicroRNA-146a, one of the inflammation-related MicroRNAs that play a crucial role in the immune system, was provoked through lipopolysaccharide and nuclear factor kappa B dependent (NF-κB) [2]. MicroRNA-146a causes negative feedback regulation of the innate immune response via targeting the pro-inflammatory adapter proteins tumor necrosis factor (TNF) receptor-associated factor 6 and interleukin-1 receptor associated kinase 1[3]. Activation of these two target proteins (IL-1 receptor-associated kinase (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) (IRAK1 and TRAF6), motivates the NF-κB which encourages the transcription of the MicroRNA-146a gene that, sequentially, down-regulates two key adapter molecules, TRAF6 and IRAK1, to decrease NF-κB activity [4]. Diabetes mellitus (DM) is a chronic inflammatory disease, chronic hyperglycemia provides a case with high concentration of pro-inflammatory mediators inducing tissue inflammation and cell death [5]. It is believed that chronic hyperglycemia enhances glucose oxidation and enhanced glycation end products, activates protein kinase C, hexosamine and polyol pathway fluxes and finally increases pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1B [6].

Many of these hyperglycemia induced mechanisms converge to activate NF-κB, a pro-inflammatory master switch, which activates pro-inflammatory cytokines genes expression [7]. NF-κB has been reported to be a basic role in the development of diabetic nephropathy [8].

Diabetic nephropathy is characterized by glomerular and tubular hypertrophy, glomerulosclerosis and tubulointerstitial fibrosis [7]. Albeit numerous systems have explained the pathogenesis of diabetic nephropathy, it stays a standout amongst
the most imperative reasons of mortality in diabetic patients [8]. In recent years, several studies suggested that MicroRNA-146a participates in immune responses, cell proliferation, cell death and inflammation through a NF-κB-dependent negative loop [9]. Some new studies have uncovered the association of MicroRNA-146a in the pathogenesis of diabetic complications [10].

The damaged MicroRNA-146a has shown expression linked subclinical inflammation and insulin resistance in diabetic patients [11]. It also participated in regulating the extracellular matrix protein production in the retinas, hearts and kidneys of diabetic rats [12]. Such observation was associated to the involvement of MicroRNA-146a in the pathogenesis of chronic renal inflammation. It is possible that MicroRNA-146a contributes in the pathogenesis of diabetic nephropathy.

Therefore, the aim of this study was to evaluate whether microRNA-146a and its effect on the expression of TRAF6 and IRAK1 were involved in metabolic disorders such as type II diabetes.

Material and Methods

Animals management

Twenty male adult albino rats aged 8 weeks and weighting 150-200 grams were used in the experimental investigation of this study. The animals were obtained from the Laboratory Animal Unit (LAU), Faculty of Veterinary Medicine, Zagazig University, and were housed under traditional laboratory circumstances through the experimental period at the Experimental Animals Research Unit (EARU). The animals were fed on a standard rat pellet diet and allowed free access to water. Rats were kept at constant environmental and nutritional condition throughout the periods of the experiment and received balanced ration. All animals were acclimatized for a minimum period of two weeks prior to the beginning of the study.

Experimental design

Rats were divided into two groups: Control group (n=10) and diabetic group (n=10), rats were fed on standard rat feed and water for two months.

Induction of diabetes

Overnight fasting adult albino rats (n=10) were rendered diabetic via a single Intra-peritoneal injection of nicotinamide (110 mg/kg) prepared in normal saline. After 15 min Intra-peritoneal injection of Streptozotocin (STZ) (Sigma –Aldrich, Chemical Cp. St. Louis, Mo, USA) 60 mg/kg dissolved in cold citrate buffer (pH 4.5) was carried out. The rats were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycemia [13].

Blood glucose levels were measured two consecutive days after STZ injection by making an injury at the tail tip, squeezing gently and using the glucose oxidase method. Rats were considered diabetic if the blood glucose level reached 200 mg/dl or more [14]. The control group rats (n = 10) were injected only with the vehicle (0.01 M citrate buffer, pH 4.5).

Sampling

Blood samples were collected from orbital venous plexus and were used for estimation of blood glucose, blood urea nitrogen and creatinine [15].

Table 1: Primers used in determination of IRAK1 and TRAF6 gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Irak1</em></td>
<td>F: 5’-GCTGTGGGACACCAGAT-3’&lt;br&gt;R: 5’-GCTACACCATCCACA-3’&lt;br&gt;&lt;br&gt;<em>Traf6</em></td>
</tr>
</tbody>
</table>
Immediately after scarifying, kidneys were removed and wrapped in aluminum foil and put immediately in liquid nitrogen container, for real time-PCR analysis and for estimating the concentrations of TNF-α, IL-1β, IL-6 and NF-κB in kidney tissues.

**Biochemical determinations**

Serum glucose concentrations were assayed enzymatically using Glucose Assay Kit (ab65333). Blood Urea Nitrogen was assayed enzymatically by using Enzymatic Kit (Cat no-5602-01). Creatinine was assayed enzymatically using Enzymatic Kit (Cat no ab65340). The tissue level of rat TNF-α of experimental groups was measured by using Abcam’s TNF-α (ab100785) Rat ELISA kit following the manufacture instruction. The tissue level of rat IL1β of experimental groups was measured by using RayBio® Rat IL-1 beta ELISA Kit (ELR-IL1beta-001C) following the manufacturer instructions. The tissue level of rat IL-6 of experimental groups was measured by using RayBio® Rat IL-6 ELISA Kit (ELR-IL6-CL) following the manufacturer instructions. The tissue level of NF-κB of the experimental groups was measured by using mybiosource Enzyme-linked Immunosorbent Assay Kit for Nuclear Factor Kappa B (NF-κB) (MBS2020410 96) following the manufacturer instructions.

**Table 2: Primers used in determination of the gene expression of miRNA-146a**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroRNA-146a</td>
<td>F: 5’-UGAGAACUGAAUCCAUUGGUU-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ACCUGUGAAGUUCAGUUCUU-3’</td>
</tr>
<tr>
<td>miR-191a</td>
<td>F: 5’-CAACGGAAUCCCAAAAGACUGUG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GCUGCAGUUGGAGUUCGUCCC-3’</td>
</tr>
</tbody>
</table>

**Molecular analysis**

**Real-time quantitative RT-PCR (qRT-PCR) Analysis of IRAK1 and TRAF6 gene expression**

To validate the magnitude of IRAK1 and TRAF6 genes with SYBR® green based data, real-time quantitative PCR analysis has been performed with tissue homogenate and specific synthesized primers (Table 1). Total RNA from tissue samples was extracted using Jena Bioscience total RNA purification kits (Cat-No. PP-210S) following the manufacturer guidelines. Reverse transcription of total RNA using Jena Bioscience SCRIPT RT-PCR Two-Step Kit (Cat.-No. PCR-506S) was carried out.

**Real-time quantitative PCR (qPCR) Analysis of miRNA-146a in experimental groups**

Extraction of miRNA-146a from the tissue using mirVana miRNA Isolation Kit (Part Numbers AM1560, AM1561) was carried out. Reverse Transcription for Quantitative Real-Time PCR using the miScript PCR Starter Kit (Cat.-No. 218193) was performed. The final reaction mixture was placed in a Rotor-Gene 3000 real time thermal cycler and real time PCR program was carried out by initial denaturation at 95°C for 15 min to activate host Taq DNA polymerase followed by 40 cycles of 94°C for 15 sec for DNA denaturation, annealing at 55°C for 30 sec, extension at 70°C for 30sec, and fluorescence data collection. The sequences of the primers are illustrated in Table (2).

**Statistical analysis**

Data were expressed as the mean±SE and were analyzed by Independent t test using PASW Statistics (computer program SPSS, Inc. version 22, IBM Corp. 2013, Armonk, NY). A level of p≤ 0.05 was considered statistically significant.
Results

Biochemical Analysis

Blood glucose

The blood glucose concentration of STZ-diabetic rats was significantly highly increased (364.65±17.69mg/dL) compared with the control one (71.15±2.62mg/dL) (Table 3).

Serum creatinine

Serum creatinine showed highly significant increase in the STZ-diabetic group (2.35±0.15mg/dl) compared with control one (0.51±0.02mg/dl) (Table 3).

Blood urea nitrogen

As shown in Table (3) BUN levels of STZ-diabetic rats were significantly high (49.37±2.02mg/dl) compared with the control one (19.67±0.59mg/dl).

Cytokine and NF-κB levels in kidney tissues

The level of the pro-inflammatory cytokines IL6, IL1β, TNFα and NF-κB in the STZ-diabetic rats were significantly increased (106.36±4.48, 49.35±2.06, 43.16±1.80 and 1.74±0.08 pg/mg), compared with the control group 66.42±2.40, 15.42±1.14, 14.93±0.82 and 0.56±0.02 pg/mg, respectively (Table 3).

Molecular Analysis

Relative quantification of IRAK1 and TRAF6 gene expression in renal tissues

Results in Figure (1A) showed that by the change in the CT number there was down regulation about the two thirds in the expression of IRAK1 in diabetic rats. Results in Figure (1B) showed that by the change in the CT number there was slightly change in the levels of gene expression of TRAF6.

Relative quantification of microRNA-146a expression in renal tissues

Results in Figure (1C) showed that by the change in the CT number there was fourfold increase in the level of MicroRNA-146a in diabetic kidney compared with the control.

Table 3: Means ± S.E. of blood glucose, blood urea nitrogen, serum creatinine and the pro-inflammatory cytokine levels in kidney homogenate of STZ-diabetic rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (mg/dl)</th>
<th>STZ-Diabetic group (mg/dl)</th>
<th>t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>71.15±2.62</td>
<td>364.65±17.69</td>
<td>-16.42</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>19.67±0.59</td>
<td>49.37±2.02</td>
<td>-14.14</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.51±0.02</td>
<td>2.35±0.15</td>
<td>-13.21</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>66.42±2.40</td>
<td>106.36±4.48</td>
<td>-7.85</td>
</tr>
<tr>
<td>Interleukin-1β</td>
<td>15.42±1.14</td>
<td>49.35±2.06</td>
<td>-14.41</td>
</tr>
<tr>
<td>Tumor necrosis Factorα</td>
<td>14.93±0.82</td>
<td>43.16±1.80</td>
<td>-14.27</td>
</tr>
<tr>
<td>Nuclear factor -kappaB</td>
<td>0.56±0.02</td>
<td>1.74±0.08</td>
<td>-13.74</td>
</tr>
</tbody>
</table>

Each value represented the means ± S.E. of 10 samples, each group. ** Highly significant difference (p<0.01)

![A] Figure 1: (A) relative gene expression of IRAK1 gene in renal tissues of diabetic and control rats (B) relative gene expression of TRAF6 gene in renal tissues of diabetic and control rats (C) relative gene expression of microRNA146a expression in renal diabetic and control rats
Discussion

Recently, chronic inflammations and oxidative stress had been recognized to play a crucial task in the commencement, promulgation, and development of metabolic syndromes [16-18]. Field studies revealed a solid correlation among pro-inflammatory biomarkers (such as IL-6, TNF-α) and perturbations in glucose homeostasis, obesity and atherosclerosis [19].

MicroRNAs were expressed not only in a tissue-specific mode, but also in cell-type specific manner that assume fundamental parts in numerous biological processes, embracing propagation, apoptosis, progress and discrimination [20]. Moreover, MicroRNA is related to inflammation, oxidative stress, damaged adipogenesis, insulin signaling, apoptosis and angiogenesis in relative to obesity. All of these routes play a role in the expansion of type II diabetes, atherosclerosis, and the linked with cardiovascular disorders [20].

In the current study we investigated the expression levels of MicroRNA-146a in kidneys of diabetic rats. Our results showed a fourfold increase in MicroRNA-146a level in the kidneys of diabetic rats which was associated with significant reduction in the expression of IRAK1 with no changes in TRAF6 expression levels.

On the contrary, significant increase in NF-κB, TNFα, IL6 and IL1β concentrations in kidney tissues of diabetic rats was observed. It was believed that the increased level of blood glucose activated protein kinase C, polyol and hexosamine pathway fluxes, enhanced the advanced glycation end products and induced oxidative/ nitrosative stress in different cells, including leukocytes and renal cells [21]. Also, it has been depicted that sustainably increased level of blood glucose enhances the blood concentration of angiotensin II [22]. All of these factors enhance activation of NF-κB to produce its effect on decreased pro-inflammatory cytokines [8].

Diabetes either of type I or type II DM is characterized by sustained hyperglycemia and chronic elevation of pro-inflammatory environment induced and perpetuated the inflammatory response [23]. However this condition was considered a low–grade inflammation, since the hyperglycemia-provoked oxidative stress upgraded inflammation via augmented endothelial cell impairment, microvascular permeability and amplified the production of pro-inflammatory cytokines, consisting of TNFα, IL6 and IL1β eventually decreased the insulin sensitivity and diabetic complications [23].

It’s well-known that, pro-inflammatory cytokines in renal tissue chip create and progress the diabetic nephropathy [7]. Thomson et al. [24] added that there was a direct relationship between the kidney weight and the renal TNFα, IL6 and IL1β expression. These data coincide with the obtained results in our study in which TNF-α, IL-6 and IL-1β and NF-κB levels were significantly increased in the kidneys of diabetic rats. Moreover, the blood urea and creatinine levels were higher in diabetic rats than in the control rats. All of these results can be considered an indication of the renal damage that results from two-month uncontrolled diabetes.

NF-κB is a transcription factor which has the ability to incorporate signaling pathways to transcriptional regulation of many genes related to inflammation, immunity, apoptosis, cell proliferation and demarcation [25]. NF-κB regulates its activation partially by a negative feedback loop during sever inflammatory processes, in which, NF-κB activation up regulated MicroRNA-146a gene that, upon processing and maturation, down regulated IRAK1 and TRAF6 (the two key adaptor molecules downstream of cytokine and Toll-like receptors) to minimize the activation of NF-κB [9].

Although the anti-inflammatory effect of MicroRNA-146a gene has been reported by several studies, the above mentioned negative loop has not been shown by some investigations. For instance, a recent study has shown an overexpression of MicroRNA-146a and TRAF6 with attending decreased
expression of IRAK1 in the peripheral mononuclear cells of patients with Sjogren syndrome [26].

Also, Liu et al. [27] reported that the up regulation of MicroRNA-146a was not accompanied with down regulation of IRAK1 and TRAF6 in helicobacter pylori induced inflammatory response in human gastric epithelial cells. The results showed highly significant increase in NF-κB associated with an increase in the expression of MicroRNA-146a. It has been suggested that the expression of MicroRNA-146a was directly controlled by NF-κB and mutation of NF-κB binding sites in the miR-146a promoter abolishes NF-κB mediated induction of MicroRNA-146a [28]. When the activation of NF-κB taken a vital occasion in the progression of diabetes complications, it was possible that a defect in the MicroRNA-146a mediated negative loop gave a situation for maintained inauguration of NF-κB and its objectives to promote cells in the direction of abnormalities.

In this regard, Balasubramanyam et al. [10] reported that the high expression of NF-κB in peripheral mononuclear cells of patients with diabetes was not accompanied by MicroRNA-146a gene overexpression resulting in elevated expression level of TRAF6 but not IRAK1. Also, the experiment showed a fourfold increase in MicroRNA-146a expression level, accompanied with a significant down regulation of IRAK1 but not in TRAF6. It may be possible that TRAF6 to be under other levels of control, or TRAF6 had different response to moderate or severe induction of MicroRNA-146a in different levels of cytokines stimulations [28]. Overall, the up regulation of MicroRNA-146a in renal inflammatory diseases has been previously reported [12].

Feng et al. [11] showed a reduction in the expression level of MicroRNA-146a in the kidneys of diabetic rats either type I or type II. At present, it is unknown whether MicroRNA-146a dysregulation is causal to diabetes or is resulted from it. However, its action may depend on tissue type, blood and tissue cytokines concentrations, timing and duration of inflammation. Alipour et al. [29] introduced the initial data on overexpression of MicroRNA-146a in the diabetic kidney; however this up regulation was not followed by significant decrease in IRAK1 and TRAF6 expressions.

**Conclusion**

It is unknown whether MicroRNA-146a up regulation is causal to diabetes or is resulted from it. However, its action may depend on tissue type, blood and tissue cytokines concentrations, timing and duration of inflammation, so all this points need further study. Also MicroRNA-146a can be used as a marker in case of diabetes.

**Conflict of interest**

The authors declare no conflict of interest.

**References**


