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Possible protective effect of procainamide as an epigenetic modifying agent in experimentally induced type 2 diabetes mellitus in rats

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KEYWORDS

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Abstract *Background:* Diabetes mellitus (DM) is a metabolic disease that is associated with disturbed carbohydrates, lipids and protein metabolism due to insulin deficiency and/or impaired insulin action. Recently epigenetic mechanisms were shown to be involved in endocrine cell differentiation and islets function. Procainamide which is a cardiac antiarrhythmic drug has been recently classified as one of the epigenetic drugs targeting DNA methylation.
Aim: The present study was designed to evaluate the effect of procainamide as a demethylating epigenetic agent on streptozotocin-induced type 2 diabetes mellitus in rats.
Methods: Fifty adult male albino rats of weight ranging from 150 to 200 g were included in this study. Rats were divided into five groups (each of 10 rats) as follows: group I: served as a normal control group, group II: diabetic rats that received 1 ml gum acacia 2% orally, daily for 4 weeks, group III: diabetic rats that received procainamide (20 mg/kg body weight)/day, orally for 4 weeks, group IV: diabetic rats that received metformin (300 mg/kg/day), orally for 4 weeks, group V: diabetic rats that received both procainamide and metformin in the same previous doses for 4 weeks. The following parameters were assessed in rats of all studied groups: fasting blood glucose level, serum insulin level, serum tumor necrosis factor alpha (TNF-α) (as a proinflammatory cytokine as well as an indirect biomarker of DNA methylation) and DNA methyltransferase enzyme (DNMT) activity in pancreatic tissues (as a direct marker of DNA methylation).
Results: The present study revealed that combined administration of both procainamide and metformin produced a statistically significant reduction of fasting blood glucose levels as compared to untreated diabetic rats as well as diabetic rats treated by either procainamide or metformin alone. In

Abbreviations: CpG, cytosine–phosphate–guanine; DM, diabetes mellitus; DNA, deoxyribonucleic acid; DNMT, DNA methyltransferase; TNF-α, tumor necrosis factor-alpha.

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addition, procainamide administration either alone or in combination with metformin resulted in a statistically significant rise of serum insulin levels. TNF-α levels were statistically elevated in diabetic untreated rats as well as those treated with metformin only while procainamide intake led to its statistical decrease. Also, procainamide administration produced a statistically significant reduction in the activity of DNA methyltransferase in pancreatic tissues reflecting its possible role as a demethylating agent that increases insulin expression and release by pancreatic cells.

Conclusion: The present work could provide a proof of concept that procainamide could be used as a possible therapeutic potential in type 2 diabetics as an epigenetic demethylating agent to increase insulin levels and it is better to be used in combination with oral hypoglycemic agent e.g. metformin to decrease insulin resistance.

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1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defect in insulin secretion, insulin action or both and it is associated with disturbed carbohydrates, lipids and protein metabolism.[1] DM can be classified according to the treatment required to control it into insulin dependent (type 1) and non-insulin dependent (type 2) diabetes or it may be classified according to the age at which the disease develops into juvenile or maturity onset diabetes. Pathogenesis of DM represents a complex of interaction between metabolic, genetic and environmental factors as well as inflammatory mediators.[2]

Evidence are emerging that several diseases and behavioral pathologies result from defects in gene functions. The best studied example is cancer but other diseases such as autoimmune diseases, asthma, type 2 diabetes and metabolic disorders display aberrant gene expression. Gene function may be altered by either change in the sequence of DNA or change in epigenetic programming.[3]

Epigenetic information is defined as heritable information other than the DNA sequence that is critical to the proper functioning of the gene .These epigenetic alterations are potentially reversible and DNA methylation represents the most characterized epigenetic modification. It is represented by methylation of cytosine residues within the context of the cytosine–phosphate–guanine (CpG) dinucleotide which is catalyzed by enzymes belonging to the DNA methyltransferases family (DNMTs). Although the density of CpG sites in the genome is very low, there are clusters of CpG sites, known as 'CpG islands', which are generally kept unmethylated. The CpG islands remain free of methylation and are associated with transcriptionally active genes, predominantly so-called 'housekeeping' genes. When a CpG island in the promoter region of a gene is methylated, expression of the gene is repressed.[4]

With epigenetic drugs, it is possible to reverse aberrant gene expression profiles associated with different disease states and several epigenetic drugs targeting DNA methylation has been tested in clinical trials. Understanding the epigenetic machinery and the differentiation roles of its components in specific disease states is essential for developing targeted epigenetic therapy.[5]

Recently, epigenetic mechanisms were shown to be involved in pancreatic endocrine cell differentiation and islet function. In depth understanding of epigenetic landscape can help to improve protocols to enhance pancreatic beta cells proliferation and lead to the discovery of novel therapeutic agents.[6]

Inhibitors of DNA methyltransferases may be either nucleoside analogs such as 5-aza-2′deoxycytidine, decetabine and zebularine or non-nucleoside such as procainamide and hydralazine. Procainamide is a cardiac antiarrhythmic drug that has been approved for treatment of life threatening ventricular arrhythmias. Recently it has been classified as one of DNA demethylating epigenetic agents with growth inhibitory effects in human cancer cells.[7] Therefore, the present study aimed to assess the possible protective effect of procainamide as a demethylating epigenetic agent on experimentally induced type 2 DM in rats.

2. Material and methods

This study was carried out on fifty adult male albino rats of body weight ranging from 150 to 200 g. They were housed under the same environmental conditions of light and temperature, fed standard diet and had free access to water. The study protocol was approved by ethics committee of Faculty of Medicine, Alexandria University.

The animals were divided into five groups (each of 10 rats) as follows:-

Group I (normal control group): This group received 1 ml of 0.1 M sodium citrate buffer (pH 4.5) intraperitoneally (i.p.).

The remaining 40 rats were subjected to induction of type 2 DM by a single i.p. injection of 40 mg/kg body weight streptozotocin (STZ) (Sigma Chemical Co., Switzerland) after being dissolved in 0.1 M sodium citrate buffer (pH 4.5).[8] One week after STZ injection, rats were assessed for hyperglycemia.[9]

When DM was developed, rats were randomly assigned into the following groups:

Group II (diabetic untreated group): This group included rats with induced DM that received 1 ml of 2% gum acacia/day (Arabic Laboratory Equipment Co.) orally for 4 weeks.

Group III (procainamide-treated diabetic group): This group included diabetic rats that were given procainamide orally for 4 weeks in a daily dose of 20 mg/kg body weight.[10]

Group IV (metformin-treated diabetic group): This group involved diabetic rats that received metformin (as oral anti-diabetic drug of biguanide class that decreases insulin resistance) in a daily dose of 300 mg/kg body weight orally for 4 weeks.[11]

Group V (procainamide- and metformin-treated diabetic group): Rats of this group were given both procainamide and

metformin orally in the same previously mentioned doses and durations.[10,11] The calculated doses of either procainamide, metformin or both were dissolved in 1 ml of 2% gum acacia and were given via an oral gavage syringe.

At the end of the experiment, rats of all groups were fasted for 8–12 h with free access to water. Rats were then anesthetized by ether and blood samples were withdrawn from medial retro-orbital venous plexus using capillary hematocrit tube.[12] Blood samples were left to coagulate at 37 °C and then were centrifuged for 15 min at 3000 rpm. Sera were separated and stored at −20 °C until being used. After taking blood samples, rats were sacrificed and pancreases were rapidly excised, immediately washed with ice-cold saline, blotted on dry filter papers, weighed and then homogenized using a kit to separate nuclear proteins (Epiquik Nuclear Extraction kit II; Epigentek, NY, USA) as follows:[13]

3. Cell pellet preparation

1. The pancreatic tissue was cut into small pieces, then placed in a clean homogenizer.
2. 10× Pre-extraction buffer (NP1) was diluted with distilled water at a 1:10 ratio, and 5 ml of diluted NP1 (1×) containing 5 µl of dithiothreitol (DTT) were added per gram of tissue and tissue pieces were homogenized (50–60 strokes), then incubated on ice for 15 min.
3. Centrifugation was done for 10 min at 12,000 rpm at 4 °C and the supernatant was removed and discarded.

4. Nuclear extract preparation

1. DTT solution and protease inhibitor cocktail (PIC) were added to extraction buffer (NP2) at a 1:1000 ratio, followed by adding extraction Pre-Cleaner (NP3) to NP2 at 1:10 ratio. Two volumes of NP2 were added to nuclear pellet (about 10 µl NP2 per 2 mg tissues). The extract was incubated on ice for 15 min. with vortex (5 sec.) every 3 min. The extract was further sonicated for 3×10 s to increase nuclear protein extraction.
2. The suspension was centrifuged for 10 min. at 14,000 rpm at 4 °C and the supernatant was transferd into a new microcentrifuge vial.
3. The Extraction Cleaner (NP4) was added to the supernatant at a 1:100 ratio (ex: 10 µl of NP4 to 990 µl of the supernatant) and incubated for 15–20 min at room temperature.
4. The suspension was centrifuged for 1 min at 14,000 rpm at 4 °C and the supernatant was transferd into a new microcentrifuge vial.
5. The protein concentration of the nuclear extract was measured.
6. The supernatant was kept at −80 °C until further use for determination of DNA methyltransferase (DNMT) activity.

The following parameters were measured in all the studied groups:

1- Serum levels of fasting blood glucose; by using glucose oxidase method.[14]
2- Serum insulin levels using a solid phase enzyme amplified sensitivity immunoassay.[15]
3- Serum levels of tumor necrosis factor-alpha (TNF-alpha) (as a proinflammatory cytokine as well as an indirect biomarker of DNA methylation) using quantitative sandwich enzyme immunoassay technique.[16]
4- DNA methyltransferase (DNMT) activity (as a direct marker of DNA methylation) in pancreatic tissues was measured colorimetrically (Epiquick DNA methyltransferase activity/inhibition assay kit) in accordance with the manufacturer's instructions.[13]

5. Statistical analysis

Data were expressed as means with their corresponding standard deviation (SD). A one way analysis of variance (ANOVA or *F* test) using the statistical package of social sciences (SPSS) was used to compare more than two means. Association between different parameters was determined using Pearson's correlation coefficient (*r*). Statistical significance was set at *P* values less than 0.05.

6. Results

6.1. Serum levels of both fasting blood glucose and insulin in the studied groups as shown in Table 1 and Figs. 1 and 2

The present study revealed that fasting blood sugar levels showed a statistically significant elevation in groups II (diabetic untreated group), III (procainamide-treated diabetic group) and IV (metformin treated diabetic group) as compared to normal healthy controls of group I as well as to rats of combined procainamide and metformin treated of group V. ($F = 75.79$ and $P = 0.0001$).

On the other hand, regarding serum insulin levels, the current study showed that there was a statistically significant reduction of serum insulin levels in group II and group IV as compared to groups I, III and V but there was no statistically significant difference between the insulin levels in rats of groups III and V. ($F = 5.931$ and $P = 0.001$).

6.2. Serum levels of tumor necrosis factor-alpha (TNF-alpha) and pancreatic tissue activity of DNA methyltransferase (DNMT) in the studied groups as shown in Table 2 and Figs. 3 and 4

The present work revealed that oral administration of procainamide either alone in diabetic rats of group III or in combination with metformin in diabetic rats of group V for 4 weeks had resulted in a statistically significant decrease in the serum levels of TNF-alpha as compared to those rats of groups I , II and IV. There was a statistically significant increase in serum levels of TNF-alpha in groups II and IV as compared to normal healthy controls of group I ($F = 5.12$ and $P = 0.002$).

Considering pancreatic tissue activity of DNA methyltransferase (DNMT) in the studied groups, the current study showed a statistically significant reduction in the activity of DNMT of pancreatic tissues of rats treated with procainamide

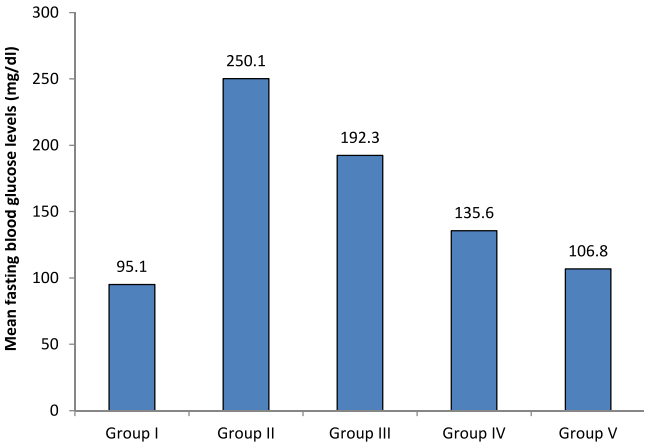

Figure 1 Serum levels of fasting blood glucose (mg/dl) in the studied groups.

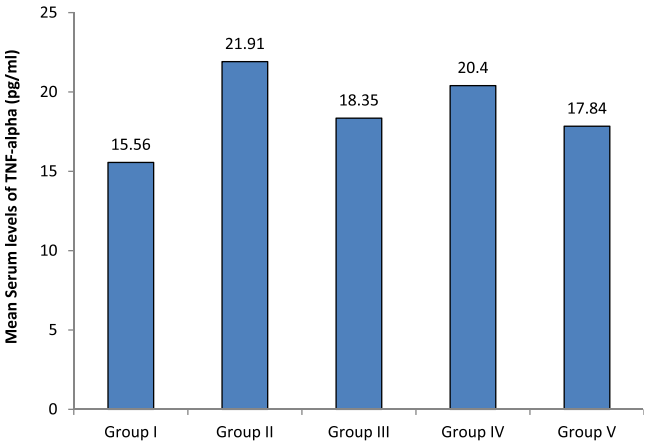

Figure 3 Serum levels of TNF-alpha (pg/ml) in the studied groups.

of groups III and V as compared to rats of the other remaining groups (groups I, II and IV) ($F = 76.4$ and $P = 0.0001$).

6.3. Correlation between serum levels of fasting blood glucose and insulin as well as tumor necrosis factor-alpha (TNF-alpha) and pancreatic tissue activity of DNA methyltransferase (DNMT) in the studied groups as shown in Table 3

The present study revealed that there was a statistically significant negative correlation between serum levels of FBS and serum insulin levels ($P = 0.015$). On the other hand, there was a statistically significant positive correlation between serum levels of FBS and serum levels of TNF-alpha as well as between pancreatic tissue activity of DNMT and serum levels of TNF-alpha ($P = .020$ and 0.0351, respectively).

N.B. the letters a, b, c, d in Tables 1 and 2 are called Duncan letters & are used to detect the least significant difference (LSD) in ANOVA study.

7. Discussion

Type 2 diabetes mellitus (DM) involves insulin resistance and relative insulin deficiency rather than absolute insulin deficiency. Insulin resistance has been proposed to be the initial step in the cascade towards diabetes. Its actual mechanism is not completely understood that may be related to several factors as leptin, TNF-alpha and other substances. Moreover, genetic polymorphism may play an important role in its pathogenesis.[17]

The mechanism of β-cell failure in type 2 diabetes is unknown, in addition to a genetic predisposition, many studies demonstrated higher rates of apoptosis and decreased mass of pancreatic β-cells.[18,19]

Changes in the normal program of gene expression are the basis for several human diseases. Gene function may be altered by either a change in the sequence of the DNA or a change in the epigenetic programming of a gene in the absence of sequence change. So, with epigenetic drugs, it is possible to reverse aberrant gene expression profile associated with different disease states.[20]

The goal of the present study was to clarify the possibility of using an epigenetic modifying agent as procainamide in cases of experimentally induced type 2 DM as a possible therapeutic potential either alone or in combination with oral hypoglycemic agent as metformin.

There was a statistically significant elevation of fasting blood glucose levels of diabetic untreated rats & those treated

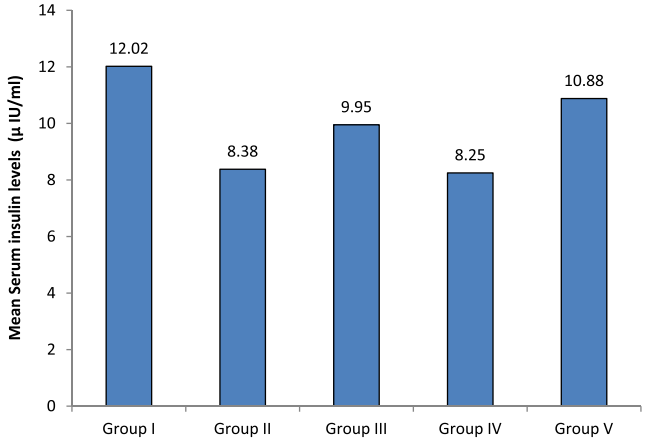

Figure 2 Serum insulin levels (μ IU/ml) in the studied groups.

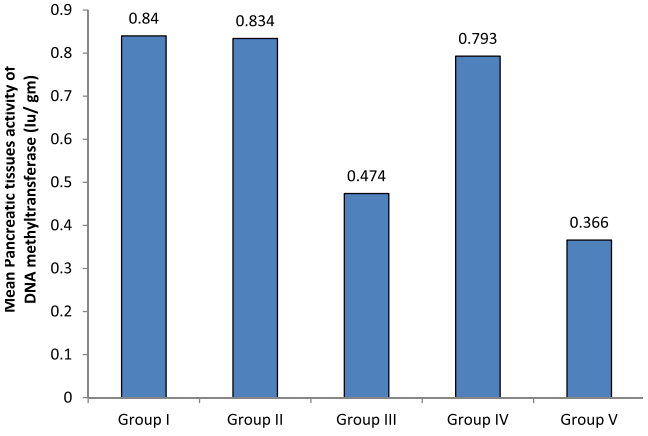

Figure 4 Pancreatic tissue activity of DNA methyltransferase (IU/gm tissue) in the studied groups.

Table 1 Serum levels of fasting blood glucose and insulin in the studied groups.

Parameter	Group I (normal controls) (n = 10)	Group II (diabetic non treated rats) (n = 10)	Group III (procainamide treated diabetic rats) (n = 10)	Group IV (metformin-treated diabetic rats (n = 10)	Group V (procainamide and metformin treated diabetic rats) (n = 10)
Fasting blood glucose level (mg/dl)					
Range	70–118	190–325	165–213	110–157	88–129
Mean ± SD	95.1 ± 16.26[a]	250.1 ± 45.59[b]	192.3 ± 15.58[c]	135.6 ± 14.21[d]	106.8 ± 13.15[a]
F	75.79				
P	0.0001*				
Serum insulin level (μ IU/dl)					
Range	7.8–16.1	4.3–11.1	6.1–12.1	5.1–10.3	7.2–12.9
Mean ± SD	12.02 ± 2.80[a]	8.38 ± 2.25[b]	9.95 ± 1.74[ab]	8.25 ± 1.73[b]	10.88 ± 1.80[ab]
F	5.913				
P	0.001*				

Different letters indicate statistically significant difference.
Statistical significance was set at P values less than 0.05.
The meaning of each group was written in the upper row of each table.

Table 2 Serum levels of tumor necrosis factor-alpha (TNF-alpha) and pancreatic tissue activity of DNA methyltransferase in the studied groups.

Parameter	Group I (normal controls) (n = 10)	Group II (diabetic non treated rats) (n = 10)	Group III (procainamide treated diabetic rats) (n = 10)	Group IV (metformin treated diabetic rats (n = 10)	Group V (procainamide and metformin treated diabetic rats) (n = 10)
Serum levels of TNF-alpha (pg/ml)					
Range	10.8–20.1	19.7–25.3	14.2–20.9	12.9–25.5	11.2–21.5
Mean ± SD	15.56 ± 3.63[a]	21.91 ± 1.97[b]	18.35 ± 2.15[c]	20.4 ± 4.51[b]	17.84 ± 4.04[c]
F	5.12				
P	0.002*				
Pancreatic tissue activity of DNA methyltransferase (IU/gm tissue)					
Range	0.7–0.93	0.72–0.99	0.33–0.67	0.7–0.89	0.25–0.49
Mean ± SD	0.84 ± 0.08[a]	0.834 ± 0.08[a]	0.474 ± 0.11[b]	0.793 ± 0.06[a]	0.336 ± 0.07[b]
F	76.4				
P	0.0001*				

Different letters indicate statistically significant difference.
Statistical significance was set at P values less than 0.05.
The meaning of each group was written in the upper row of each table.

Table 3 Correlation between serum levels of fasting blood glucose and insulin as well as tumor necrosis factor-alpha (TNF-alpha) and pancreatic tissue activity of DNA methyltransferase (DNMT) in the studied groups.

		Fasting blood glucose	Serum insulin	Serum level of TNF-alpha
Serum insulin	Pearson Correlation (r)	−.343*		
	P	.015		
Serum level of TNF-alpha	Pearson Correlation (r)	.329*	−.105	
	P	.020	.469	
DNA methyltransferase activity of pancreatic tissues	Pearson Correlation (r)	.146	−.094	.235*
	P	.311	.517	.0351

* Correlation is significant at the 0.05 level (2 - tailed).

by either procainamide or metformin alone in comparison to normal or those treated by both procainamide and metformin. Serum insulin levels were significantly elevated in rats treated with procainamide either alone or in combination with metformin, when compared to those treated only with metformin. These results could reflect the possible role of procainamide as an agent that increased expression and release of insulin from pancreatic β-cells due to its epigenetic modifying effect in diabetes treatment. These findings are consistent with results of Bramswig et al.[21] study that reported that epigenetic

mechanisms were shown to be involved in endocrine cell differentiation and islet function and could enhance β-cell proliferation and may lead to the discovery of novel therapeutic target.

Regarding serum levels of TNF-alpha, results of the current work showed a significant elevation in diabetic untreated rats and those treated by metformin alone but procainamide treatment resulted in a significant reduction of TNF-alpha levels. These findings indicate the probable inflammatory role of TNF-alpha as a mediator in the pathogenesis of DM.

Our results were in accordance to the finding reported by Hu et al.[22] Recent research done by Mirza et al [23] reported that diabetes as whole was strongly associated with elevated levels of IL-6, leptin, C-reactive protein and TNF-alpha and there was an association between low-grade inflammation and quality of glucose control.

Also, the same finding was reported in a study done by Jatla et al.[24]who stated that TNF-alpha inhibits insulin transduction and has an effect on glucose metabolism. Moreover, it was found that TNF-α could be a contributing factor to diabetic complications such as atherosclerosis[25] and dyslipidemia.[26]

Similar to our findings, Tousoulis et al [27] reported that TNF-α serum levels remained unchanged in diabetic patients treated by metformin alone but, these levels were reduced in those treated by a combination of metformin and atorvastatin. Also, it was found that metformin alone did not improve the plasma concentrations of inflammatory markers and adipokines in patients with type 2 DM.[28] But, in contrary to our findings, Derosa et al [29] reported that metformin led to a significant reduction in inflammatory state parameters in poorly controlled type 2 diabetic patients.

However, no studies were found that spoke on the effect of PA on TNF-alpha level or expression.

Considering pancreatic tissue activity of DNA methyltransferase (DNMT), the results of the present study revealed that it was higher in both diabetic untreated rats and metformin alone treated ones, but procainamide either alone or in combination with metformin reduced its pancreatic activity. These results could suggest that DNA methylation during insulin synthesis by β-cells may be implicated in the pathogenesis of decreased insulin levels and consequently diabetes mellitus, whereas procainamide could emerge as a potential demethylating agent for clinical translation.

Many lines of evidence indicate that procainamide inhibits DNMT by reducing its affinity with its two substrates which are hemimethylated DNA and adenosyl methionine.[30]

Moreover, in a study performed on adult rats with diabetes, it was found that there was an interaction between diabetes and status of gene methylation. Thus a demethylating agent could down regulate the expression of DNMT and hence, may guide the development and evaluation of new treatment modalities for patients with diabetes.[31]

Epigenetics provide a mechanism which may explain the etiology of type 2 diabetes, obesity and other human diseases such as cancer.[32] DNA methylation is an epigenetic modification that plays a key role in various biological processes and it is believed to be modulated by environmental and nutritional factors and essentially functioning as a molecular switch to turn genes on or off.[33]

In the current work, there was a significant positive correlation between serum levels of TNF-alpha and pancreatic tissue levels of DNMT. This finding was in accordance to the study performed in 2009[34] and reported that baseline TNF-alpha circulating levels were positively correlated with total methylation and could be a good marker for DNA methylation.

Finally, to sum up, our data provide a proof of concept that procainamide could be pharmacologically exploited to develop a novel DNA methylation inhibitor that may be used in type 2 diabetes to increase insulin expression and release. It could also be used in combination with other anti-diabetic drugs that decrease insulin resistance as metformin. The exact roles of procainamide as an epigenetic demethylating agent in different body systems deserve further investigations in the future researches.

Conflict of interest

None declare.

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