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Original Article

Investigating the Role of the Histone Deacetylases-Inhibitor Suberanilohydroxamic Acid in the Differentiation of Stem Cells into Insulin Secreting Cells

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Abstract

Introduction: The United Arab Emirates has the second incidence of diabetes in the world. The current diabetes management plans are associated with many complications and do not provide a cure. Stem cells offer hope for permanent alleviation of this health problem through the possible differentiation into insulin-secreting cells. The current methods for the differentiation do not produce homogeneous beta-cell populations. Histone deacetylation is an epigenetic silencing mechanism that can render many genes irresponsive to the induction protocols. This study aimed at investigating the effect of the histone deacetylase inhibitor suberanilohydroxamic acid (SAHA) on the production of functional beta cells, based on a mesenchymal stem cells model. **Methods:** MG63 cells were treated for three consecutive days with SAHA, followed by a three-steps of beta cells differentiation protocol, with media-contained retinoic acid, epidermal growth factor, nicotinamide and exendin-4 at different stages. Then, glucose-stimulated insulin secretion was conducted to assess the functional state of the differentiated cells. **Results:** Pretreating the cells with SAHA enhanced the insulin production and secretion in comparison to the control (PBS) and the vehicle dimethyl sulfoxide, as shown by the immunofluorescence detection of insulin and the transcription factor "PDX1", as well as an increase in insulin secretion in the media. Gene expression analysis showed that SAHA pretreated cells had more induction of the studied markers when challenged with high glucose level. **Conclusion:** Such findings indicate a novel approach to enhance the ability of stem cells to differentiate into insulin-producing cells with potential therapeutic implications.

Keywords: Diabetes, epigenetics, histone acetylation, insulin, stem cells

Introduction

Diabetes affects millions around the world which are estimated to be 415 million in 2015. In the United Arab Emirates, over 1 million cases of diabetes were reported in 2015, and the prevalence of diabetes in adults is more than double the international prevalence (19.3% and 8.8% respectively).[1]

In spite of the continuous development in the medical field, there is no current cure for diabetes. The management plan includes insulin injections for Type 1 diabetes and/or oral hypoglycaemic drugs for Type 2. Considering the limitations of the current management strategies that do not provide a final treatment, several diabetes cure-focused researches were established trying to find an ultimate cure. Currently, islet transplantation represents the only treatment for diabetes in spite of many limitations.[2-4]

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The possibility of stem cells for differentiation into insulin-secreting cells could represent an inexhaustible source for transplantation and a great hope as a potential permanent cure of diabetic patients.[5] In the embryo, stem cells undergo different development stages to become functional and mature β-cells. Each of these stages is controlled by specific key transcriptional factors such as FOXA2, SOX17, PDX1, NKX6.1, NEUROD1 and MafA.[6]

Unfortunately, the proportion of the differentiated cells, according to previously used protocols, is insufficient and

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the cells can be immature. These cells either do not properly respond to glucose stimulation, fail to express β cell markers, co-express other hormones, need a long difficult process or combine >1 of these defects.[7-9] There have been multiple reports suggesting that the stem cells differentiation is controlled by epigenetic factors.[10]

Suberanilohydroxamic acid (SAHA) is a histone deacetylases inhibitor (HDAC-inhibitor) agent that binds to zinc ions in the active site of HDAC enzymes and disturb its function, which render the chromatin to loosen state and the genes more available for transcription.[11]

In this study, we investigated the role of the epigenetic modifier SAHA in the differentiation of stem cells into insulin-secreting cells.

Materials and Methods

Cell culture

The MG63 cell line (American Type Culture Collection;) has been extensively used for their mesenchymal stem cells (MSCs) characteristics.[12-14] The cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) media (Sigma-Aldrich) contained 25 mM glucose and supplemented with 10% foetal bovine serum (Himedia), 100 μg/ml streptomycin (Sigma-Aldrich), 100 I.U./ml penicillin (Sigma-Aldrich), 200 mM L-Glutamine (Gibco) and 10 mM HEPES (Himedia) in humidified air with 5% CO_2, at 37°C.

Cell treatment protocol

MG63 cells were cultured until reached 50% confluent, then serum starved for 24 h and divided into three groups. Each group was incubated with complete DMEM (25 mM glucose) and treated daily with either 1 μM SAHA (Sigma-Aldrich) for three consecutive days, an equivalent amount of dimethyl sulfoxide (DMSO) as a vehicle or PBS (Himedia) as control.

Cell differentiation protocol

Differentiation was carried out according to a previously described, 3-step protocol,[15] using retinoic acid (Sigma-Aldrich), epidermal growth factor (EGF) (Sigma-Aldrich), nicotinamide (Sigma-Aldrich) and exendin-4 (Sigma-Aldrich).

Glucose stimulated insulin secretion assay

Two glucose concentrations (5.5 mM, 16.5 mM) were used. The cells incubated in serum-free DMEM containing 5.5 mM glucose for 4 h at 37°C. Then complete DMEM media with 5.5 mM or 16.5 mM glucose were added for 4 h. The final media were collected and frozen at −20°C until they were assayed for insulin content with ELISA.

Immunofluorescence

The fixed cells on coverslips were permeabilised using 0.05% Triton X-100 (Sigma-Aldrich), saturated with 3% BSA (Sigma-Aldrich) and incubated with primary rabbit anti-human insulin monoclonal antibody (1:500) (Abcam) or anti-human PDX1 monoclonal antibody (1:500) (Abcam). Subsequently, the cells were incubated with a secondary antibody, goat anti-rabbit IgG (1:800) (Abcam). Nuclei were counterstained with DAPI (Invitrogen).

RNA extraction

Total RNA was extracted for all the three groups using the all-in-one Purification Kit (Norgen Biotek) according to the manufacturer's instructions. Briefly, the cell pellets were lysed and applied to a spin column attached to a collecting tube, after multiple steps of washing and drying the membrane, large RNA was released by applying a specific RNA elution solution to the column.

Reverse transcription

Reverse transcription (RT) was performed with the total RNA using GoScript RT system (Promega) according to the manufacturer's protocol. cDNA concentration was determined by Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific) and diluted accordingly.

Quantitative reverse transcription-polymerase chain reaction analysis

RT-polymerase chain reaction (RT-PCR) reaction included 2X SYBR Green Master mix (Promega) and 5–6 pM of each the forward and reverse primers (Macrogen), as well as 100 ng of cDNA. The final reaction volume was 20 μl. The reaction was performed in triplicates using the real-time PCR thermal cycler "Rotor-gene Q" (Qiagen) under the following conditions: 95°C for 2 min followed by 40 cycles of 95°C for 15 s then 60°C for 30 s and 60°C for 30 s. The relative expression levels of the target genes were normalised to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. The figures represent the relative fold change between the gene expression before and after glucose stimulation in correspondence to the control group. The sequences of the primers are enlisted in Table 1.

Measurement of insulin secretion

The culture supernatant was collected after glucose stimulation and used to measure the secreted insulin by the human insulin ELISA kit (Abcam). The incubation and washing steps were performed according to the manufacturer's protocol, and the final colour was read immediately at 450 nm by Varioskan Flash multimode reader (Thermo Fisher Scientific).

Results

Suberanilohydroxamic acid enhanced the production of insulin and PDX1 at the cellular level

The cells, which received PBS or vehicle, had a basal level of insulin and PDX1 production under standard glucose concentration [Figure 1]. In SAHA pretreated cells, both markers were further expressed in comparison to the other two groups. These results suggested that the vehicle (DMSO) had no additive effect over the control (PBS) on the classical differentiation protocol, while SAHA induced the expression of the studied markers.

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Table 1: The primers sequence

Gene	Primer sequences	Reference
INS	Forward: 5'-GCAGCCTTTGTGAACCAACAC -3' Reverse: 5'-CCCCGCACACTAGGTAGAGA -3'	[16]
NKX6.1	Forward: 5'-TCAACAGCTGCGTGATTTTC -3' Reverse: 5'-CCAAGAAGAAGCAGGACTCG -3'	[17]
MafA	Forward: 5'-AGCGAGAAGTGCCAACTCC -3' Reverse: 5'-TTGTACAGGTCCCGCTCTTT -3'	[18]
GCK	Forward: 5'-GCAGATGCTGGACGACAG -3' Reverse: 5'-TCCTGCAGCTGGAACTCTG -3'	[18]
SOX17	Forward: 5'-GACGACCAGAGCCAGACC-3' Reverse: 5'-CGCCTCGCCCTTCACC -3'	[19]
FOXA2	Forward: 5'-GGGAGCGGTGAAGATGGA -3' Reverse: 5'-TCATGTTGCTCACGGAGGAGTA -3'	[20]
GAPDH	Forward: 5'-CCAGGTGGTCTCCTCTGACTTC -3' Reverse: 5'-TCATACCAGGAAATGAGCTTGACA -3'	[21]

Gene expression analysis

On the molecular level [Figure 2], SAHA has enhanced the relative gene expression of insulin in response to high glucose challenge by 248% in comparison to the control and 161% to the vehicle. Similarly, SAHA induced the signalling molecules NKX6.1, MafA, SOX17 and FOXA2 and by 304%, 321%, 285% and 246% in response to glucose stimulation as well as GCK by 342%. All the markers were statistically higher than the vehicle ($P < 0.05$).

Suberanilohydroxamic acid enhanced basal insulin secretion but not in response to high glucose stimulation

As shown in Figure 3, insulin secretion was evident in all studied groups. However, for both PBS and DMSO treated cells, there was no statistically significant difference between the insulin secretion before (8.5 and 9.8 μlU/ml) or after high glucose stimulation (11.5 and 14.4 μlU/ml). Interestingly, SAHA treated cells secreted almost double amount of insulin (17.5 μlU/ml) than PBS and vehicle. However, these cells did not respond appropriately to the high glucose challenge, as their insulin secretion showed no statistical difference than the nonstimulated cells.

Discussion

Diabetes mellitus represents a major public health concern that affects a large number of patients worldwide. The current management plan includes lifelong insulin injection and/or oral hypoglycaemic agents, while there is no current cure for diabetes. Diabetes can lead to many complications due to the high level of blood glucose and the subsequent glycosylation reaction for many proteins and the consequent disturbance of the lipids metabolism. The control of the disease and the management of the complications added economic burden to the diabetic patients, families as well as the healthcare system. The direct annual cost of diabetes worldwide was >827 billion US dollars in 2016, according to the World Health Organisation global report on diabetes.[22]

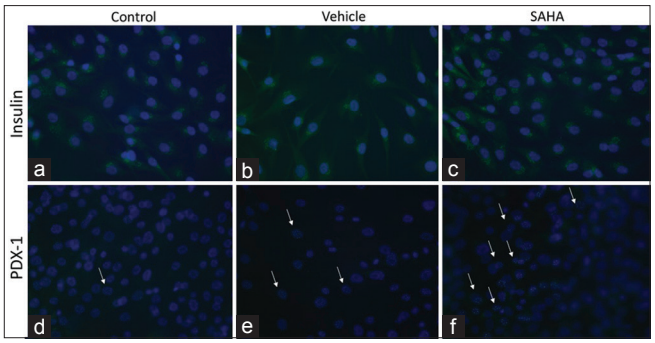

Figure 1: Immunodetection of insulin (a-c) and PDX-1 (d-f) has showed basal expression of both markers in control (a and d) and vehicle (b and e) groups. Suberanilohydroxamic acid pretreated cells showed increased intensity of insulin in the cytoplasm (c) and PDX-1 in the nucleus (f). Photos were taken at ×60

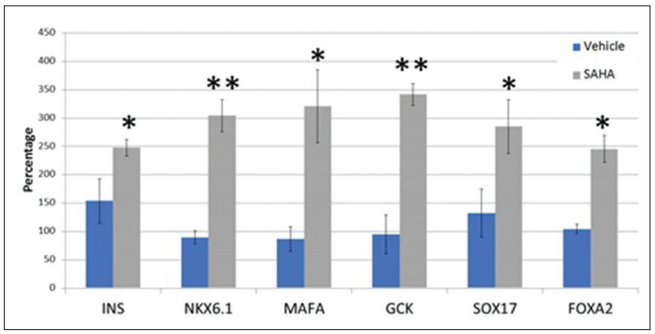

Figure 2: Relative changes of gene expression upon high glucose challenge. Suberanilohydroxamic acid pretreated cells upregulated all the studied markers in comparison to the vehicle. *$P < 0.05$, **$P < 0.01$

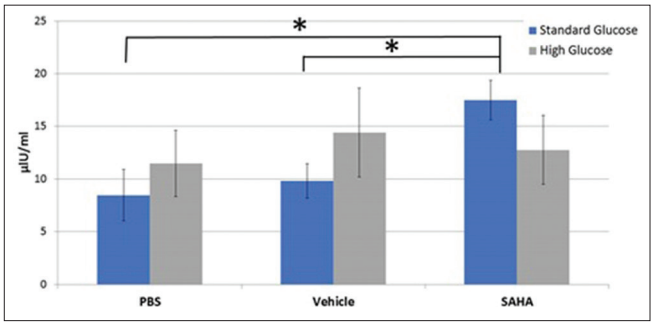

Figure 3: Insulin secretion in the media was measured by ELISA. At the standard glucose level, suberanilohydroxamic acid enhanced the production of insulin in comparison to the control by 205% and vehicle by 178%, while there was no statistically significant difference between the control and vehicle. After high dose glucose stimulation, there was no significant difference between any of the groups. *$P < 0.05$

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Pancreatic or pancreatic islets transplantation represents the only hope for physiological glycaemic control. However, the number of donors is limited and the transplantation-related complications are still major obstacles. Therefore, finding an alternative source of insulin-secreting cells may solve this dilemma. Regenerative medicine is an emerging branch of medicine which aims at replacing lost or failed organs, based on the stem cells technology.[23,24]

In this study, we aimed at generating insulin-secreting cells that would help diabetic patients and function as the normal beta cells in response to changing glucose levels in the blood. The main target was to provide a feasible source for insulin-secreting cells that may replace the islets or pancreatic transplantation. Furthermore, successful autologous stem cells differentiation into insulin-secreting cells represents an attractive target, as these cells would lack the immune response which can be triggered by the allogenic pancreatic and islets transplantation.

Despite the intensive researches in this field, the generated differentiated cells according to previously reported protocols are not completely satisfactory.[8] A recent study, compared three protocols for the differentiation of MSCs into insulin-secreting cells, reached to a conclusion that the used protocols were not very efficient and further development was required. Therefore, the authors suggested the modification of the current protocols to achieve more efficient differentiation.[7] Other research groups showed better differentiation of insulin-secreting cells, but the process was very long and difficult to be routinely applied.[9]

Epigenetic mechanisms, like histone acetylation, are well known to be involved in the stem cell differentiation.[21,25] In this study, we used an epigenetic modifying agent to improve the responsiveness of the cells to the differentiation protocols. The basic concept is activating the genome, rendering the target genes more accessible for transcription factors and other signalling molecules activated by the differentiation protocol components.[26] SAHA is a HDAC-inhibitor. Similar derivatives were used effectively in improving the differentiation of stem cells into several lineages including neurogenic, osteogenic, chondrogenic lineages.[27,28]

The differentiation of stem cells in our study was carried out according to a three-steps differentiation protocol, which required relatively fewer reagents and involves fewer steps than classical protocols.[15] This protocol depended on providing the crucial elements that enhance stem cell differentiation into the endocrine lineage. Retinoic acid, nicotinamide, EGF, and exendin-4 were applied to the culture media through three consequent steps, over 16 days. Retinoic acid induces the expression of transcription factors that control endocrine cell differentiation, such as PDX-1 and Ngn3 that are expressed in the foregut endoderm.[29] Nicotinamide and EGF induces maturation of the pancreatic endocrine cells and sustains PDX1 expression.[30] Exendin-4 is a potent glucagon-like peptide-1 agonist that increases insulin and PDX1 expression.[31,32] Unfortunately, the original protocol produced immature insulin-producing cells.[15]

In our system, control group PBS showed similar results to Gao *et al*. 2008.[15] There was evidence of insulin synthesis (by immunofluorescence), secretion (by ELISA) and gene expression (by RT-PCR). Our data suggested that pretreating the cells with SAHA, the HDAC-inhibitor, could enhance the process of insulin production. In addition, the cells were able to respond to the high glucose challenge by inducing the genes coding for the various molecules involved in the insulin production as well as the insulin gene. The latter can be induced by the direct action of SAHA or under the effect of an upregulated signalling molecule, which can be one of the reported upregulated genes. Epigenetic regulation of insulin production and secretion can be achieved through multiple approaches. For example, PDX1 can stimulate insulin gene expression by hyperacetylating histone H4 at the insulin gene promoter.[33] Nevertheless, all four histone acetyltransferases are important for insulin gene expression.[34] Therefore, pretreating the cells with SAHA showed enhancement of insulin production, as showed by immunofluorescence, but the cells appeared to be at an earlier stage of maturation regarding insulin secretion in response to glucose stimulation.

SOX17 has multiple roles in beta cells development and function. While the classical role was in the endoderm lineage development, some reports showed that knocking down SOX17 did not influence the pancreatic development, but it affects insulin trafficking and secretion.[35-37] In our system, SAHA has enhanced the gene expression of SOX17 in response to high glucose stimulation in comparison to the vehicle and control.

The transcription factor Nkx6.1 is important for β-cells development and insulin production, through the regulation of a gene network involved in insulin production and processing.[16] In our study, SAHA pretreated cells showed the highest NKX6.1 expression.

MafA is considered as a key marker that is expressed only in mature β-cells, and it regulates glucose-stimulated insulin secretion as well as drives for beta cells proliferation in the postnatal life.[17,18] Different cell groups followed the same pattern of increasing the expression of this marker when they were in high glucose. In addition, MafA has a role in the regulation of many pancreatic β-cells associated genes such as the glucose transporter Type 2 (GLUT2) and GCK. GLUT2 is the main transporter of glucose through the β-cells plasma membrane while GCK is the enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate and regulates the carbohydrate metabolism. Moreover, in beta cells, it serves as a glucose sensor, amplifying insulin secretion as blood glucose rises. The gene expression for this enzyme was upregulated in response to high glucose in SAHA pretreated cells.

The collective evidence suggested that SAHA enhanced the insulin production as could be observed by immunohistochemistry

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as well as ELISA. These cells responded to the high glucose stimulation by overexpressing many transcription factors in the insulin production pathway. The cells could be at an earlier stage of development that needed more time to respond by further enhancement of the insulin gene expression and consequently regulated secretion of insulin into the media. Further studies that assess the secretion at different time points, over a longer duration, could help in explaining this part of our findings.

Conclusion

SAHA represents a potential agent to improve stem cells differentiation into insulin-secreting cells, when used according to our protocol. Further investigation is recommended to improve the cell response to increasing concentration of glucose. In addition, the effect of pretreatment with SAHA on other beta cells differentiation protocols could also be considered.

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Conflicts of interest

There are no conflicts of interest.

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