Swimming training attenuates pancreatic apoptosis through miR-34a/Sirtu1/P53 Axis in high-fat diet and Streptozotocin-induced Type-2 diabetic rats

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Abstract

Objective The present study sought to evaluate the miR-34a/Sirtuin1/p53 pro-apoptotic pathway, and reveal its modulation in diabetic rats undergoing swimming exercise.

Methods Twenty-eight male Wistar rats were divided into four groups. They were induced to develop diabetes by injection of streptozotocin. After 12 weeks of swimming, the pancreatic tissue of these rats were removed to be evaluated for the expression level of Sirtuin1/P53/miR-34a through qPCR.

Results Findings indicated a marked rise in the expression of miR-34 and P53 ($P < 0.01$) as well as a significant decrease in expression of Sirtuin1 ($P < 0.01$) in the diabetic group. In contrast, swimming resulted in a significant decrease in miR-34a expression ($P < 0.01$), and a prominent rise in the level of Sirtuin1 in the swimming-trained-diabetic group ($P < 0.01$). Additionally, high, moderate and low apoptosis rate were observed in the pancreatic tissue of the diabetic, swimming-trained diabetic, and control groups, respectively.

Conclusion Our findings suggested a correlation between pancreatic tissue apoptosis rate and miR-34a/Sirtuin1/p53 signaling, that was subject to modulation by training.

Keywords Diabetes · Swimming training · Apoptosis · miR-34a · Sirtuin1 (SIRT-1) · P53

Introduction

Showing an exponential rise in the incidence rate on a global scale, type 2 diabetes (T2D) is an insidious systemic disease with ever-expanding epidemic proportions [1]. T2D develops as a result of combined genetic predisposition and environmental

factors, that include hyper caloric diet, obesity and sedentary lifestyle [2, 3]. The disease, as a progressive metabolic disorder, is characterized by hyperglycemia, peripheral resistance to insulin, and insulin insufficiency. Peripheral insulin resistance occurs long before the development of hyperglycemia, and is characterized by the reduced response to insulin in some tissues [4, 5].

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In the skeletal muscles, resistance to insulin leads to a hallmark decrease in glucose uptake. In the liver, resistance to the insulin-mediated inhibition of hepatic gluconeogenesis and glycogenolysis results in the increase of glucose output by this organ. On the other hand, resistance to the anti-lipolytic function of insulin in the fat tissue enhances lipolysis and the release of free fatty acids (FFA) into the circulation. In addition, chronic exposure to high concentrations of glucose induces beta-cell (β -cell) glucose toxicity and apoptosis [5, 6]. Combined T2D and obesity in patients is frequently associated with increased serum FFA, that can result in pancreatic β -cell death. It has also been confirmed that apoptosis might be a culprit in β -cell loss as a result of T2D [6]. Nevertheless, the exact mechanisms involved in instigation of β -cell failure through the natural history of T2D have not been elucidated.

Today, many studies tend to focus on the key role of non-protein coding RNAs in the development and progression of T2D [3]. MicroRNAs (miRNAs), a subtype of non-coding RNAs [7], are small endogenous and highly conserved RNAs involved in important biological and physiological events [8, 9] such as proliferation, development and differentiation [10–12], inflammation, oxidative stress, cell damage, insulin signaling, cardiovascular disease, diabetes [10, 13] and tumorigenesis [14–16]. T2D-related miRNAs are miR-34a, miR-146a, miR-29a, miR-124a, miR-375 and miR-9 [17]. Up-regulation of miR-34a has been reported to be correlated with an impairment in the function of β -cells, and their increased rate of apoptosis [18].

Previous studies reported Sirtuin1 (SIRT1) as an important target of miR-34a, while demonstrating a negative correlation between the expression of miR-34a and SIRT1 [19]. SIRT1 is a nicotinamide adenine dinucleotide (NAD⁺-dependent deacetylase) with potential useful effects on glucose homeostasis and peripheral sensitivity to insulin. This molecule is actually an intermediate by which miR-34a induces its effects on the P53 pathway, and ultimately apoptosis [20]. The sirtuin class is recognized as a novel family of enzymes with important implications in the emergence of several ailments, such as cancer [19]. Presumably, the induction of apoptosis through miR-34a is determined by the expression level of its target proteins that mediate cell death. The most recognized direct target of miR-34a, SIRT1 mediates apoptosis whenever oxidative and genotoxic stresses are present [19]. Inhibition of SIRT1 may result in a miR-34a-induced increase in p53 acetylation and transcription, that can eventually lead to up-regulation of pro-apoptotic molecules such as “p53 upregulated modulator of apoptosis” (PUMA). Since the miR-34 family are direct transcriptional targets of p53, they can also initiate a positive feedback loop [21]. Exacerbation of inflammation in T2D positively regulates p53, which is thought to be a biologically active molecule in mitochondrial pathways of apoptosis observed in diabetes [22].

Recently, certain studies have indicated that regular physical activity could alleviate hyperglycemia in diabetes. In 2016, scientists reported that exercise training had beneficial effects on lipid metabolism and insulin sensitivity through musclin downregulation and GLUT4 upregulation in the animal models of insulin resistance [23]. In a previous study, in 2019, we showed that swimming training induced a considerable increase in the pancreatic production of SIRT1, serum albumin, antioxidant enzymes, and metabolic parameters in diabetic rats receiving high-fat diet (HFD) [24]. Accordingly, more evidence on the importance of exercise in the molecular pathways involved in the pathogenesis of diabetes, especially the ones mediated by miRNAs, are required for a better understanding of the role of SIRT1 in this metabolic disease [25].

Hence, in this study, we evaluated whether swimming training through miR-34a and SIRT1 axis could modulate p53 level in the pancreas, and affect pancreatic cell apoptosis.

Materials and methods

28 male Wistar rats were purchased from the Animal Conservation Center of Tabriz University of Medical Sciences. They were randomly assigned to 4 different groups: diabetic (Dia), control (Con), swimming-trained diabetic (Dia + Exe), and swimming-trained control (Exe), each with 7 subjects. The rats had free access to water and food, while being exposed to 12 h of light and darkness each at a temperature of 22–25°C throughout the study. The protocol for this study was planned to follow the guidelines of NIH, and be in agreement with the code of conduct defined by the Ethics Committee for the Use of Animals in Research at Tabriz University of Medical Sciences.

Induction of T2D

For induction of T2D, a combination of HFD and low-dose streptozotocin (STZ) was used [26]. Based on this method, the test subjects received HFD for 4 weeks (48% carbohydrate, 22% fat, 20% protein). In the end of this period, the rats were intraperitoneally (i.p.) injected with 35 mg/kg of STZ. Seven days later, diabetic rats were selected for further investigations provided that they had a non-fasting basal plasma glucose (PGL) of ≥ 300 mg/dL.

Swimming training

A 60 × 100 cm cylindrical tank was filled with 35°C water up to 35–45 cm in height, and used for training. During the course of the training, exercise groups were allowed to swim for 60s once a day (for 60 min/5 days/weekly for 12 weeks) [26, 27].

Preparation of tissue samples

As a final step to the intervention, the rats were given deep anesthesia through the i.p. injection of 60 mg/kg of ketamine sodium and 4 mg/kg of xylazine following a period of fasting overnight. The pancreatic tissue was immediately removed, and homogenized after being washed with cold normal saline, as described by Carrillo et al. [28]. The extracted tissue was evaluated for the expression of miR-34a, SIRT1, and p53, along with apoptosis.

Real-time PCR

- Real-time PCR was adopted for the measurement of mRNA and miRNA expression in the pancreatic tissue [26]. With miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark), total RNA was extracted from 300 mg of pancreatic tissue. The quality and quantity of the total RNA was estimated by Nanodrop 1000 spectrophotometer (Thermo scientific, Wilmington, DE, USA) based on the relative absorbance ratio at A260/A280 and A260/A230 ratios. The samples were utilized in a concentration of 522 ng/μl.
- A cDNA synthesis kit was used for production of cDNA (Fermentas GmbH, Leon-Rot, Germany). For this purpose we followed the primer-extension method. Using the enzyme poly (A) polymerase, a poly A tail was added to the 3' of miR-34, followed by the addition of oligodT adapter primer and reverse transcriptase. 3 μg of total RNA was mixed with a total of 52 μl reaction buffer containing 500 U Moloney murine leukemia virus (MMLV) reverse transcriptase, 5 μl dNTPs, 60 U RNase inhibitor, 10 μl buffer 10X 8 μl and 5 μl Random hexamer primers. The reaction was initiated at 65 °C for 5 min, and then cooled on ice for 5 min. The mixture was then incubated for 12 min at 85 °C for hybridization, and 48 min at 48 °C for elongation. Final denaturation and deactivation was attained within 5 min at 25 °C, and then the samples were stored at 82 °C.
- Real-time PCR was initiated using the SYBR Green master mix (Exiqon, Vedbaek, Denmark) and a Bio-Rad iQ5 detection system (Bio-Rad, Richmond, CA, USA). The PCR reaction was performed using 4 μl of cDNA, 5 μl of SYBR Green PCR master mix and 1 μl of primer mixture in a final reaction volume of 12 μl. Initial denaturation and enzyme activation were attained with a Roche light cycler 96 set to 95 °C for 10 min and 45 cycles, 95 °C for 10 s and 60 °C for 60 s. The amount of PCR products were normalized with housekeeping gene (β-actin and miR-191) to determine the relative expression ratios of the gene. Finally, melt curve analysis was performed to determine the specificity of the PCR reaction. The relative expressions of SIRT1 and P53 mRNA, and miR-34a

were determined based on their threshold cycles (Ct) compared to that of the housekeeping genes (β-actin and miR-191). Relative quantification was analyzed by the 2- $\Delta\Delta C_t$ method. Fold change was calculated; (Mean expression of gene in therapy group- mean expression of gene in control group)/mean expression of gene in control group OR Mean expression of gene in therapy group/ (mean expression of gene in control group – 1)

Apoptosis assessment by TUNEL assay

Terminal deoxynucleotide transferase mediated dUTP nick end labeling (TUNEL) [29] staining was performed to detect apoptosis of Langerhans islets by means of an in situ Cell Apoptosis Detection Kit (Roche, In Situ Cell Death Detection Kit, Fluorescein). 5 μm-thick slides were prepared from paraffin-embedded pancreatic tissues via microtome apparatus, and then the purification steps and removal of paraffin from the tissues were done. Briefly, tissues embedded with paraffin were stripped of their paraffin and then hydrated, and then incubated for 5 min in 20 g/mL proteinase K at room temperature. Following a two-round washing, the samples were immersed for 5 min in a pH = 6 sodium citrate buffer comprising 10 mmol/L trisodium citrate and 2 mmol/L citric acid with a temperature of 37°C. After a two-round washing with phosphate-buffered saline (PBS) for 5 min each, 20 mL TUNEL reaction mixture (1 mL digoxin-labelled d-UTP, 1 mL terminal deoxynucleotidyl transferase, and 18 mL Labeling Buffer) was added to the samples, and the resulting mixture was incubated for 60 min at 37°C. After being washed, the mixture was incubated with biotin-anti-digoxin antibody for 30 min at 37°C, and then processed with DAB Substrate Kit. The resulting slides were then counterstained with hematoxylin lightly, dehydrated and then mounted. Each tissue section was examined under the microscope with a magnification of ×400.

Statistical analysis

Data were statically analyzed by ANOVA and Tukey's test. Results were presented as Mean ± SEM. A $p < 0.05$ was considered to be statistically significant.

Results

Effect of swimming training on the expression of miR-34a in the pancreatic tissue of diabetic rats

As shown in Fig. 1, miR-34a was significantly up-regulated in the pancreatic tissue of diabetic rats compared to the Con group ($p < 0.01$). However, swimming training markedly attenuated the pancreatic expression of miR-34a in the Dia-Exe group in relation to the Dia group ($p < 0.01$).

Fig. 1 Pancreatic expression levels of miR-34a in studied groups: diabetic (Dia), control (Con), diabetic swimming-trained (Dia + Exe), swimming-trained control (Exe) rats. Data are presented as mean ± SEM. * $p < 0.01$ significant difference vs control group and # $p < 0.01$ significant difference vs Dia group

The Fold change of miR-34 was calculated Exe/Con: -0.6 , Dia/Con: $+1.6$, Dia-Exe/con: -0.62 .

Effect of swimming training on the mRNA expression of SIRT1 and p53 in the pancreatic tissue of diabetic rats

A comparison between the four groups indicated a significantly lower expression of SIRT1 (Fig. 2a) in the Dia group compared with the Con group ($p < 0.01$). However, p53 expression (Fig. 2b) was found to be significantly increased in the Dia group compared with that of the Con group. Meanwhile, as can be inferred from the Fig. 2a, the swimming training remarkably elevated the SIRT1 expression in the Dia-Exe group in comparison to the Dia group ($p < 0.01$). The Fold change of SIRT-1 was found; Exe/Con: $+0.01$, Dia/Con: -0.2 , Dia-Exe/con: -0.05 .

Also, swimming training highly decreased the p53 expression level in the Dia-Exe group compared to that of the Dia group (Fig. 2b) ($p < 0.01$). The Fold change of p53 mRNA was found Exe/Con: $+0.05$, Dia/Con: $+0.3$, Dia-Exe/con: $+0.15$.

Fig. 2 Pancreatic expression levels of SIRT1 (A) and P53 (B) mRNA in studied groups: diabetic (Dia), control (Con), diabetic swimming-trained (Dia + Exe), swimming-trained control (Exe) rats. Data are shown as mean ± SEM. * $p < 0.01$ significant difference vs control group and # $p < 0.01$ significant difference vs Diabetic group

The effect of swimming training on apoptosis in the pancreatic tissue of diabetic rats

As shown in Fig. 3, high, moderate and low apoptosis rate were observed in the pancreatic tissue of the diabetic, swimming-trained diabetic, and control groups, respectively. This suggests that diabetes may lead to increased apoptosis in the pancreatic tissue (TUNEL-positive cells), which could be counter-regulated by swimming training (Table 1).

Discussion

In the present study, miR-34a and p53 were upregulated, while the expression of SIRT1 was downregulated in response to HDF-STZ-induced T2D in the pancreatic tissue. Based on our findings, the upregulation of SIRT1, and downregulation of miR-34a and p53 might suppress the diabetes-induced apoptosis in pancreatic tissue. According to another study, SIRT1 may potentially play a protective role against diabetes-induced apoptosis [30]. By and large, this study revealed that the miR-34a-SIRT1/p53 axis may play significant role in the pathogenesis of diabetes, and the consequential elevated apoptosis rate in the pancreatic tissue, which might as well serve as a target pathway in treatment of diabetic. Several *in vivo* investigations, including our study, have supported the theory that diabetes can lead to an increased incidence of apoptosis [31]. Importantly, hyperglycemia, as the hallmark sign of diabetes, might accelerate apoptosis in the pancreatic tissue [30]. Moreover, one study suggests that hyperglycaemia can lead to oxidative stress, which may be an influential factor in the pathogenesis of diabetes-associated complications [32]. It has also been documented that oxidative stress is a primary cause of apoptosis [33].

The occurrence of apoptosis and abnormal β -cell function in the presence of fatty acids (FAs) is a common finding among several studies [34, 35]. Many factors are involved in the T2D-induced apoptosis. Chronic hyperglycemia is toxic to the β -cells, and eventually causes apoptosis in the Langerhans islets [36]. HFD and obesity are frequently linked with

Fig. 3 Apoptotic pancreatic cells detected by TUNEL staining. Representative photomicrographs shown here demonstrate TUNEL staining of pancreas tissue sections from the control (Con) group (A), swim trained (Exe) group (B), the diabetic (Dia) group (C), and the diabetic-swim trained (Dia + Exe) group (D). TUNEL-positive cells are discerned by brown staining

elevated levels of FFAs and blood glucose may promote β -cell dysfunction and death [35].

As mentioned earlier, our findings showed considerable overexpression of miR-34a in the pancreatic tissue of rats with T2D. Interestingly, increased levels of miR-34a had previously been reported in β -cells treated with FAs [37]. Previous studies demonstrated that miR-34a was also correlated with metabolic diseases, e.g. diabetes and obesity [38, 39]. miR-34a is considered a pro-apoptotic miRNA, that may lead to cell death if upregulated [40, 41]. In other words, the transient knockdown of miR-34a induces inhibitory effects on β -cell line MIN6 apoptosis [41, 42].

In this context, the primary findings of this study highlighted the inhibitory effect of swimming exercise on the pancreatic production of miR-34a, along with a positive regulatory effect on the SIRT1 expression. Consistent with the present

study, there are several accounts of the pro-diabetic and pro-obesity functions of miR-34a through the repression of SIRT1 (13, 35). One study suggested that miR-34a overexpression dwindled SIRT1 expression, and accelerated the rate of free fatty acid-induced apoptosis in murine hepatocytes (16). It is thought that upregulation of SIRT1 may hinder the progression rate of complications caused by diabetes [43].

Recent investigations suggest that miR-34a can trigger apoptosis through the SIRT1/P53 axis. In 2018, scientists found that hippocampal neural apoptosis may arise as a consequence of high blood glucose in the streptozotocin-induced diabetic mice in response to increased acetylation of p53 and down-regulation of SIRT1 [44]. Another report indicated that miR-34a targeted *SIRT1* mRNA, which induced apoptosis in the testicular tissue of diabetes-induced C57BL/6 male mice [45]. In accordance with these findings, we noticed that the

Table 1 The effect of swimming training on apoptosis in diabetic pancreas tissue

Studied groups				
	Con	Dia	Exe	Dia-Exe
Tunnel positive apoptotic cells	±	++++	±	++

Abbreviations;

±: rare apoptotic cells, ++++: numerous apoptotic cells, ++: medium apoptotic cells

control (Con), diabetic (Dia), swimming-trained control (Exe) and swimming-trained diabetic rats (Dia + Exe)

expression of miR-34a in the pancreas of diabetic rats was associated with the concomitant up-regulation of SIRT1 and P53, and acceleration of apoptosis. Our findings suggested that the miR-34a/SIRT1/P53 pathway might have contributed to apoptosis of pancreatic cells. We also found a significant down-regulation in the SIRT1 mRNA level in the pancreatic cells. A recent study by Sun et al. reported down-regulation of SIRT1 in insulin-resistant C2C12 myo-tubes and mice receiving HFD [46]. Our reports were in agreement with another study that indicated attenuated expression and activity of SIRT1 in DM [47].

Additionally, we showed that swimming training induced miR-34a downregulation, SIRT1 up-regulation and prevented P53 acetylation and apoptosis in the pancreatic tissue of rats with T2D. Attenuation of pancreatic apoptosis is arguably one of the most effective strategies to preserve Langerhans islets against cell death and reverse an upset metabolic status. It should be noted that physical activity is a rather well-known and strong inhibitor of pancreatic apoptosis in diabetes [48]. Previous studies reported that exercise induced protective anti-apoptotic signals [49] through increasing the anti-ageing genes like SIRT1. It has been showed that SIRT1 is involved in several cellular functions such as cell cycle regulation, gene silencing, apoptosis and energy homeostasis [50]. Most notably, SIRT1 is capable of deacetylating P53, thus, attenuating its effect in transactivation of the downstream target genes, e.g., p21 and Bax in cell cycle and apoptosis, respectively [51]. The effects of physical activity on serum and tissue levels of SIRT1 have not extensively been investigated. In this regard, Saremi et al. showed that exercising for two months led to a significant increase in the serum level of SIRT1 [3]. In another study, Chi Huang et al. revealed that swimming enhanced SIRT1 level in gastrocnemius and soleus muscles of the mice [52]. Likewise, Causu et al. reported that SIRT1 levels were increased in healthy mice after six weeks of training [53]. Conversely, Marton et al. findings revealed that physical activity had no effect on the serum levels of SIRT1 [54].

Exercise training alters the expression profile of microRNAs that brings about beneficial regulatory effects in T2D [55]. In other words, the beneficial effect of exercise is a decrease in the pancreatic level of miR-34a. Excessive production of miR-34a may promote apoptosis [18]. Previous studies concluded that down-regulation of miR-34a might attenuate apoptosis. Both physical activity and exercise modulate the expression of mRNAs and miRNAs involved in cell growth, proliferation, regulation of cell cycle and apoptosis in several tissues [11]. In several studies, 10 weeks of swimming training led to up-regulation of miR-126 [11, 56]. An experimental study reported that in the acute response following exercise, expression of miR-222 in the cardiac muscle tissue was increased [11]. Similarly, 10 weeks of swimming led to up-regulation of miR-16, miR-21, and miR-126 in the muscle tissue of rats with hypertension. This process was affected by both anti-apoptotic and apoptotic factors [11, 57]. In 2017, an investigation concluded that swimming training might delay

brain aging in D-gal-induced aging rats by reversing the impairment of miR-34a-mediated autophagy and abnormal mitochondrial dynamics. In this regard, miR-34a could serve as a novel therapeutic target for ailments associated with aging such as Alzheimer's disease [58].

The present study, for the first time, reports the negative regulatory effect of swimming training in the expression of miR-34a in pancreatic tissue, which is believed to be a beneficial effect that may counteract elevated rate of diabetes-induced apoptosis.

Conclusion

Our study, for the first time, evaluated the miR-34a, P53 and SIRT1 axis, and swimming training effects on the pancreatic tissue of rats with T2D. Our findings demonstrated a significant up-regulation of miR-34a and P53, and a considerable down-regulation of SIRT1 with elevated apoptosis in the pancreatic tissue. We found that up-regulation of miR-34a might result in dysfunctional cell apoptosis as a result of increased p53 function, which is not uncommon in diabetes. In contrast, interventions such as swimming exercise are shown to be effective in attenuating the apoptotic process by repressing the activity of miR-34/p53, and enhancing the expression of SIRT1. Based on our findings, regular exercise may reverse the expression of miR-34a, P53 and SIRT1, and diminish the pancreatic cell death.

In general, the present findings demonstrate an eloquent link between pancreatic cell apoptosis and miR-34a/SIRT1/P53 signaling pathway modulated by swimming training. Through its regulatory effect on the expression of p53 and SIRT1, miR-34a might be involved in the pathogenesis of pancreatic cell apoptosis. Hopefully, further investigations on the functional roles of miR-34a will provide us with a novel therapeutic strategy for management of neurodegenerative diseases including diabetes mellitus.

Future perspective

Regular exercising, like swimming, could be considered a new strategy not only as a complement to the routine diabetes therapeutics, but also as a primary therapy in treatment of diabetes. Farther studies in large cohorts are required to evaluate the effects of short-term, long-term, aerobic and anaerobic exercises on the pancreatic tissue and molecular axis of miR-34a/SIRT1/P53.

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Author's contributions Mohammad Reza Alipour and Rafighe Ghiasi designed the project. Rafighe Ghiasi, Roya Naderi and Alireza Alihemmati performed the required tests. Rafighe Ghiasi and Roghayeh Sheervalilou performed statistical analysis, and prepared the manuscript draft and revised it.

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Compliance with ethical standards

Conflict of interest The authors have affirmed that there is no conflict of interest.

Ethical issues The protocol for this study was planned to follow the guidelines of NIH, and be in agreement with Ethics Committee for the Use of Animals in Research at Tabriz University of Medical Sciences (project No: 5/4/610).

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