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SIRTUIN 1, a novel approach in the treatment of type 2 diabetes mellitus

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ABSTRACT
Type 2 diabetes mellitus (T2DM) was a chronic metabolic disorder in which prevalence has been increasing progressively all over the world. The innovate therapeutic approaches against T2DM are required in order to inhibit and treat T2DM. On the other hand, previous research has proven that activation of SIRT1 might prevent T2DM, demonstrating the SIRT1 may consider as a novel curative target for T2DM treatment. So, in this study, SIRT1 aptamer as activator of SIRT1enzyme was used as a pharmacological model to prevent T2DM by management the expression of SIRT1, FOXO3a and PGC-1α, genes in INS-1 832/13 rat insulinoma high fat cell lines (a type of model that used in order to study the pancreatic islet beta-cell role and for insulin excretion organization). The outcomes of RT-PCR showed that 10 μM of SIRT1 aptamer can up regulates PGC-1α, SIRT1 and FOXO3a expression in rat insulinoma cell lines. Moreover, the outcomes of western blot showed that palmitic acid (PA) modified the expression of lipid metabolism-related genes and mitochondrial biogenesis-related, while SIRT1 aptamer treatment improvement the PA that prompted alterations in the expression of previous genes through SIRT1. To sum up, this study provides important information regarding the effect of SIRT1 aptamer on modifiable the expression of mitochondrial genes related with biogenesis. So, SIRT1 aptamer may be considered as a prospective future therapy for mitigates T2DM.

Keywords: SIRT1, aptamer, FOXO, Gene expression, RT-PCR.

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INTRODUCTION
Diabetes mellitus was one of the major public chronic diseases that threatens human health in worldwide populations. [1] The development of T2DM was related with alterations in some factors that involving lipid metabolism, mitochondrial biogenesis, in addition to β-cells development.[2] In spite of huge efforts invested in the study of diabetes mellitus, the progress of accurate diagnoses and treatments for this disease stay problematic because of the restrictions of modern techniques. For that reason, more effective treatments and novel therapeutic strategies are required to prevent T2DM. Aptamer is oligonucleotides that have ability to bind to specific target molecules [3, 4] .Aptamer recognised to have high stability [5, 6], specificity with high affinity [7, 8], easily modified by various chemical reaction [9, 10], low immunogenicity [11], and low-toxic [12, 13] molecule. It has been proved in this research that the novel linear SIRT1 aptamer [14] which can enter the insulinoma cell lines to activate SIRTI and mitigates T2DM through targeting SIRT1 to regulate the expression of mitochondrial genes associated with biogenesis. SIRT1 define as a nicotinamide adenosine dinucleotide (NAD^+)-dependent histone and it's basically located in the nucleus and cytoplasm. [15] The main substrates of SIRT1 involved FOXO, PGC-1α. So, SIRT1 was capable of regulate the deacetylase activity of unlike transcription factors [16]. Moreover, SIRT1 is expecting a promising future in the pharmacological therapeutic target in order to treatment of insulin-resistance and consequent T2DM [17]. In various studies proposed that SIRT1 have ability to contribute in the organization of insulin excretion from pancreatic β-cells. So, SIRT1 might contribute in the control of glucose homeostasis within the subsequent mechanisms: adjustment insulin secretion [18] in addition to protecting pancreatic β-cells [19] improved insulin resistance as a result of the adjustment of post insulin receptor signalling; reducing lipid mobilization, inflammation, and adiponectin secretion [20] controlling mitochondrial biogenesis and fatty acid oxidation [21] and regulating circadian rhythms and hepatic glucose production skeletal muscle, monocytes/macrophages, adipose tissue and the liver. To discover the relationship among SIRT1 and mitochondrial biogenesis that means it is necessary to evaluate lipid metabolism and β-cells development and recognize the fundamental technique through which linear SIRT1 aptamer mitigates T2DM. In current study, INS-1 832/13 rat insulinoma high fat cell lines were used, it was the same INS-1 832/13 rat insulinoma cell line but the INS-1 832/13 HF rat insulinoma cell line were exposed either to palmitate and oleate complexed to BSA (bovine serum albomin) and added in media containing 1% FBS [22] .The results of RT-PCR showed that 10 μM of SIRT1 aptamer can up regulates PGC-1α, SIRT1 and FOXO3a genes expression in INS-1 832/13 HF rat insulinoma cell lines. Furthermore, the SIRT1 aptamer which treated to INS-1 832/13 HF rat insulinoma cell lines improvement the palmitic acid-induced changes in the expression of FOXO3a PGC-1α and SIRT1 genes through SIRT1. To sum up, this study provides important information regarding the effect of SIRT1 activator by aptamer on modifiable the expression of mitochondrial genes related by biogenesis and this SIRT1 aptamer may be used as a treatment to relieve T2DM.

Material &Methods
Material: Chemicals were purchased from Sigma-Aldrich Ltd. USA, lonza, Thermo Fisher Scientific, USA, Invitrogen Germany, and Qiagen. SIRT1 ptamer (5'-CGGACTGCAACCTATGCTATCGTTGATGTCTGTCCAAGCA-3'), SIRT1 siRNA (5′-CACCCCAGCAACTCAGCATTCATCGAAATGAATGCTGAGTT GCTGG-3**)** and siRNA (5'-TTCTCCGAACGTGTCACGT-3') were purchased from Bonier-Korea.

Cell culture: The experimentations were done in the tissue culture laboratory of the department of Pharmacology and Toxicology at College of Pharmacy / Mustansiriya University. Insulinoma cell line (INS-1 832/13 Rat Insulinoma cell line) was purchased from sigma Aldrich (Merck, USA). The cell culture medium was Roswell Park Memorial Institute -1640 (RPMI-1640) (Thermo Fisher, UK) accompanied with 10% fetal bovine serum (Fisher Scientific, USA), 11 mM glucose (Lonza, UK) , 10 mM HEPES (Lonza, UK) , 1mM sodium pyruvate (Lonza, UK) , 50 µM 2-mercaptoethanol (β-mercaptoethanol was a critical for the continued propagation of the cell line) (Thermo Fisher, UK) , 50 µg/ml penicillin and 100 µg/ml streptomycin(Lonza, UK) . Cells were cultured in 75cm tissue culture flask (Falcon, USA) and incubation carried out at 37°C in a humidify atmosphere 95% having 5% CO_2 in RPMI .Until the cells reached 90% confluence ,tissue culture flask reserved under without sterile condition. .Cells were passage into a new tissue culture flask (T75 cm) every 4 days by washing with 5 ml of phosphate buffer saline (PBS) (Sigma-Aldrich Ltd) without Ca^{+2}/Mg^{+2} because lead to destruction the membrane, followed by addition of 3 ml of trypsin-EDTA (Thermo Fisher Scientific) solution in order to isolated the cells from flask bottom, then rotate the flask and incubated at 37°C incubator for 3-2 minutes. Gently tapping the side of the flask with the palm of hand in order to confirm the complete detachment of cells. 7ml of INS-1 832/13 expansion medium was added to the plate and the suspension cells was moved into a 15 ml conical tube. Centrifugation of cells was done at 1200 rpm for 3minutes. The supernatant was isolated, and the cell pellet undergo re-suspended in fresh supplemented growth media. Cells were counted under the microscope (Optika, Italy) on a haemocytometer and an equalled volume of 0.4% trypan blue (Qiagen) was added in order to determine the number of viable cells that was present in a cell suspension. The cells were stored for 24h at -80 °C and moved to liquid nitrogen. Thereafter, the cells were taken from the liquid nitrogen tank, thawed in incubator at 37 °C [23]. The cells were transferred gradually to T75cm flask with 15 ml of fresh medium.

Effect of SIRT1 aptamer on the expression of FOXO3a PGC-1α and SIRT1 in insulinoma cell lines: In order to study the effect of SIRT1 aptamer on the expression of PGC-1α, SIRT1 and FOXO3a in insulinoma cell lines, INS-1 832/13 and INS-1 832/13 HF cell lines were seeded into 6-well plates at a density of 5×10^5 cells/well and divided into 3 groups: INS-1 832/13 + media**,** INS-1 832/13 HF + media and INS-1 832/13 HF cells treated with 10µM SIRT1 aptamer+ media. There are several steps to carry out the effect of SIRT aptamer on the expression of PGC-1α, SIRT1 and FOXO3a in insulinoma cell lines include: RNA extraction, agarose gel electrophoresis, quantitative real-time polymerase chain reaction technique and western blotting.

RNA Extraction: After INS-1 832/13 cells and INS-1 832/13 HF cell lines were growth, media was removed and added 0.3–0.4 ml of TRIzol reagent (BioNeer, Korea) per 1×10^5 cells directly to the culture dish to lyse the cells. Pipetted the lysate up and down several times to homogenize. The high fat cells group were centrifuged the lysate for 5 minutes at 12,000 × g at 4 –10°C, then transferred the clear supernatant to a new tube. Incubated for 5 minutes to allow completed dissociation of the nucleoproteins complex, then added 0.2 ml of chloroform (Sigma, USA) per 1ml of TRIzol reagent used for lysis, securely cap the tube, and then thoroughly mixed by shaking. After that, incubated for 2–3 minutes and centrifuged the sample for 15 minutes at 12,000 × g at 4°C. The blend separated inside as a lower red phenol-chloroform, an interphase, and a colourless upper aqueous phase. The aqueous phase that containing the RNA was transfer to a clean tube through angling the tube at 45° and the solution was pipetted out. At the bottom of the tube, total RNA precipitate and forms a white gel-like pellet and discarded the supernatant with a micropipette. Re-suspended the pellet in 20µl of RNase-free water (Qiagen), at that time measured absorbance at 260 nm and 280 nm by Nano Drop (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). The RNA can be stored in 75% ethanol (Fisher Ltd. USA) for at least 1 year at -20°C.

Agarose Gel Electrophoresis: The quality of RNA is determining through agarose electrophoresis and the concentration was measured by a Nano Drop at 260 nm and 280 nm (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Agarose gel electrophoresis was a generally utilized technique designed for separating DNA, proteins or RNA [24]. The molecule of Nucleic acid was size separated through the assistance of an electric field wherever the negative charge molecules immigrate to anode positive pole. Exclusively determination of the migration flow was via the molecular weight anywhere tiny weightiness molecules immigrate quicker than bigger ones [25]. Furthermore, to size separation, utilize of agarose gel electrophoresis via nucleic acid fractionation be able to be a first stage for additional cleansing of a band of attention. Expansion of the method comprises eliminating the wanted "band" from a stained gel observed through a UV trans illuminator [26]. Agarose gel prepared by mixed of 2% agarose gel powder (Fisher Ltd. USA) with Tris-acetate-EDTA (Sigma, Aldrich Ltd. USA) (electrophoresis buffer was essentially for migration of RNA in the gel and it has the lowest buffering capacity but provides the best resolution for larger RNA). Then after prepared the agarose gel heated in the microwave until all the mixture dissolved. 3µl of RNA samples mixed with 0.5 µg/ ml loading dye Ethidium bromide (Lonza) (2, 7-diamino-10-ethyl-9-phenylphenanthridiniumbromide for staining RNA in agarose gels) and was placed in the wells [27]. Agarose gel was illuminated by the ultraviolet lamp (GiBox Syngene, USA) by placing the gel on the light box the maximum excitation and fluorescence emission of EtBr was obtained from 500- 590 nm, and photographs were taken by a digital camera.

Quantitative Real-time Polymerase Chain Reaction technique: RT-qPCR was a biochemistry and molecular biology technique intended for amplification of target DNA through some instructions of magnitudes, creating millions or additional copies of target DNA pieces. In the initial 1990s, the RT-qPCR technique was established [28]. Reverse Transcription (cDNA Synthesis) (BioNeer) use an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction. In table 1, shown the primers that utilized for reverse transcription-quantitative polymerase chain reaction. All primers were purchased from BioNeer-Korea.

Table I. showed the primers that utilized for reverse transcription-quantitative polymerase chain reaction

Genes	Forward Primers(5'-3')	Reverse primers(5'-3')
SIRT1	GCTGACGACTTCGACGACG	TCGGTCAAGAGGAGGTTGTCT
PGC1-α	TATGGAGTGACATAGAGTGTGCT	GTCGCTACACCACTTCAATCC
FOXO3	TCTTACGCCGACCTCATCAC	TCTTACGCCGACCTCATCAC
INS	CCCTGTTGGTGCACTTCCT	TCCCAGCTCCAGTTGTTCC
β-actin	CCTCTATGCCAACACAGTGC	GTACTCCTGCTTGCTGATCC

qPCR was performed by used SYBR-Green qPCR Super Mix (BioNeer, Korea) on a real-time RT-PCR system (BioNeer, Korea), added 5µl from 1 µg total RNA to 200 µl for tube of SYBR-Green qPCR Super Mix that contained DNA genetic code to be copied (AGCT) and nucleotide used to create the cDNA strand, Tag polymerase was an enzyme that read original DNA sequence and made a complimentary copy, Probe (Taqman required a specially labelled probe), reaction buffer, water and finally primers specific to section of DNA to be copied. After that, added to each tubes 2µl from forward primer and 2µl from reverse primers that specific to FOXO3a, PGC-1α and SIRT1 proteins, then completed the mixture to 220µl by added 11µl deionized water. The thermo cycling circumstances were involve : 94˚C for 5 minute subsequently 35 cycles of 94˚C for 30 second, 56˚C for 30 second , then 72˚C for 30 second , with a final elongation step at 72˚C for 5 minute [29]. Wholly, the amplification reactions were achieved in triplicate.

Western blotting: Western blotting is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions). The blot was first developed by Towbin *et al*. in 1979 [30]. The proteins were separated by SDS acrylamide electrophoresis. The specific proteins (FOXO3a, PGC-1α and SIRT1) were identified from a complex mixture of proteins extracted from cells by five steps of western blot technique, it includes: [31]

Cell lysis to extract protein: INS-1 832/13 rat insulinoma cell line and INS-1 832/13 HF rat insulinoma cell line were washed in the tissue culture flask by added 3 ml of cold phosphate buffered saline (PBS) (Gibco, UK) with rocking gently, then the flask kept on ice, after that the PBS was discarded. 3 ml of cold phosphate buffered saline was added and used the cell scraper to dislodge the cells. Pipetted the mixture into micro centrifuge tubes, then the mixture was centrifuged at 1500 rpm for 5 minutes and discarded the supernatant. RIPA buffer (Gibco, UK) (25 mM tris-HCl pH 7.6, 150 mM NaCl, 1%NP-40, 1% sodium deoxycholate, 0.1% SDS) with 20 µl fresh protease inhibitor cocktail (Sigma-Aldrich Ltd. USA) was added to the tube, and incubated for 30 minutes on ice. By spinning for 30 minutes at 15,000 rpm at 4°C, the lysate was clarified. The supernatant (protein) was transferred to a fresh tube and stored at -80°C. Finally, the spectrophotometer was used to measure the concentration of protein

Sample preparation: 5µl of buffer was added to the samples, then made the volume in each lane equalized by used dd.H_2O, the total volume was 15 µl per lane. After that, samples were heated by dry plate for five min. at 100˚C.

Gel preparation: Gel included 10% stacking gel solution and 6% separating gel were prepared in BioRad mini tank. These gels were then run in 1x TBE (Tris-Borate EDTA) (Fisher Ltd. USA) at 60 V/cm. Contents for gels:

Solution	Materials	Volume
10% Stacking gel	H_2O	3ml
	1M Tris-Hcl	2.1ml (PH 8.9)
	30% AcrBis	2.8ml
	10% SDS	80 µl
	10%APS	56 µl
	TEMED	6 µl
6% separating gel	H_2O	2 µl
	1M Tris-Hcl	400 µl (pH 6.7)
	30% AcrBis	600 µl
	10%SDS	36 µl
	10%APS	24 µl
	TEMED	4 µl

After prepared the 10% stacking gel solution, assembled the rack for gel solidification. After that, added stacking gel solution carefully until the level is equal to the green bar holding the glass plates. Added H_2O to the top and remind for 15–30 minutes until the gel turning solidified. Then removed the water to and inserted the comb. Waited until the gel is solidified.

Electrophoresis: Separation of proteins happen according to isoelectric point, molecular weight, electric charge, or all of these factors. [32] The running buffer was added into the electrophoretic

Electro transfer and blotting: Consequently , proteins were electrotransferred to polyvinylidene fluoride membrane (PDVF) (Fisher Scientific, USA) .Then, the transfer occurred by an electric field concerned with perpendicular to the surface of the gel, triggering proteins to travel out of the gel and upon the membrane.

Blocking and antibody: After proteins were electrotransferred to polyvinylidene fluoride membrane, the membrane was blocked with 5% non-fat milk in Tris-buffer saline (Fisher Ltd. USA) with 0.05% Tween-20 (Sigma, Aldrich Ltd. USA) for 1 h at room temperature. Primary antibody that involve: mouse anti-SIRT1, anti-FOXO3a, anti-PGC-1α and anti-β-actin antibodies (all 1:1,000 dilution) (Severn Biotech Ltd. UK) was added in 5% bovine serum albumin (BSA) and the membrane incubated overnight at 4ºC. The membrane washed with Tris-buffer saline with 0.05% Tween-20 for 5 minutes three times, then secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse) (Severn Biotech Ltd. UK) was added in 5% skim milk in 0.05% Tween-20, and incubated for 1 hour. The membrane was washed with 0.05% Tween-20 for 5 min. The development of membrane band by utilizing an improved chemiluminescence kit (Takara, Japan) by added 1 drop of oxidant reagent to 1ml of substrate reagent (125µl of reagent covers 1cm2 membrane). This solution was used immediately. Immersed the membrane in chemiluminescent reagent for 1 to 10 minutes then, the excesses reagent was removed and covered the membrane with a plastic sheet. Putted the plastic covered membrane into contact with light sensitive film in an exposure chamber. Developed the film after 30 seconds to 10 minutes exposure. A relative density of bands (target protein) were normalize versus β-actin utilizing Quantity One software (Bio-Rad, USA). [31]

Palmitic acid suppresses expression of SIRT1 in a dose and time dependent manner in INS-1 832/13 rat insulinoma cell lines: Previous research established that Palmitic acid has a role in obesity and T2DM [33]. When

density of INS-1 832/13 rat insulinoma cell lines reached 80%, cells were pretreated with 0, 0.125, 0.2, 0.5, 1, 1.5 and 2 mM PA for 24 h to test the effect of PA on expression of SIRT1in dose response. About the study the effect of PA on expression of SIRT1 in time response, INS-1 832/13 rat insulinoma cell lines were treated with 0.5 mM PA for 0, 12, 24, 48 or 72 h. The expression of SIRT1 mRNA was assessed using RT-qPCR. The purpose of pretreated INS-1 832/13 rat insulinoma cell line with palmitic acid in order to suppression the expression of SIRT1 that be useful to know the effected of SIRT1 aptamer on the expression of this gene.

SIRT1 aptamer affects the expression of PGC-1α and FOXO3a via SIRT1 in Palmitic acid induced INS-1 832/13 HF cell lines: In this experiment, cell transfection was used. The main purpose of transfection is to study the function of genes or gene products, by enhancing or inhibiting specific gene expression in cells, and to produce recombinant proteins in mammalian cells.

Cell Transfection: In this experiment, INS-1 832/13 and INS-1 832/13 HF cell lines were used. Cells were seeded into 6-well plates at a density of 5×10^5 cells/well and divided into 5 groups: INS-1 832/13 + media, INS-1 832/13 HF cells + media (+ve control), INS-1 832/13 HF cells treated with 10μM SIRT1 aptamer + media, INS-1 832/13 HF cells treated with 10μM SIRT1 aptamer + 2μg/ml SIRT1 small interfering (siRNA) + media, and INS-1 832/13 HF cells treated with 10μM SIRT1aptamer + 2μg/ml siRNA (-ve control) + media. In order to transfect cells with SIRT1 siRNA or negative control siRNA (SIRT1 siRNA was designed to knockdown the SIRT1 gene expression), INS-1 832/13 HF cells + 10 μM SIRT1 aptamer + 2 μg/ml SIRT1 siRNA and INS-1 832/13 HF cells + 10 μM SIRT1 aptamer + 2 μg/ml siRNA groups were incubated in 1 ml RPMI 1640 with SIRT1 2 μg/ml siRNA(5′CACCCCAGCAACTCAGCATTCATCGAAATGAATGC TGAGTTGCTGG-3′), 2 μg/ml siRNA (5′-TTCTCCGAACGTGTCACGT-3′) respectively, and 4 μg/ml Lipofectamine 2000 (Lipofectamine 2000 is a cationic liposome based reagent and a common transfection reagent that manufactured and sold by Invitrogen , utilized in molecular and cellular biology to increase the transfection efficiency of RNA (including mRNA and siRNA). [34] Subsequent 48 h incubation at 37°C, cells in the INS-1 832/13 HF cells + 10 μM SIRT1 aptamer, INS-1 832/13 HF cells + 10 μM SIRT1 aptamer + SIRT1 siRNA and INS-1 832/13 HF cells + 10 μM SIRT1 aptamer + negative control siRNA groups were treated with 0.5 mM Palmitic acid +10 μM SIRT1 aptamer, (Palmitic acid was dissolved at 10 mmol/l in M-199 medium containing 11% bovine serum albumin (BSA), shaken overnight at 37°C, sonicated for 15 min, and filtrated under sterile conditions (stock solution)). The high fat group received the same amount of Palmitic acid (Gibco, UK) and negative control cells are treated with the similar quantity of vehicle. Subsequent 24 h incubation at 37°C, the total RNA was extracted for more experimentations and determined the expression by RT-PCR and western blot as described above.

Statistical analysis: Wholly statistical analyses were done using SPSS 19 and Graph pad prism 8. All results are presented as the mean ± standard error of the mean. Significant differences in mean values were evaluated using one-way analysis of variance with Turkey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of SIRT1 aptamer on the expression of PGC-1α, SIRT1 and FOXO3a in insulinoma cell lines.

a- By RT-qPCR: To investigate the mechanism of action of the SIRT1 aptamer in DM, level of PGC-1α, SIRT1 and FOXO3a mRNA were evaluated via RT-qPCR. The level of PGC-1α, SIRT1 and FOXO3a mRNA were significantly decreased in the INS-1 832/13 HF cell lines as compared with INS-1 832/13 cell lines as shown in figure 1, p< 0.0005. INS-1 832/13 HF rat insulinoma cell lines treatment with 10μM SIRT1 aptamer significantly attenuated the decrease (P<0.001; figure 1). These results suggest that SIRT1 aptamer treatment could up regulated PGC-1α, SIRT1 and FOXO3a expression in INS-1 832/13 HF rat insulinoma cell line.

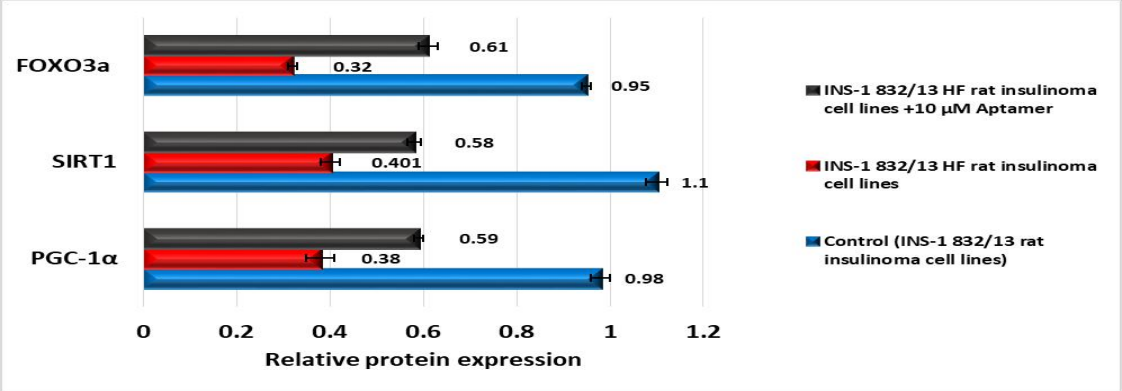


Figure 1: SIRT1 aptamer modulates PGC-1α, SIRT1 and FOXO3a in mRNA as evaluated via reverse transcription-quantitative polymerase chain reaction. All amplification reactions were performed in triplicate. p< 0.001.

b- By western blot: As shown in figure 2, the level of PGC-1α, SIRT1 and FOXO3a protein were significantly suppressed in the INS-1 832/13 HF cell lines as compared with the control (INS-1 832/13 rat insulinoma cell lines). On the other hand, treatment with 10 μM SIRT1 aptamer significantly mitigated these decreases (P<0.05). These statistics propose that SIRT1 aptamer management up regulates PGC-1α, SIRT1 and FOXO3a expression in INS-1 832/13 HF rat insulinoma cell line.

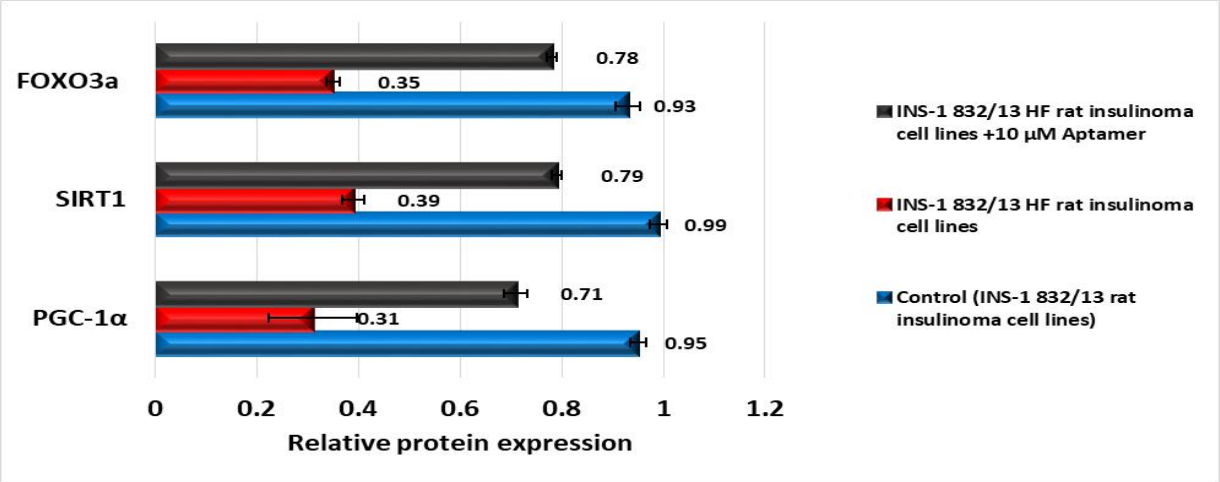


Figure 2: Aptamer modulates PGC-1α, SIRT1 and FOXO3a protein as assessed by western blot. p< 0.05.

As revealed in figure 3, the western blot results showed a decrease in the density of target protein bands (PGC-1α, SIRT1 and FOXO3a) of INS-1 832/13 HF cell lines compared with the control (INS-1 832/13 cell line) bands. Additionally, INS-1 832/13 HF rat insulinoma cell line treatment with 10 µM SIRT1 aptamer showed increased the density of target protein bands (PGC-1α, SIRT1 and FOXO3a). The target protein bands (relative density) were normalized versus β-actin using Quantity One software v4.4.

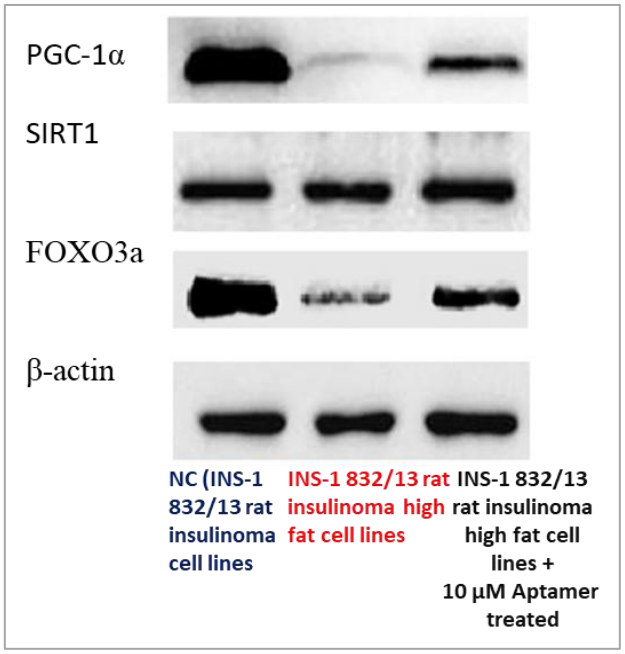


Figure 3: Protein levels (PGC-1α, SIRT1 and FOXO3a) as assessed by western blot showed the target protein bands (relative density) were normalized versus β-actin using Quantity One software v4.4.

Palmitic acid suppresses expression of SIRT1 in a dose and time dependent manner in INS-1 832/13 rat insulinoma cell lines.

a- Relative SIRT1 mRNA expression (Concentration response): previous research established that PA has a role in obesity and T2DM [33]. The outcomes showed that PA significantly suppressed SIRT1 mRNA in a dose-dependent manner as shown in figure 4 p< 0.05, when INS-1 832/13 cell lines treated with different concentrations 0, 0.125, 0.2, 0.5, 1, 1.5 and 2 mM of PA.

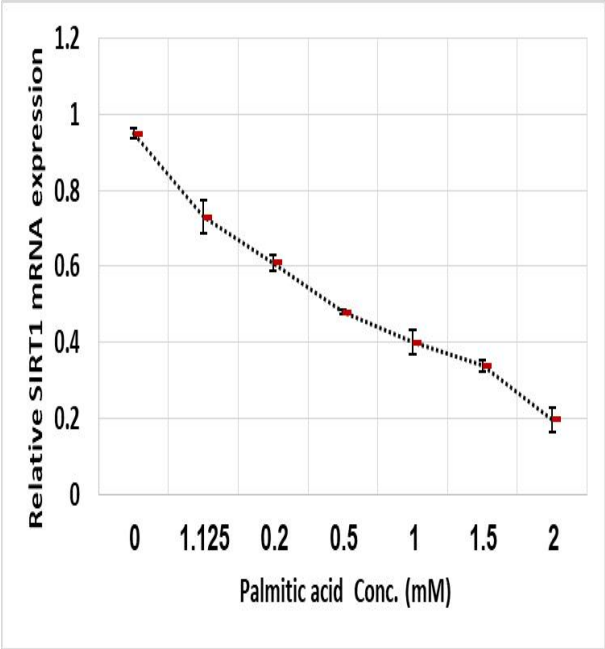


Figure 4: Palmitic acid repressed the expression of SIRT1 in a dose response. INS-1 832/13 rat insulinoma cell lines were treated with 0, 0.125, 0.2, 0.5, 1, 1.5- and 2-mM PA for 24 h and the expression of SIRT1 mRNA was assessed using RT-qPCR. P<0.05 vs. 0 mM PA.

b- Relative SIRT1 mRNA expression (Time response): The relationship between SIRT1 expression and PA induction time in INS-1 832/13 rat insulinoma cell lines was consequently investigated and the outcomes showed that 0.5mM PA management as well significantly reduced SIRT1 mRNA expression in a time-dependent manner (0, 24, 48 and 72 h) p< 0.0001 as showed in figure 5.

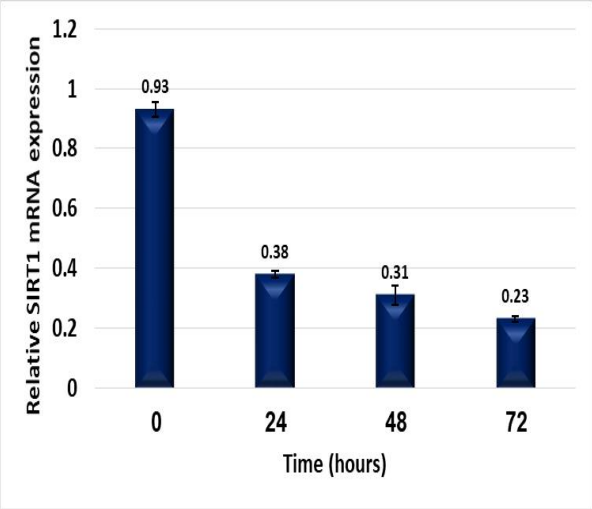
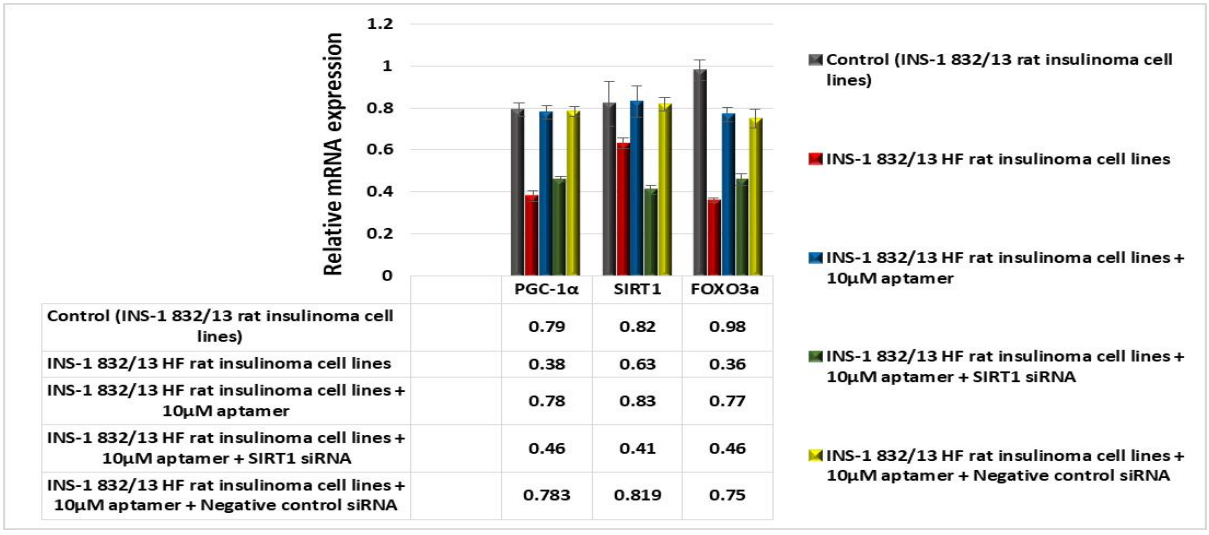


Figure 5: INS-1 832/13 rat insulinoma cell lines were treated with 0.5 mM PA for 0, 24, 48 and 72 h, correspondingly and SIRT1 mRNA was measured using RT-qPCR. P<0.0001 vs. 0 h.

SIRT1 aptamer affects the expression of PGC-1α and FOXO3a via SIRT1 in Palmitic acid induced INS-1 832/13 HF cell lines.

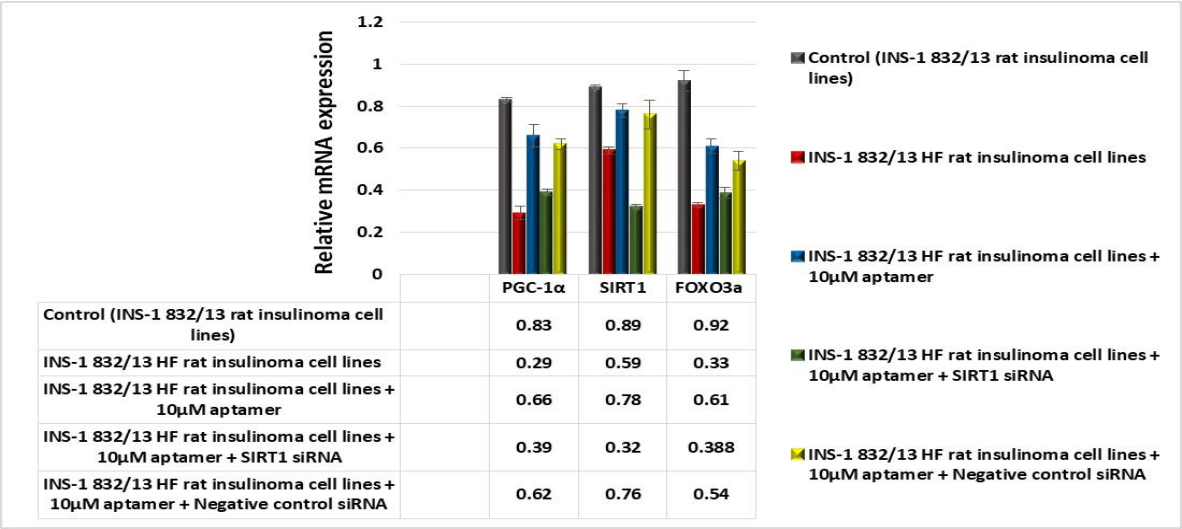
a- Relative SIRT1mRNA expression: To investigate the role of aptamer *in vitro*, level of PGC-1α, SIRT1 and FOXO3a in INS-1 832/13 HF rat insulinoma cell lines were evaluated after treated this cell with 0.5μM PA. The results showed that 0.5μM PA significantly suppressed the level of PGC-1α, SIRT1 and FOXO3a mRNA and protein compared with the INS-1 832/13 rat insulinoma cell lines without treated with 0.5μM PA, P<0.001; figure 3-6. However, administration of aptamer significantly attenuated this effect (P<0.001; figure 6). Furthermore, transfection with SIRT1 siRNA in conjunction with PA significantly reduced the expression of PGC-1α, SIRT1 and FOXO3a compared with the INS-1 832/13 HF rat insulinoma cell lines +10μM SIRT1 aptamer + negative control siRNA group (P<0.05; figure 6). These statistics propose that the SIRT1 aptamer mitigated the high fat that induced the inhibition of PGC-1α and FOXO3a expression via SIRT1.



	PGC-1α	SIRT1	FOXO3a
Control (INS-1 832/13 rat insulinoma cell lines)	0.79	0.82	0.98
INS-1 832/13 HF rat insulinoma cell lines	0.38	0.63	0.36
INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer	0.78	0.83	0.77
INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer + SIRT1 siRNA	0.46	0.41	0.46
INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer + Negative control siRNA	0.783	0.819	0.75

Figure 6: SIRT1 aptamer modulates the expression of PGC-1α and FOXO3a expression via SIRT1in INS-1 832/13 HF rat insulinoma cell lines. mRNA levels of PGC-1α and FOXO3a in INS-1 832/13 HF rat insulinoma cell lines assessed by RT-qPCR, p< 0.05.

b- Relative protein expression: Compared with the INS-1 832/13 rat insulinoma cell lines as showed in figure 7; PA significantly suppressed the levels of PGC-1α, SIRT1 and FOXO3a protein, p< 0.001. On the other hand, administration of SIRT1 aptamer significantly diminished this effect. Also, transfection with SIRT1 siRNA in combination with PA significantly reduced the expression of PGC-1α, SIRT1 and FOXO3a compared with the INS-1 832/13 HF rat insulinoma cell lines + 10μM SIRT1 aptamer + negative control siRNA group. These results advise that aptamer mitigated the high fat that induced the inhibition of PGC-1α and FOXO3a expression via SIRT1 as the same suggestion in mRNA expression.



	PGC-1α	SIRT1	FOXO3a
Control (INS-1 832/13 rat insulinoma cell lines)	0.83	0.89	0.92
INS-1 832/13 HF rat insulinoma cell lines	0.29	0.59	0.33
INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer	0.66	0.78	0.61
INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer + SIRT1 siRNA	0.39	0.32	0.388
INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer + Negative control siRNA	0.62	0.76	0.54

Figure 7: Protein levels of PGC-1α and FOXO3a affected by aptamer as modulates the expression of PGC-1α and FOXO3a expression via SIRT1in INS-1 832/13 HF rat insulinoma cell lines. Protein levels assessed by western blotting. P< 0.001

As revealed in figure 8, the western blot results showed a decrease in the density of target protein bands (PGC-1α and FOXO3a) of INS-1 832/13 HF compared with INS-1 832/13 cells bands of internal control. On the other hand, INS-1 832/13 HF cell line treatment with 10μM SIRT1 aptamer showed increased in the density of target protein bands (PGC-1α, SIRT1 and FOXO3a). Also, transfection with SIRT1 siRNA in combination with PA significantly reduced the density of PGC-1α, SIRT1 and FOXO3a compared with the INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer + negative control siRNA group. The target protein bands (relative density) were normalized versus β-actin using Quantity One software v4.4.

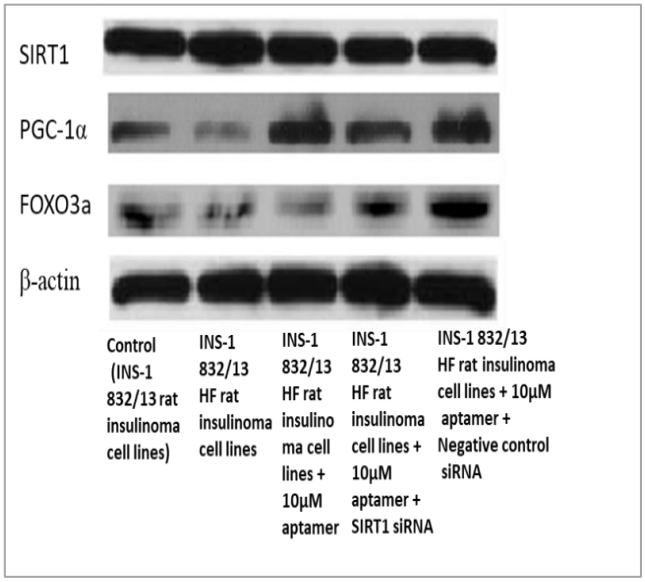


Figure 8: The relative densities of target proteins PGC-1α, SIRT1 and FOXO3a bands were normalized against β-actin.

Effect of SIRT1 aptamer on the expression of insulin gene (INS gene) in insulinoma cell lines: To study the mechanism through which SIRT1 functions, the expression of gene was investigated including β-cells-associated (INS gene). 0.5mM PA treatment significantly suppressed mRNA levels of SIRT1 and INS. Treatment the INS-1 832/13 HF rat insulinoma cell lines with 10μM SIRT1 aptamer significantly attenuated these PA-induced alterations in gene expression .As compared with control (INS-1 832/13 rat insulinoma cell lines), INS-1 832/13 HF rat insulinoma cell lines and INS-1 832/13 HF rat insulinoma cell lines + 10μM SIRT1 aptamer + SIRT1 siRNA were demonstrated reduced mRNA expression level of SIRT1. INS-1 832/13 HF rat insulinoma cell lines + 10μM SIRT1aptamer + negative control siRNA and INS-1 832/13 HF rat insulinoma cell lines + 10μM aptamer showed modulation of mRNA expression level of SIRT1 (P< 0.001; figure 9).

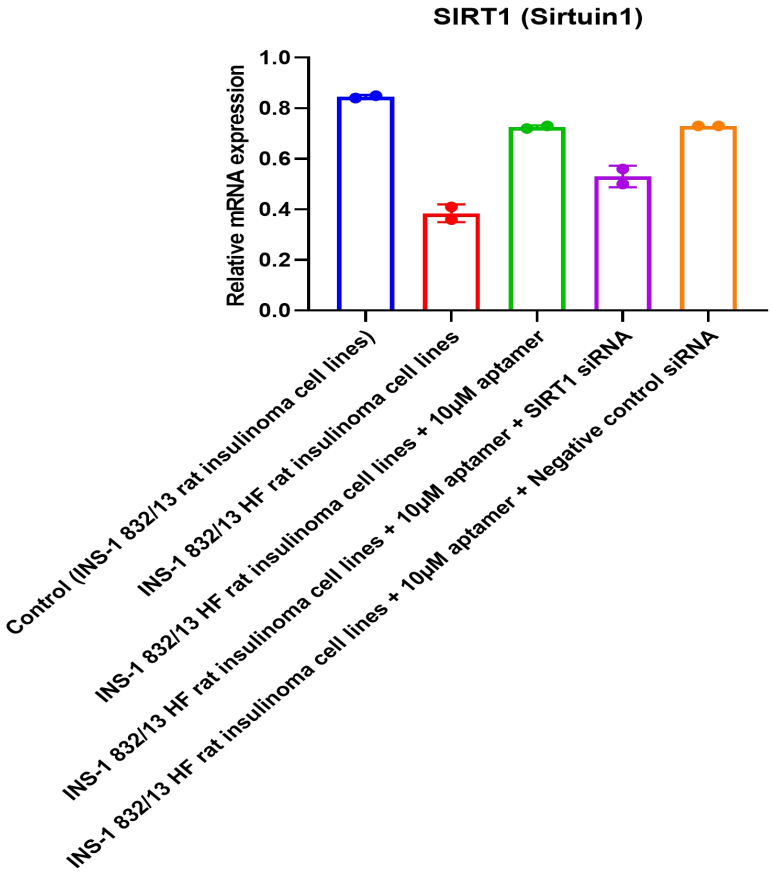


Figure 9: Effect of SIRT1 aptamer and palmitic acid on the expression of SIRT1 mRNA in INS-1 832/13 cell lines and INS-1 832/13 HF, p< 0.001. The figure was drawn by Graph pad prism 8.1.

PA treatment significantly suppressed mRNA levels of INS mRNA. Management with aptamer significantly mitigated these PA-induced changes in gene expression. As compared with control (INS-1 832/13 rat insulinoma cell lines), INS-1 832/13 HF rat insulinoma cell lines and INS-1 832/13 HF rat insulinoma cell lines + 10µM SIRT1 aptamer + SIRT1 siRNA were demonstrated reduced mRNA expression level of INS gene. INS-1 832/13 HF rat insulinoma cell lines + 10µM aptamer + negative control siRNA and INS-1 832/13 HF rat insulinoma cell lines + 10µM SIRT1 aptamer showed modulation of mRNA expression level of INS gene (p< 0.001; figure 10). These data suggest that aptamer might influence β-cells functionality by SIRT1in insulinoma cell line.

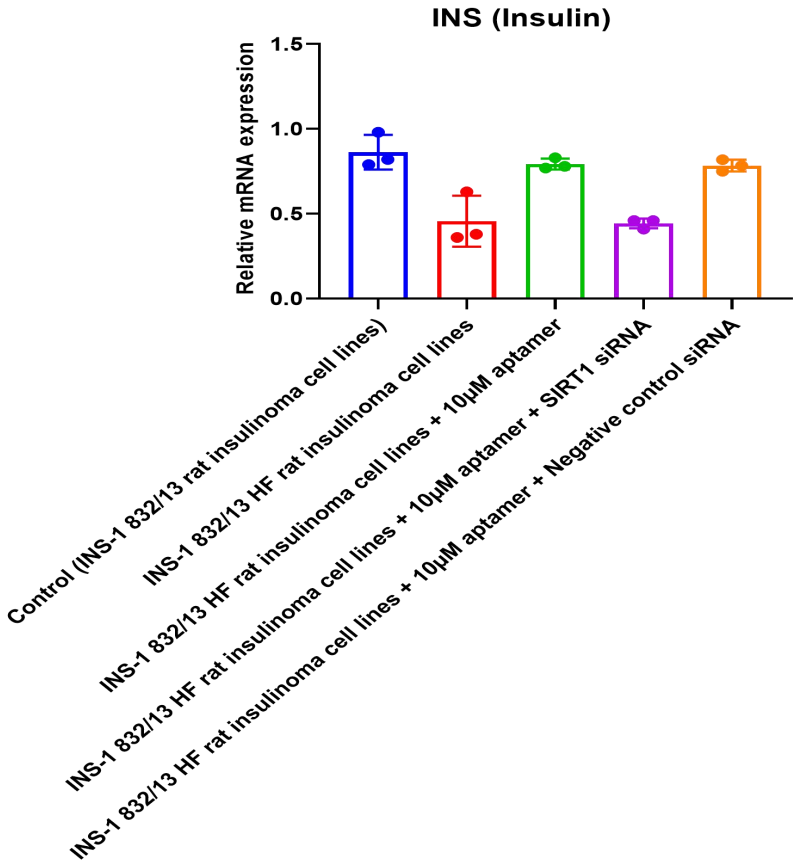


Figure 10: RT-qPCR for measuring the relative INS gene expression level. Effect of SIRT1 aptamer and palmitic acid on the relative INS gene expression level in INS-1 832/13 cell lines and INS-1 832/13 HF, p< 0.001. The figure was drawn by Graph pad prism 8.1.

Discussion

Diabetes mellitus is one of the major public chronic diseases that threatens human health in worldwide. [1] The development of T2DM was related with alterations in some factors that involving lipid metabolism, mitochondrial biogenesis, in addition to β-cells development.[2] In spite of huge efforts invested in the study of diabetes mellitus, the progress of accurate diagnoses and treatments for this disease stay problematic because of the restrictions of modern techniques. For that reason, more effective treatments and novel therapeutic strategies are required to prevent T2DM. More importantly, SIRT1 activators, comprising resveratrol is established to have useful special effects on glucose homeostasis and insulin sensitivity in animal models of insulin resistance [17]. Consequently, SIRT1 may be a innovate curative target for the prevention of T2DM. Additionally, SIRT1 has been demonstrated to interact directly with PGC-1 to increase PGC-1α expression and mitochondria biogenesis [35]. The present study examined the potential effect of SIRT1 aptamer as SIRT1 activator as a promising therapeutic target for T2DM through modifiable the expression of mitochondrial genes related with biogenesis in insulinoma cell lines. Some studies have demonstration that SIRT1 can adjust mammalian FOXO and PGC-1α transcription factors by either direct binding or deacetylation [36]. On the other hand, relations among SIRT1, PGC-1α and FOXO3a beneath diabetic circumstances are undistinguishable [36] . In this experiment, up regulated the SIRT1 expression by SIRT1 aptamer in insulinoma cell lines as shown in figures 1 and 3 led to overexpression the FOXO3a and PGC-1α proteins in these cells. A unique probable mechanism, the improve activity of the FOXO by SIRT1 through their nuclear translocation [37] and perhaps adjusts the gene-specific transcription [38, 39, 40, 41].In response to oxidative stress, the deacetylation of FOXO3a by SIRT1lead to translocation of FOXO3a from the cytoplasm to the nucleus [41, 42]. Between FOXO families, FOXO3a known it keeps quiescent cells from oxidative stress through directly rising their amounts of manganese superoxide dismutase (MnSOD) messenger RNA and protein [41]. In recent times, Barthel *et al* [37] have proposed that the FOXO3a genotype was suggestively related with plasma insulin levels in addition to cancer, and Type 2 diabetes occurrence. The outcomes of the present study expose that elevated the level of FOXO3a in INS-1 832/13 HF insulinoma cell lines by SIRT1 aptamer is an essential controller effecting obesity and diabetes. Moreover, several lines of evidence have indicated that PGC-1α activated by SIRT1 also modulates the glucose homeostasis [43] and this evidence have agreement with our results. For a long period of time, palmitic acid (PA) has been negatively showed for its recognised harmful health effects, shadowing its multiple critical physiological activities. The most common saturated fatty is PA that comprise (20–30%) of whole fatty acids in the human body and they are provided in the diet or manufactured endogenously through *de novo* lipogenesis [44] .In current study, PA utilized to induce insulin secretion in INS-1 832/13 insulinoma cell lines. The outcomes established that PA suppressed the expression

of SIRT1 in a different dose- and time-dependent manner as shown in figures (4 and 5), suggesting that SIRT1 utilities a main part in the adjust of lipid metabolism. It has been reported that SIRT1 adjusts a variety of cellular roles influencing metabolic homeostasis and the relation among metabolism and insulin secretion be influenced by mitochondrial role [45]. One of the most essential improvements in biology has been the detection of siRNA (small interfering RNA) which is capable of adjust the expression of genes, via a phenomenon identified as RNAi (RNA interference). [46] In this study, SIRT1 siRNA was designed to knockdown the SIRT1 gene expression in INS-1 832/13 HF rat insulinoma cell line, SIRT1 siRNA is specifically inhibit SIRT1 expression by RNA interference, a technique by means of gene expression be able to selectively silenced via the delivery of double stranded RNA molecules into the cell. [47, 48] Negative control siRNA used as a non-silencing siRNA helps to identify non-specific changes in gene expression [49]. Furthermore, the results of genes expression by mRNA and western blot as shown in figures 6 and 7 for mRNA and 3-8 for protein expression by western blot demonstrated that palmitic acid inhibited PGC-1α and FOXO3a expression. SIRT1 knockdown mitigated the special effects of SIRT1 aptamer on PA that repressed PGC-1a and FOXO3a expression. The deacetylation of definite targets was intermediates by SIRT1 and comprising FOXO3a, PGC-1α and p53, in lipid metabolism mitochondrial biogenesis and inflammation [50, 51]. The outcomes of this experiment propose that SIRT1 aptamer roles via SIRT1 in palmitic acid induced INS-1 832/3 HF rat insulinoma cell lines. SIRT1 aptamer regulated the level of insulin gene via SIRT1 as shown in figure (9 and 10). The insulin gene was found almost exclusively in pancreatic β-cells.[52] In the blood , glucose was the main stimulant that adjusts the insulin gene expression and allows the beta cells to manufacture insulin and keep an suitable store of intracellular insulin to tolerate the metabolic request [53]. 0.5mM PA treatment significantly suppressed mRNA levels of SIRT1 and INS. Treatment of the INS-1 832/13 HF rat insulinoma cell lines with 10μM SIRT1 aptamer significantly mitigated these PA-induced changes in gene expression (figure 9 and 10). On the other hand, SIRT1 knockdown significantly mitigated the PA-induced increases in SIRT1and INS expression. These data suggest that SIRT1 aptamer may affect β-cells functionality via SIRT1in INS-1 832/13 HF rat insulinoma cell lines.

Conclusion

It has been proved in this research that the novel linear SIRT1 aptamer at dose 10 micromolar can enter the cells to activate SIRTI and mitigates T2DM through targeting SIRT1 via regulated the expression of mitochondrial genes related with biogenesis, lipid metabolism and β-cells in insulinoma cell lines. Our results demonstrations that activation of SIRT1 via linear SIRT1 aptamer may be considered as a prospective future therapy for mitigates T2DM.

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