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Aberrant *PDK4* Promoter Methylation Preceding Hyperglycemia in a Mouse Model

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Abstract
Diabetic prevalence is at speedy increase globally. Previous studies stated that other than genetics, factors such as environment, lifestyle, and paternal-maternal condition play critical roles in diabetes through DNA methylation in specific areas of the genome. The purpose of this study is to investigate the methylation pattern of the *PDK4* promoter in streptozotocin-induced diabetic mice until the 12th week of the observation. The methylation pattern in the blood samples was analyzed periodically, while the pattern in the muscle sample was only analyzed at the end of the experiment using the blood of the sacrificed animals. Three methylated CpG site 1, CpG site 6, and CpG site 7 were analyzed and quantified based on the band density using bisulfite treatment and methylation-specific polymerase chain reaction (PCR). The hyperglycemia period was developed at the 9th week of experiment. However, there was a significant increase of methylation, specifically on CpG site 6 started from week 6 to week 12. This peculiar methylation on CpG site 6 of *PDK4* promoter in the blood sample before the hyperglycemic period might serve as a potential biomarker for early detection of diabetes in the patients. No significant difference was found between the methylation level of streptozotocin (STZ)–treated mice and of the control group in the muscle sample.

Keywords Blood · Epigenetics · Methylation pattern · *PDK4* · STZ-induced diabetic mice

Introduction

From 151 million in 2000 to 451 million in 2017, global diabetic prevalence has been an increasing trend [1]. Das and Elbein mentioned in their review that parental diabetes is descended on the

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children through gene mutation [2]. Recent studies show that genetics is not the only factor in the emergence of diabetes. Environmental factors such as maternal health during pregnancy, diets, and lifestyles are more dominant in contributing to the new events of diabetes [3].

Genetic and environmental factors interact, drive the gene expression, and lead to certain phenotypes. The interaction between environment and gene is called epigenetics [4], and one of the most studied epigenetic mechanisms is DNA methylation. DNA methylation refers to the addition of a methyl group to the cytosine residues located on the CpG sites in the DNA [5]. This type of methylation in the promoter of the gene may lead to the gene repression since its pattern regulates specific gene expression in a specific cell at a certain time. Thus, the regulation of methylation level can meet normal metabolism process.

Aberrant DNA methylation pattern causes abnormal gene expression and leads to diseases, such as diabetes [6]. Our previous study showed that aberrant high DNA methylation levels in the placenta independently correlated with gestational diabetic mothers [4]. Aberrant DNA methylation pattern of specific genes is also reported to have affected the metabolic activity [7].

One of the reasons for diabetes is the imbalance between energy production and its consumption. Less energy consumption leads to the new onset of diabetes. Cells have the flexibility to select and to transform the sources of energy based on the availability of the sources in the body [8]. These cells tend to use glucose as an energy source once the cells have rich glucose environment. Conversely, when there is no glucose intake for some time, the cell breaks lipid molecules to generate energy. Failure of cell flexibility in transforming glucose into lipid as energy source molecule may lead to serious health problems followed with several symptoms such as insulin resistance and hyperglycemia which serve as the markers of diabetes [9].

One enzyme that plays an important role in maintaining cell flexibility between glucose and lipid molecule as an energy source is the pyruvate dehydrogenase complex (PDC) [10]. This enzyme plays a role in the decarboxylation of pyruvate into acetyl-CoA which is a molecule that can enter the citric acid cycle. PDC is an enzyme that can link glucose to lipid metabolism. The increased activity of the PDC enzyme can reduce glucose levels by accelerating the formation of pyruvic acid to acetyl-CoA which will produce energy after the citric acid cycle. Conversely, in low glucose levels, PDC activity is also low, and acetyl-CoA is mostly produced by the oxidation of free fatty acids as a result of lipolysis.

The PDC activity is regulated by the pyruvate dehydrogenase kinase (PDK) through the regulation of the phosphor level in PDC. Currently, there are several PDK enzymes that work specifically in different tissues [11]. The previous study showed that excessive expression of *PDK4* causes hyperglycemia and is associated with diabetes [12]. Furthermore, animal studies show that a high-fat diet correlates with a high level of fasting blood sugar concentration. Conversely, animals with high-fat diet can keep the normal sugar levels when the expression of *PDK4* is simultaneously suppressed. This shows that *PDK4* expression is essential to the contribution of hyperglycemia in the individual diabetes through the maintenance of the balanced expression in using glucose or lipid molecules as energy sources [12].

The previous study showed that the *PDK4* methylation in the 5′ UTR decreases, and the expression is increased in the patients' obesity from the blood sample [13]. Other studies have shown an increase in metabolism of *PDK4* in patients undergoing gastric bypass to control body weight. As seen there, epigenetic regulation plays a role in the weight of the patients with gastric bypass [14]. Direct observation was conducted by Kulkarni et al. who reduced methylation and increased expression of *PDK4* in diabetic patients from the muscle sample [15]. However, the abovementioned studies have so far been confined to the exploration of the correlation between diabetes and the *PDK4* methylation level. In contrast, further exploration

of the first-onset phenotype between hyperglycemia as a symptom of diabetes and aberrant methylation pattern of *PDK4* is required. The aim of this study is to answer whether the aberrant methylation pattern of *PDK4* or hyperglycemia is the first detected phenotype in the diabetic mouse model. The study is designed to increase the savior of diabetes using periodic streptozotocin (STZ) injection to mouse models. The blood sample was periodically taken until the hyperglycemia was observed. DNA methylation was determined afterwards.

Method

Animal Experiment

The experimental procedures have passed the guidelines of institutional ethical committee, University of Surabaya, Indonesia (No. 012/KE/III/2018). This research used experimental animals of 5-week-old male Swiss Webster mice that have almost the same weight. There were two groups: the control ($n = 8$) and the STZ ($n = 7$) groups. Both groups were fed with common pellet diet. The STZ group underwent the STZ injection 60 mg/kg body weight intraperitoneally [16]. The injection was carried out from the 5th week of the experiment and repeated weekly until the 9th week of the experiment to develop type 2 diabetes mellitus (T2D) mice. Blood sampling was carried out at the beginning of the experiment and repeated every 3 weeks until the end of the experiment within the same hour. The sampling schedule and the STZ injection can be seen in Fig. 1. At the end of the experiment, the mice were sacrificed. Then, blood and muscle samples were taken.

Glucose Tolerance Test

Glucose tolerance tests were carried out orally using the previous study as the reference [17]. The glucose dose was given at 2 g glucose/kg of body weight. Blood sugar levels were determined after 0, 15, 30, 60, and 120 min prior to oral gavage glucose solution. A total of 0.3 μl of blood samples was needed for blood measurement using a digital blood sugar measuring device. Blood samples were taken from the tail vein. The tail was wiped with EtOH 70%. Blood was collected from the tip of the mice's tail by scarring using scissor, and the blood was drip on the autocheck® glucometer.

Determination of Methylation Level

With the exception of the last week of experiment in which the muscle and blood samples were obtained by animal sacrifice, blood sampling was carried out for every 3 weeks to observe the

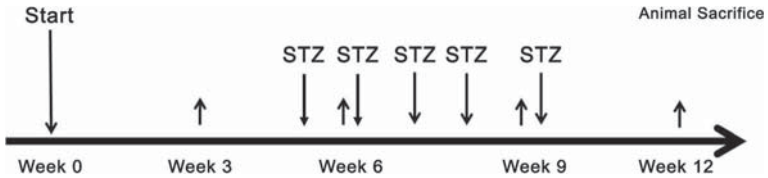

Fig. 1 Sampling schedule (upwards arrow) and injection of STZ (downwards arrow). The horizontal arrow indicates the time of sampling and STZ injection. Week 0 to week 12 indicated the week of experiments

methylation dynamics of *PDK4*. Blood was sampled by retroorbital from the venous sinus and collected in an EDTA tube. Methylation level was determined using several steps starting with the isolation of the DNA continued with the bisulfite treatment of the DNA samples [18], the amplification of bisulfite-treated DNA in the *PDK4* promoter segment (Fig. 2), and finally the quantification of the treated DNA using specific software performed using several primers listed in Table 1.

DNA Isolation

DNA isolation was performed in several samples including the blood, pancreas, and muscle. The isolation was carried out using a commercially DNA isolation kit according to the method developed by the kit manufacturer (Tiangen®).

Bisulfite Treatment

Bisulfite treatment was carried out using a Zymo research bisulfite conversion kit. The DNA conversion was carried out based on the procedures developed by the kit sales company. In general, 500 ng of DNA reacted with bisulfite to convert the cytosine base into thymine resulting in 10 μl of the converted DNA solution in the end.

Primer Design

The design of the methyl-specific PCR (MSP) primer was carried out at the *PDK4* promoter area (*Mus musculus PDK4* gene Locus AF239176). This locus included the regulatory element or promoter for 2312 bp, followed with the untranslated region, and the coding sequences until 15,534 bp. The primer was designed to be the promoter to yield important CpG for *PDK4* expression regulation. Prior to designing the primer, bioinformatics analysis was performed to search for certain cofactor binding sides in the promoter area. The primer was designed to cover the amplification of the important area of *PDK4* promoter. The amplified area is displayed in Fig. 2. Three CpG sites were targeted in this study: CpG site 1 (the 1855th sequence), CpG site 6 (the 1922nd sequence), and CpG site 7 (the 1969th sequence). PCR was performed using optimal condition to meet the requirements of the DNA denaturation and extension. The annealing temperature for every primer is listed in Table 1.

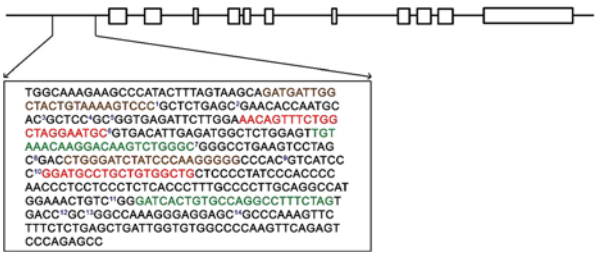

Fig. 2 Schematic diagram of *PDK4* promoter area (regions 1802–2215) where targeted CpG sites were located. Brown letters are the primer sequence used for CpG site 1 methylation determination. Red and green letters represent the sequences of the primer used in the determination of CpG site 6 and CpG 7 methylation levels, respectively

Table 1 Primers used in the study

No.	Primer name	Sequence	$T_{annealing}$
1	D-F_MSP_Met_*PDK4*	TAAATAAGGATAAGTTTGGGC	51 °C
2	D-F_MSP_Non_*PDK4*	TAAATAAGGATAAGTTTGGGT	
3	D-R_MSP_*PDK4*	CTAAAAAAACCTAACACAATAATC	
4	F-F_MSP_Met_*PDK4*	GATGATTGGTTATTGTAAAAGTTTC	53 °C
5	F-F_MSP_Non_*PDK4*	GATGATTGGTTATTGTAAAAGTTTT	
6	F-R_MSP_*PDK4*	CCCCCTTAAAATAAATCCCAA	
7	S-F_MSP_Met_*PDK4*	AATAGTTTTTGGTTAGGAATGC	51 °C
8	S-F_MSP_Non_*PDK4*	AATAGTTTTTGGTTAGGAATGT	
9	S-R_MSP_*PDK4*	CAACCACAACAAACATCC	
10	F1_*PDK4*_Outer	AAGGGGGATTATAGGGTTAGTTAA	55 °C
11	R1_*PDK4*_Outer	CTTAAAACCACACCAATCAACTC	

Methylation Quantification

Methylation level was quantified based on band density of the MSP result with alpha ease expressed in the following formula:

$$\%\ \text{Methylation} = \frac{\text{The band intensity of MSP methylated CpG site}}{\text{The band intensity of MSP non-methylated CpG site}}$$

PDK4 Expression Quantification

PDK4 gene expression was quantified from the muscle organ samples of both the control and STZ groups obtained from the last week of experiment through animal sacrifice. Muscle was frozen in liquid nitrogen and powdered using organ pulverizer. Total RNA of the muscle organ was isolated using a protocol based on FavorPrep™ Tissue Total RNA Mini Kit (Favorgen®). cDNA was made using oligo (dT) and random hexamer concepts and carried out using protocol based on ExcelRT™ Reverse Transcription Kit II (SMOBIO®). qPCR was done by mixing 10 times diluted cDNA with SYBR Green and Primers (F-primer: 5′-ACTA GTGATGTGCGTGAGGC-3′; R-primer: 5′-CTTCCCTCGCTCCTCGTTTT-3′). cDNA was initially denatured (95 °C, 3 min) followed by 40 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 10 s), and elongation (72 °C, 15 s). RT-qPCR assays were carried out using MyGoPro real-time PCR instrument. ΔCt of both the control and STZ groups was measured by differentiating *PDK4* Ct and GAPDH Ct as the endogenous control. ΔΔCt was measured by differentiating the ΔCt sample and ΔCt mean of the control. PDK4 expression of the control group was normalized by using ΔΔCt calibrator measurement. Each sample was measured thrice. Data were represented in relative quantification (rq) with a formula of $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Statistical analysis was conducted by comparing certain data in two groups, namely, the control group and STZ group. Normality test was determined by the Shapiro-Wilk test at $P > 0.05$ while the homogenous of the data variances was analyzed by Levene's test. All experiment data was analyzed for the significant difference using Student's *t* test with the *P* values written in the table and figure description. The statistical analysis was performed using SPSS version 22.

Results

This study was designed to determine the first observed phenotype between hyperglycemia and aberrant methylation at the *PDK4* promoter area. To achieve this goal, the fasting glucose level of the control group and the STZ animal experiment group was observed and recorded every 3 weeks. Once the difference of the fasting glucose levels was obtained, the glucose tolerance test was performed to further confirm the glucose performance in the mice. Figure 3 shows the fasting glucose level difference between the control and the STZ groups. The STZ group yielded significantly higher glucose levels since week 9, and this continued to the 12th week of the experiment. Moreover, glucose tolerance test results from week 9 support the fact that in every time point post glucose injection, the blood glucose level of the STZ group is always significantly higher compared with that of the control group (Table 2). The similar trend was observed in the 12th week of the experiment (Table 3).

The next step in this study is to determine the level of methylation using the MSP method. CpG sites 1, 6, and 7 were located at the *PDK4* promoter area. Nested PCR was designed to generate optimal MSP result of 179 bp of amplicon length for CpG site 1, whereas amplicon lengths for CpG site 6 and CpG site 7 detection were 142 and 190 bp, respectively (Fig. 4).

The dynamics of the methylation level during the experiment was shown in Fig. 4. No significant difference was observed in week 0 and week 3 between the control group and the STZ group. However, it is interesting that only the methylation level from CpG site 6 is different between the control group and the STZ group in week 6 (Fig. 5). The difference of the methylation levels in CpG site 6 was firstly observed and compared with the significant difference of the fasting glucose levels firstly observed in the 9th week of the experiment. Moreover, there was no significant difference in CpG site 1 and CpG 7 methylation levels between the control group and the STZ group along the experiment time (Fig. 5).

DNA methylation level is unique for every different cell. *PDK4* is an enzyme that is actively expressed in muscles and helps regulate blood sugar levels by keeping the *PDK4* expression levels low. The above results show that the methylation level of CpG site 6 of blood cells is significantly higher in the STZ group compared with that in the control group. However, we found no significance on methylation level of CpG site 1, CpG site 6, and CpG site 7 of the muscle cells (Fig. 6). The trend of lower methylation level of the STZ group compared with that of the control group was observed, but it did not reach the significant level.

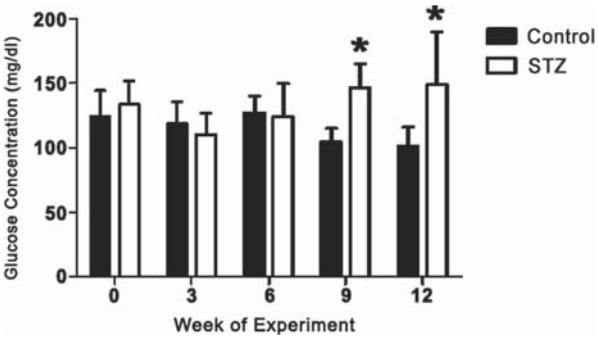

Fig. 3 Fasting blood glucose level on different weeks during the experiment (control group, $n = 8$; STZ group, $n = 7$). The comparison between the averages of the glucose concentration for each week of the experiment was analyzed using t test, $^*P < 0.05$

Table 2 Glucose tolerance test of week 9 experiment

Treatment	Glucose concentration (mg/dl) at a certain time (in minutes) after oral glucose intervention				
	0 min	15 min	30 min	60 min	120 min
Control	$100.6^{a} \pm 11.8$	$184.8^{a} \pm 38.1$	$196.0^{a} \pm 51.1$	$129.4^{a} \pm 34.9$	$88.3^{a} \pm 14.2$
STZ	$136.5^{b} \pm 23.3$	$286.5^{b} \pm 60.3$	$344.6^{b} \pm 93.9$	$301.5^{b} \pm 109.3$	$156.3^{b} \pm 61.9$

Different superscript letters in different columns symbolize significantly different on the blood glucose level using t test between the control and STZ groups at $P < 0.05$ (control group, $n = 8$; STZ group, $n = 7$)

However, the *PDK4* expression in the muscle organ of the STZ group was significantly increased to about threefold compared with the control group (Fig. 7). The difference between methylation level and *PDK4* expression might be because methylation level was analyzed using site-specific primers; thus, these primers cannot give a bigger picture of total methylation in *PDK4* promoter. Nevertheless, increase of *PDK4* expression in the muscle organ affirmed that hyperglycemia has occurred [12].

Discussion

This study shows that the periodic injection of the STZ can be used to generate hyperglycemia in the mouse model. Fasting blood glucose level was found significantly higher in the STZ group compared with that in the control group since the 9th week of the experiment. Fasting blood glucose concentration in the STZ group continued to increase at the end of the 12th week of the experiment. The observation of methylation dynamics during the experiment revealed a continuous increase of *PDK4* promoter area methylation in the STZ group. The comparison of the methylation level between the STZ group and the control group resulted in significantly higher methylation of CpG site 6 at the promoter area of *PDK4* observed during the 6th week of the experiment. No significant difference was found between the STZ group and the control group on CpG site 1 and CpG site 7 at the *PDK4* promoter area. There was also a positive correlation between the methylation level of CpG site 6 and the blood glucose concentration.

Hyperglycemia was observed in the STZ group after the STZ multiple injections. This result was in accordance with the results of several previous studies [19–21]. The gradual injection leads to the gradual damage to the pancreatic beta cells by means of necrosis. In the previous study, the multiple injections were used to develop type 2 diabetes mellitus (T2D) in combination with the diet on an animal experiment model [22]. Gradual damage in pancreatic beta cells due to multiple STZ injections is expected to mimic the physiological effects of T2D individuals who experience

Table 3 Glucose tolerance test of week 12 experiment

Treatment	Glucose concentration (mg/dl) at a certain time (minutes) after oral glucose intervention				
	0 min	15 min	30 min	60 min	120 min
Control	$97.9^{a} \pm 12.7$	$179.7^{a} \pm 50.4$	$134.6^{a} \pm 35.5$	$115.6^{a} \pm 26.1$	$91.3^{a} \pm 16.1$
STZ	$167.4^{b} \pm 75.0$	$311.2^{b} \pm 69.9$	$329.6^{b} \pm 90.2$	$297.2^{b} \pm 125.0$	$212.4^{b} \pm 125.0$

Different superscript letters in different columns symbolize significantly different on the blood glucose level using t test at $P < 0.05$ (control group, $n = 8$; STZ group, $n = 7$)

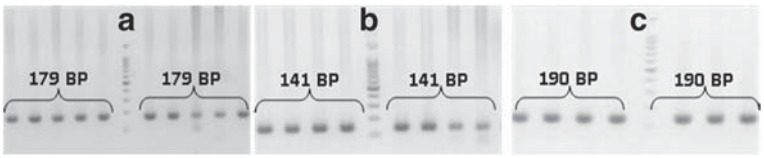

Fig. 4 MSP result of CpG site 1, CpG site 6, and CpG site 7 of *PDK4* area promoter (A, B, and C, respectively)

increased damage in their pancreatic beta cells over time. Another method to achieve hyperglycemia is by using single high-dosed STZ injection. A single STZ injection in large doses may generate adequate levels of pancreatic beta cell damage at once and lead to hyperglycemia. Large doses of a single STZ injection are not applicable here since this study requires only a slight increment in glucose concentration during the experimental time to achieve the purpose of the study.

In this study, CpG site 6 methylation level at the *PDK4* promoter area was significantly higher in the STZ group than that in the control group. This difference was initially observed during the 6th week of the experiment—3 weeks prior to the 9th week which was the initial development of hyperglycemia. There was no significant difference on CpG site 1 and CpG site 7 methylation levels between the STZ group and the control group. However, the methylation level on CpG site 6 was increased until the 12th week of the experiment. de la Rocha et al. used 5 sites of CpG of 5′ UTR of *PDK4* from blood samples of overweight patients as a target of interest [13]. In contrast, Kulkarni et al. analyzed the muscle sample of *PDK4* promoter region + 160 to + 446 from T2D patients [15] and reported a reduction in the methylation level. Another study also showed that only one out four loci in *PDK4* gene was

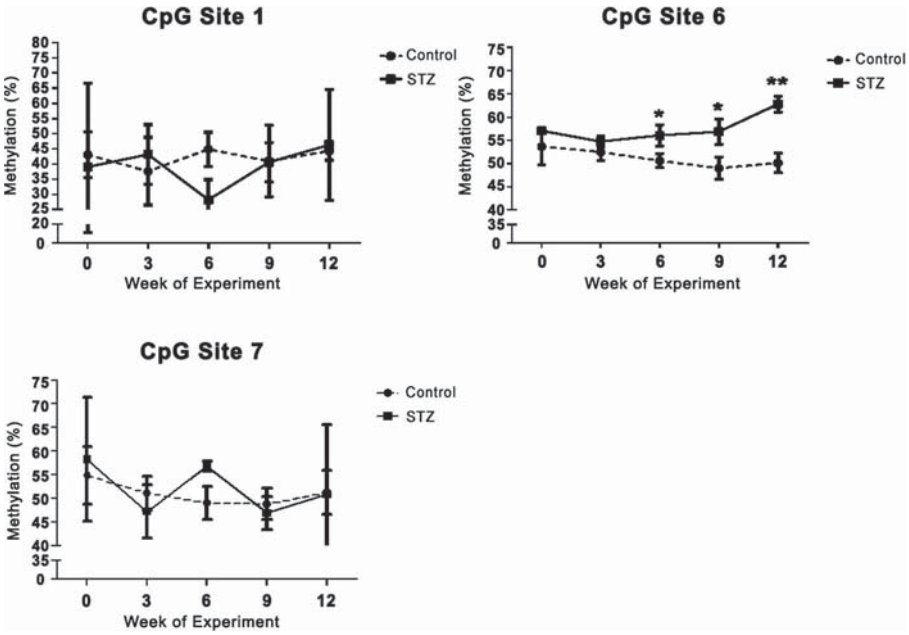

Fig. 5 Methylation dynamics of CpG site 1, CpG site 6, and CpG site 7 during the experiment (control group, $n = 8$; STZ group, $n = 7$). Comparison between the averages of the methylation level for each week of the experiment was analyzed using t test. $^{*}P < 0.05$, $^{**}P < 0.01$

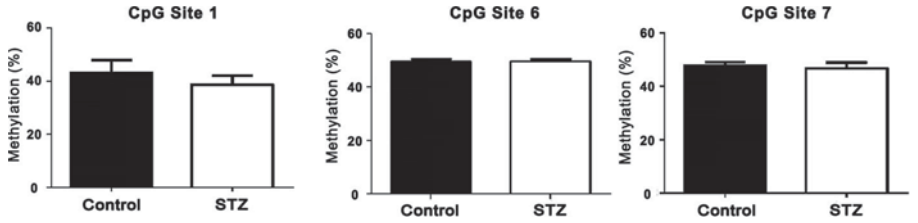

Fig. 6 Methylation level of CpG site 1, CpG site 6, and CpG site 7 of *PDK4* promoter of the muscle cell at week 12 of the experiment (control group, $n = 8$; STZ group, $n = 7$). Comparison between the averages of the methylation level was analyzed using t test. $P < 0.05$

significantly reduced [23]. The different trend compared with some previous studies could be due to the difference between the analyzed region and the cell sample [24].

Nevertheless, since the significant difference of the blood glucose level between the STZ group and the control group was observed during the 9th week of the experiment, this result gives insight to the use of CpG site 6 methylation level as a molecular marker of hyperglycemia. The previous study had reported candidate gene methylation or global methylation that could be used as a molecular marker for diabetes. The aberrant methylation of *TCF7L2*, *KCNQ1*, *ABCG1*, *TXNIP*, *PHOSPHO1*, *SREBF1*, *SLC30A8*, and *FTO* in the blood cells was reproducibly associated with diabetes [25]. Global methylation of pancreatic cells was also shown to be associated with diabetes [4]. However, most of the studies were only performed in the correlation analysis method. The report in this study which shows that aberrant methylation of *PDK4* came before hyperglycemia can be very useful to anticipate the upcoming unfavorable events. This experiment result benefits the impacted individuals through the management of their diets or lifestyles in order to avert hyperglycemia.

PDK4 is a gene that is expressed in pancreatic cells slightly and in several other cells such as the muscles, brain, liver, lungs, and kidneys. Mostly, the active site of *PDK4* is the muscle cell. *PDK4* activated the β-oxidation pathway and could be repressed by insulin. However, *PDK4* is used to maintain homeostasis between glucose and fat levels while in short-term fat diet, increase of *PDK4* expression helped to protect muscle cells from fatty acid–induced oxidative stress [26]. *PDK4* works effectively in muscle cells to remove glucose from the bloodstream. Methylation is specifically considered for use in different cells, and the methylation analysis was conducted to

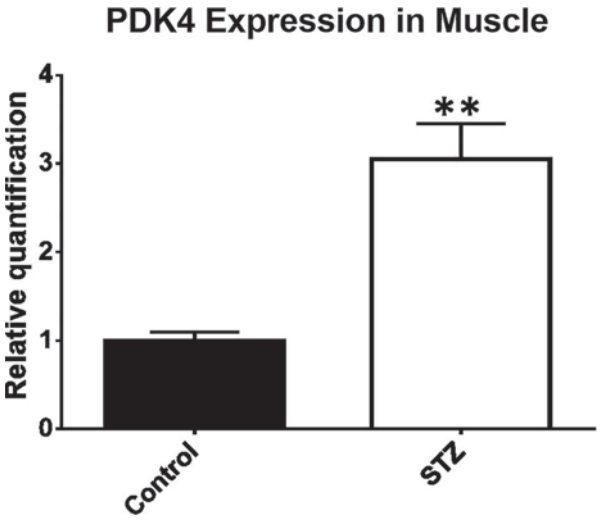

Fig. 7 *PDK4* expression in the muscle organ at week 12 of the experiment analyzed with RT-qPCR (control group, $n = 4$; STZ group, $n = 5$). Comparison between the averages of the *PDK4* expression was analyzed using t test. $^{**}P < 0.01$

analyze muscle cells. There was no significant reduction in the methylation level at the *PDK4* promoter area between the STZ group and the control group. Regulatory damage to *PDK4* expression in the muscle cells is associated with insulin resistance [27]. Although the reduction in the methylation level was not observed in this study, *PDK4* expression in the muscle sample was repressed in the STZ group compared with the control group. This difference can be explained since our study employed the specific-site PCR of bisulfite conversion, in contrast to bisulfite sequencing which could read whole regulatory sequence [28]. Only three CpG sites were analyzed through numerous CpG sites in the *PDK4* promoter.

The investigation of metabolic-related gene expression and methylation such as *PDK4* in blood can potentially result in a production of marker for early detection of metabolic disorder. The blood *PDK4* promoter methylation level and *PDK4* expression in the muscle sample were not coherent. However, this might be because one protein behaves differently in different organs. *Scd1* overexpression in the liver was associated with insulin resistance [29]. In contrast, activation of *Scd1* in the skeletal muscle cell induced fatty acid oxidation and increased cell metabolism, thus protecting mice from getting obese [30]. Previous study stated that methylation at a specific locus of *ABCG1* positively correlated with the future risk of diabetes mellitus. This result is in contrast with the methylation that negatively correlated at specific CpG locus of *PHOSPHO1* [23]. One of the CpG loci in the *PPARγ* and two CpG loci FTO from peripheral blood cells showed almost a significant methylation increase in T2D-diagnosed people [24].

CpG site 6 of *PDK4* methylation level in blood cell and the blood glucose level observed in this study correlate positively. Furthermore, the methylation had appeared before the hyperglycemia period occurred. This occurrence indicated the mechanistic link between the methylation level of CpG site 6 at the *PDK4* promoter of blood cell and the blood glucose concentration. Further studies, however, are needed to determine the mechanistic link between *PDK4* methylation level of blood cell and hyperglycemia.

Conclusion

Changes in the methylation at the *PDK4* promoter area of blood cells successfully occurred before the observation of hyperglycemia in the STZ group compared with those in the control group. No methylation level difference was observed between the STZ group and the control group in muscle cells.

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Compliance with Ethical Standards The experimental procedures have passed the guidelines of institutional ethical committee, University of Surabaya, Indonesia (No. 012/KE/III/2018).

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Cho, N. H., Shaw, J. E., Karuranga, S., Huang, Y., da Rocha Fernandes, J. D., Ohlrogge, A. W., & Malanda, B. (2018). IDF diabetes atlas: global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice, 138*, 271–281.

2. Das, S. K., & Elbein, S. C. (2006). The genetic basis of type 2 diabetes. *Cellscience., 2*(4), 100–131.
3. Kolb, H., & Martin, S. (2017). Environmental/lifestyle factors in the pathogenesis and prevention of type 2 diabetes. *BMC Medicine, 15*(1), 131.
4. Reichetzeder, C., Dwi Putra, S. E., Li, J., & Hocher, B. (2016). Developmental origins of disease - crisis precipitates change.*Cellular Physiology and Biochemistry, 39*(3), 919–938.
5. Mamrut, S., Harony, H., Sood, R., Shahar-Gold, H., Gainer, H., Shi, Y.-J., et al. (2013). DNA methylation of specific CpG sites in the promoter region regulates the transcription of the mouse oxytocin receptor. *PLoS One, 8*(2), e56869.
6. Chen, X., Liu, L., Mims, J., Punska, E. C., Williams, K. E., Zhao, W., et al. (2015). Analysis of DNA methylation and gene expression in radiation-resistant head and neck tumors. *Epigenetics., 10*(6), 545–561.
7. Shao, W.-J., Tao, L.-Y., Gao, C., Xie, J.-Y., & Zhao, R.-Q. (2008). Alterations in methylation and expression levels of imprinted genes H19 and Igf2 in the fetuses of diabetic mice. *Comparative Medicine, 58*(4), 341–346.
8. Pang, J., Xi, C., Huang, X., Cui, J., Gong, H., & Zhang, T. (2016). Effects of excess energy intake on glucose and lipid metabolism in C57BL/6 mice. *PLoS One, 11*(1), e0146675.
9. Galgani, J. E., Moro, C., & Ravussin, E. (2008). Metabolic flexibility and insulin resistance. *The American Journal of Physiology - Endocrinology and Metabolism, 295*(5), E1009–E1017.
10. Tareen, S. H., Kutmon, M., Adriaens, M. E., Mariman, E. C., de Kok, T. M., Arts, I. C., & Evelo, C. T. (2018). Exploring the cellular network of metabolic flexibility in the adipose tissue. *Genes & Nutrition, 13*(1), 17.
11. Zhang, S., Hulver, M. W., McMillan, R. P., Cline, M. A., & Gilbert, E. R. (2014). The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutrition and Metabolism, 11*, 10.
12. Jeoung, N. H., & Harris, R. A. (2010). Role of pyruvate dehydrogenase kinase 4 in regulation of blood glucose levels. *Korean Diabetes Journal, 34*(5), 274–283.
13. de la Rocha, C., Pérez-Mojica, J. E., León, S. Z.-D., Cervantes-Paz, B., Tristán-Flores, F. E., Rodríguez-Ríos, D., et al. (2016). Associations between whole peripheral blood fatty acids and DNA methylation in humans. *Scientific Reports, 6*:25867.
14. Kirchner, H., Nylen, C., Laber, S., Barrès, R., Yan, J., Krook, A., et al. (2014). Altered promoter methylation of *PDK4*, IL1 B, IL6, and TNF after Roux-en Y gastric bypass. *Surgery for Obesity and Related Diseases, (4)*, 671–678.
15. Kulkarni, S. S., Salehzadeh, F., Fritz, T., Zierath, J. R., Krook, A., & Osler, M. E. (2012). Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. *Metabolism., 61*(2), 175–185.
16. Wang, K., Tang, Z., Zheng, Z., Cao, P., Shui, W., Li, Q., & Zhang, Y. (2016). Protective effects of Angelica sinensis polysaccharide against hyperglycemia and liver injury in multiple low-dose streptozotocin-induced type 2 diabetic BALB/c mice. *Food & Function, 7*(12), 4889–4897.
17. Andrikopoulos, S., Blair, A. R., Deluca, N., Fam, B. C., & Proietto, J. (2008). Evaluating the glucose tolerance test in mice. *American Journal of Physiology. Endocrinology and Metabolism, 295*(6), E1323–E1332.
18. Yang, A. S., Estécio, M. R., Doshi, K., Kondo, Y., Tajara, E. H., & Issa, J. P. J. (2004). A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Research, 32*(3), e38–e38.
19. Chaudhry, Z. Z., Morris, D. L., Moss, D. R., Sims, E. K., Chiong, Y., Kono, T., & Evans-Molina, C. (2013). Streptozotocin is equally diabetogenic whether administered to fed or fasted mice. *Laboratory Animals, 47*(4), 257–265.
20. Furman, B. L. (2015). Streptozotocin-induced diabetic models in mice and rats. *Current Protocols in Pharmacology, 70*(1), 5–47.
21. Vatandoust, N., Rami, F., Salehi, A. R., Khosravi, S., Dashti, G., Eslami, G., Momenzadeh, S., & Salehi, R. (2018). Novel high-fat diet formulation and streptozotocin treatment for induction of prediabetes and type 2 diabetes in rats. *Advanced Biomedical Research, 7*, 107.
22. Barrière, D. A., Noll, C., Roussy, G., Lizotte, F., Kessai, A., Kirby, K., Belleville, K., Beaudet, N., Longpré, J. M., Carpentier, A. C., Geraldes, P., & Sarret, P. (2018). Combination of high-fat/high-fructose diet and low-dose streptozotocin to model long-term type-2 diabetes complications. *Scientific Reports, 8*(1), 424.
23. Dayeh, T., Tuomi, T., Almgren, P., Perfilyev, A., Jansson, P. A., de Mello, V. D., Pihlajamaki, J., Vaag, A., Groop, L., Nilsson, E., & Ling, C. (2016). DNA methylation of loci within ABCG1 and PHOSPHO1 in blood DNA is associated with future type 2 diabetes risk. *Epigenetics., 11*(7), 482–488.
24. van Otterdijk, S. D., Binder, A. M., vel Szic, K. S., Schwald, J., & Michels, K. B. (2017). DNA methylation of candidate genes in peripheral blood from patients with type 2 diabetes or the metabolic syndrome. *PLoS One, 12*(7), e0180955.

25. Willmer, T., Johnson, R., Louw, J., & Pheiffer, C. (2018). Blood-Based DNA Methylation biomarkers for type 2 diabetes: potential for clinical applications. *Frontiers in Endocrinology, 9*, 744..
26. Chokkalingam, K., Jewell, K., Norton, L., Littlewood, J., Van Loon, L. J. C., Mansell, P., MacDonald, I. A., & Tsintzas, K. (2007). High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: an important role for skeletal muscle pyruvate dehydrogenase kinase 4. *The Journal of Clinical Endocrinology and Metabolism, 92*(1), 284–292.
27. Zhou, Z., Sun, B., Li, X., & Zhu, C. (2018). DNA methylation landscapes in the pathogenesis of type 2 diabetes mellitus. *Nutrition & Metabolism (London), 15*, 47.
28. Kurdyukov, S., & Bullock, M. (2016). DNA methylation analysis: choosing the right method. *Biology., 5*(1), 3.
29. Drąg, J., Goździalska, A., Knapik-Czajka, M., Gawędzka, A., Gawlik, K., & Jaśkiewicz, J. (2017). Effect of high carbohydrate diet on elongase and desaturase activity and accompanying gene expression in rat's liver. *Genes & Nutrition, 12*(1), 2.
30. Rogowski, M. P., Flowers, M. T., Stamatikos, A. D., Ntambi, J. M., & Paton, C. M. (2013). SCD1 activity in muscle increases triglyceride PUFA content, exercise capacity, and PPARδ expression in mice. *Journal of Lipid Research, 54*(10), 2636–2646.

aberrant_pdk4_promoter_methylation.pdf

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Aberrant *PDK4* Promoter Methylation Preceding Hyperglycemia in a Mouse Model

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Abstract

Diabetic prevalence is at speedy increase globally. Previous studies stated that other than genetics, factors such as environment, lifestyle, and paternal-maternal condition y critical roles in diabetes through DNA methylation in specific areas of the genome. The purpose of this study is to investigate the methylation pattern of the *PDK4* promoter in streptozotocin-induced diabetic mice until the 12th week of the observation. The methylation pattern in the blood samples was analyzed periodically, while the pattern in the muscle sample was only analyzed at t end of the experiment using the blood of the sacrificed animals. Three methylated CpG site 1, CpG site 6 and CpG site 7 were analyzed and quantified based on the band density using bisulfite treatment and methylation-specific polymerase chain reaction (PCR). The hyperglycemia period was developed at the 9th week of experiment. However, there was a significant increase of methylation, specifically on CpG site 6 started from week 6 to week 12. This peculiar methylation on CpG site 6 of *PDK4* promoter in the blood sample before the hyperglycemic period might serve as a potential biomarker for early detection of diabetes in the patients. No significant difference was found between the methylation level of streptozotocin (STZ)–treated mice and of the control group in the muscle sample.

Keywords Blood · Epigenetics · Methylation pattern · *PDK4* · STZ-induced diabetic mice

Introduction

From 151 million in 2000 to 451 million in 2017, global diabetic prevalence has been an increasing trend [1]. Das and Elbein mentioned in their review that parental diabetes is descended on the

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children through gene mutation [2]. Recent studies show that genetics is not the only factor in the emergence of diabetes. Environmental factors such as maternal health during pregnancy, diets, and lifestyles are more dominant in contributing to the new events of diabetes [3].

Genetic and environmental factors interact, drive the gene expression, and lead to certain phenotypes. The interaction between environment and gene is called epigenetics [4], and one of the most studied epigenetic mechanisms is DNA methylation. DNA methylation refers to the addition of a methyl group to the cytosine residues located on the CpG sites in the DNA [5]. This type of methylation in the promoter of the gene may lead to the gene repression since its pattern regulates specific gene expression in a specific cell at a certain time. Thus, the regulation of methylation level can meet normal metabolism process.

Aberrant DNA methylation pattern causes abnormal gene expression and leads to diseases, such as diabetes [6]. Our previous study showed that aberrant high DNA methylation level in the placenta independently correlated with gestational diabetic mothers [4]. Aberrant DNA methylation pattern of specific genes is also reported to have affected the metabolic activity [7].

One of the reasons for diabetes is the imbalance between energy production and its consumption. Less energy consumption leads to the new onset of diabetes. Cells have the flexibility to select and to transform the sources of energy based on the availability of the sources in the body [8]. These cells tend to use glucose as an energy source once the cells have rich glucose environment. Conversely, when there is no glucose intake for some time, the cell breaks lipid molecules to generate energy. Failure of cell flexibility in transforming glucose into lipid as energy source molecule may lead to serious health problems followed with several symptoms such as insulin resistance and hyperglycemia which serve as the markers of diabetes [9].

One enzyme that plays an important role in maintaining cell flexibility between glucose and lipid molecule as an energy source is the pyruvate dehydrogenase complex (PDC) [10]. This enzyme plays a role in the decarboxylation of pyruvate into acetyl-CoA which is a molecule that can enter the citric acid cycle. PDC is an enzyme that can link glucose to lipid metabolism. The increased activity of the PDC enzyme can reduce glucose levels by accelerating the formation of pyruvic acid to acetyl-CoA which will produce energy after the citric acid cycle. Conversely, in low glucose levels, PDC activity is also low, and acetyl-CoA is mostly produced by the oxidation of free fatty acids as a result of lipolysis.

The PDC activity is regulated by the pyruvate dehydrogenase kinase (PDK) through the regulation of the phosphor level in PDC. Currently, there are several PDK enzymes that work specifically in different tissues [11]. The previous study showed that excessive expression of *PDK4* causes hyperglycemia and is associated with diabetes [12]. Furthermore, animal studies show that a high-fat diet correlates with a high level of fasting blood sugar concentration. Conversely, animals with high-fat diet can keep the normal sugar levels when the expression of *PDK4* is simultaneously suppressed. This shows that *PDK4* expression is essential to the contribution of hyperglycemia in the individual diabetes through the maintenance of the balanced expression in using glucose or lipid molecules as energy sources [12].

The previous study showed that the *PDK4* methylation in the 5′ UTR decreased and the expression is increased in the patients' obesity from the blood sample [13]. Other studies have shown an increase in metabolism of *PDK4* in patients undergoing gastric bypass to control body weight. As seen there, epigenetic regulation plays a role in the weight of the patients with gastric bypass [14]. Direct observation was conducted by Kulkarni et al. who reduced methylation and increased expression of *PDK4* in diabetic patients from the muscle sample [15]. However, the abovementioned studies have so far been confined to the exploration of the correlation between diabetes and the *PDK4* methylation level. In contrast, further exploration

of the first-onset phenotype between hyperglycemia as a symptom of diabetes and aberrant methylation pattern of *PDK4* is required. The aim of this study is to answer whether the aberrant methylation pattern of *PDK4* or hyperglycemia is the first detected phenotype in the diabetic mouse model. The study is designed to increase the savior of diabetes using periodic streptozotocin (STZ) injection to mouse models. The blood sample was periodically taken until the hyperglycemia was observed. DNA methylation was determined afterwards.

Method

Animal Experiment

The experimental procedures have passed the guidelines of institutional ethical committee, University of Surabaya, Indonesia (No. 012/KE/III/2018). This research used experimental animals of 5-week-old male Swiss Webster mice that have almost the same weight. There were two groups: the control ($n=8$) and the STZ ($n=7$) groups. Both groups were fed with common pellet diet. The STZ group underwent the STZ injection 60 mg/kg body weight intraperitoneally [16]. The injection was carried out from the 5th week of the experiment and repeated weekly until the 9th week of the experiment to develop type 2 diabetes mellitus (T2D) model. Blood sampling was carried out at the beginning of the experiment and repeated every 3 weeks until the end of the experiment within the same hour. The sampling schedule and the STZ injection can be seen in Fig. 1. At the end of the experiment, the mice were sacrificed. Then, blood and muscle samples were taken.

Glucose Tolerance Test

Glucose tolerance tests were carried out orally using the previous study as the reference [17]. The glucose dose was given at 2 g glucose/kg of body weight. Blood sugar levels were determined after 0, 15, 30, 60, and 120 min prior to oral gavage glucose solution. A total of 0.3 μl of blood samples was needed for blood measurement using a digital blood sugar measuring device. Blood samples were taken from the tail vein. The tail was wiped with EtOH 70%. Blood was collected from the tip of the mice's tail by scarring using scissor, and the blood was drip on the autocheck® glucometer.

Determination of Methylation Level

With the exception of the last week of experiment in which the muscle and blood samples were obtained by animal sacrifice, blood sampling was carried out for every 3 weeks to observe the

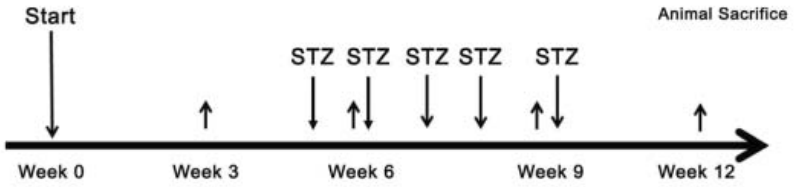

Fig. 1 Sampling schedule (upwards arrow) and injection of STZ (downwards arrow). The horizontal arrow indicates the time of sampling and STZ injection. Week 0 to week 12 indicated the week of experiments

methylation dynamics of *PDK4*. Blood was sampled by retroorbital from the venous sinus and collected in an EDTA tube. Methylation level was determined using several steps starting with the isolation of the DNA continued with the bisulfite treatment of the DNA samples [18], the amplification of bisulfite-treated DNA in the *PDK4* promoter segment (Fig. 2), and finally the quantification of the treated DNA using specific software performed using several primers listed in Table 1.

DNA Isolation

DNA isolation was performed in several samples including the blood, pancreas, and muscle. The isolation was carried out using a commercially DNA isolation kit according to the method developed by the kit manufacturer (Tiangen®).

Bisulfite Treatment

Bisulfite treatment was carried out using a Zymo research bisulfite conversion kit. The DNA conversion was carried out based on the procedures developed by the kit sales company. In general, 500 ng of DNA reacted with bisulfite to convert the cytosine base into thymine resulting in 10 μl of the converted DNA solution in the end.

Primer Design

The design of the methyl-specific PCR (MSP) primer was carried out at the *PDK4* promoter area (*Mus musculus PDK4* gene Locus AF239176). This locus included the regulatory element or promoter for 2312 bp, followed with the untranslated region, and the coding sequences until 15,534 bp. The primer was designed to be the promoter to yield important CpG for *PDK4* expression regulation. Prior to designing the primer, bioinformatics analysis was performed to search for certain cofactor binding sides in the promoter area. The primer was designed to cover the amplification of the important area of *PDK4* promoter. The amplified area is displayed in Fig. 2. Three CpG sites were targeted in this study: CpG site 1 (the 1855th sequence), CpG site 6 (the 1922nd sequence), and CpG site 7 (the 1969th sequence). PCR was performed using optimal condition to meet the requirements of the DNA denaturation and extension. The annealing temperature for every primer is listed in Table 1.

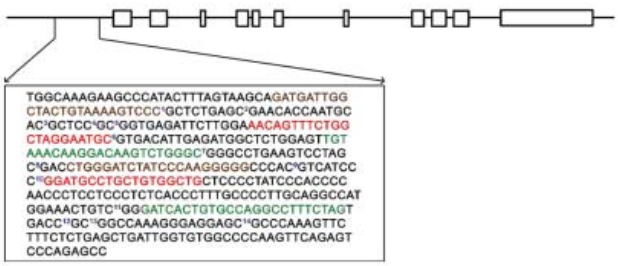

Fig. 2 Schematic diagram of *PDK4* promoter area (regions 1802–2215) where targeted CpG sites were located. Brown letters are the primer sequence used for CpG site 1 methylation determination. Red and green letters represent the sequences of the primer used in the determination of CpG site 6 and CpG 7 methylation levels, respectively

Table 1 Primers used in the study

No.	Primer name	Sequence	$T_{annealing}$
1	D-F_MSP_Met_*PDK4*	TAAATAAGGATAAGTTTGGGC	51 °C
2	D-F_MSP_Non_*PDK4*	TAAATAAGGATAAGTTTGGGT	
3	D-R_MSP_*PDK4*	CTAAAAAAACCTAACACAATAATC	
4	F-F_MSP_Met_*PDK4*	GATGATTGGTTATTGTAAAAGTTTC	53 °C
5	F-F_MSP_Non_*PDK4*	GATGATTGGTTATTGTAAAAGTTTT	
6	F-R_MSP_*PDK4*	CCCCCTTAAAATAAATCCCAA	
7	S-F_MSP_Met_*PDK4*	AATAGTTTTTGGTTAGGAATGC	51 °C
8	S-F_MSP_Non_*PDK4*	AATAGTTTTTGGTTAGGAATGT	
9	S-R_MSP_*PDK4*	CAACCACAACAAACATCC	
10	F1_*PDK4*_Outer	AAGGGGGATTATAGGGTTAGTTAA	55 °C
11	R1_*PDK4*_Outer	CTTAAAACCACACCAATCAACTC	

Methylation Quantification

Methylation level was quantified based on band density of the MSP result with alpha ease expressed in the following formula:

$$\%\ \text{Methylation} = \frac{\text{The band intensity of MSP methylated CpG site}}{\text{The band intensity of MSP non-methylated CpG site}}$$

PDK4 Expression Quantification

PDK4 gene expression was quantified from the muscle organ samples of both the control and STZ groups obtained from the last week of experiment through animal sacrifice. Muscle was frozen in liquid nitrogen and powdered using organ pulverizer. Total RNA of the muscle organ was isolated using a protocol based on FavorPrep™ Tissue Total RNA Mini Kit (Favorgen®). cDNA was made using oligo (dT) and random hexamer concepts and carried out using protocol based on ExcelRT™ Reverse Transcription Kit II (SMOBIO®). qPCR was done by mixing 10 times diluted cDNA with SYBR Green and Primers (F-primer: 5′-ACTA GTGATGTGCGTG[illegible]GC-3′; R-primer: 5′-CTTCCCTCGCTCCTCGTTTT-3′). cDNA was initially denatured (95 °C, 3 min) followed by 40 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 10 s), and elongation (72 °C, 15 s). RT-qPCR assays were carried out using MyGoPro real-time PCR instrument. ΔCt of both the control and STZ groups was measured by differentiating *PDK4* Ct and GAPDH Ct as the endogenous control. ΔΔCt was measured by differentiating the ΔCt sample and ΔCt mean of the control. PDK4 expression of the control group was normalized by using ΔΔCt calibrator measurement. Each sample was measured thrice. Data were represented in relative quantification (rq) with a formula of $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Statistical analysis was conducted by comparing certain data in two groups, namely, the control group and STZ group. Normality test was determined by the Shapiro-Wilk test at $P > 0.05$ while homogenous of the data variances was analyzed by Levene's test. All experiment data was analyzed for the significant difference using Student's *t* test with the *P* values written in the table and figure description. The statistical analysis was performed using SPSS version 22.

Results

This study was designed to determine the first observed phenotype between hyperglycemia and aberrant methylation at the *PDK4* promoter area. To achieve this goal, the fasting glucose level of the control group and the STZ animal experiment group was observed and recorded every 3 weeks. Once the difference of the fasting glucose levels was obtained, the glucose tolerance test was performed to further confirm the glucose performance in the mice. Figure 3 shows the fasting glucose level difference between the control and the STZ groups. The STZ group yielded significantly higher glucose levels since week 9, and this continued to the 12th week of the experiment. Moreover, glucose tolerance test results from week 9 support the fact that in every time point post glucose injection, the blood glucose level of the STZ group is always significantly higher compared with that of the control group (Table 2). The similar trend was observed in the 12th week of the experiment (Table 3).

The next step in this study is to determine the level of methylation using the MSP method. CpG sites 1, 6, and 7 were located at the *PDK4* promoter area. Nested PCR was designed to generate optimal MSP result of 179 bp of amplicon length for CpG site 1, whereas amplicon lengths for CpG site 6 and CpG site 7 detection were 142 and 190 bp, respectively (Fig.

The dynamics of the methylation level during the experiment was shown in Fig. 4. No significant difference was observed in week 0 and week 3 between the control group and the STZ group. However, it is interesting that only the methylation level from CpG site 6 is different between the control group and the STZ group in week 6 (Fig. 5). The difference of the methylation levels in CpG site 6 was firstly observed and compared with the significant difference of the fasting glucose levels firstly observed in the 9th week of the experiment. Moreover, there was no significant difference in CpG site 1 and CpG 7 methylation levels between the control group and the STZ group along the experiment time (Fig. 5).

DNA methylation level is unique for every different cell. *PDK4* is an enzyme that is actively expressed in muscles and helps regulate blood sugar levels by keeping the *PDK4* expression levels low. The above results show that the methylation level of CpG site 6 of blood cells is significantly higher in the STZ group compared with that in the control group. However, we found no significance on methylation level of CpG site 1, CpG site 6, and CpG site 7 of the muscle cells (Fig. 6). The trend of lower methylation level of the STZ group compared with that of the control group was observed, but it did not reach the significant level.

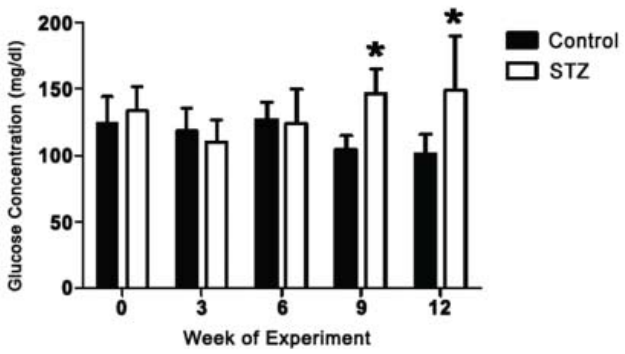

Fig. 3 Fasting blood glucose level on different weeks during the experiment (control group, $n = 8$; STZ group, $n = 7$). The comparison between the averages of the glucose concentration for each week of the experiment was analyzed using t test, $^*P < 0.05$

Table 2 Glucose tolerance test of week 9 experiment

Treatment	Glucose concentration (mg/dl) at a certain time (in minutes) after oral glucose intervention				
	0 min	15 min	30 min	60 min	120 min
Control	$100.6^{a} \pm 11.8$	$184.8^{a} \pm 38.1$	$196.0^{a} \pm 51.1$	$129.4^{a} \pm 34.9$	$88.3^{a} \pm 14.2$
STZ	$136.5^{b} \pm 23.3$	$286.5^{b} \pm 60.3$	$344.6^{b} \pm 93.9$	$301.5^{b} \pm 109.3$	$156.3^{b} \pm 61.9$

Different superscript letters in different columns symbolize significantly different on the blood glucose level using *t* test between the control and STZ groups at $P < 0.05$ (control group, $n = 8$; STZ group, $n = 7$)

However, the *PDK4* expression in the muscle organ of the STZ group was significantly increased to about threefold compared with the control group (Fig. 7). The difference between methylation level and *PDK4* expression might be because methylation level was analyzed using site-specific primers; thus, these primers cannot give a bigger picture of total methylation in *PDK4* promoter. Nevertheless, increase of *PDK4* expression in the muscle organ affirmed that hyperglycemia has occurred [12].

Discussion

This study shows that the periodic injection of the STZ can be used to generate hyperglycemia in the mouse model. Fasting blood glucose level was found significantly higher in the STZ group compared with that in the control group since the 9th week of the experiment. Fasting blood glucose concentration in the STZ group continued to increase at the end of the 12th week of the experiment. The observation of methylation dynamics during the experiment revealed a continuous increase of *PDK4* promoter area methylation in the STZ group. The comparison of the methylation level between the STZ group and the control group resulted in significantly higher methylation of CpG site 6 at the promoter area of *PDK4* observed during the 6th week of the experiment. No significant difference was found between the STZ group and the control group on CpG site 1 and CpG site 7 at the *PDK4* promoter area. There was also a positive correlation between the methylation level of CpG site 6 and the blood glucose concentration.

Hyperglycemia was observed in the STZ group after the STZ multiple injections. This result was in accordance with the results of several previous studies [19–21]. The gradual injection leads to the gradual damage to the pancreatic beta cells by means of necrosis. In the previous study, the multiple injections were used to develop type 2 diabetes mellitus (T2D) in combination with the diet on an animal experiment model [22]. Gradual damage in pancreatic beta cells due to multiple STZ injections is expected to mimic the physiological effects of T2D individuals who experience

Table 3 Glucose tolerance test of week 12 experiment

Treatment	Glucose concentration (mg/dl) at a certain time (minutes) after oral glucose intervention				
	0 min	15 min	30 min	60 min	120 min
Control	$97.9^{a} \pm 12.7$	$179.7^{a} \pm 50.4$	$134.6^{a} \pm 35.5$	$115.6^{a} \pm 26.1$	$91.3^{a} \pm 16.1$
STZ	$167.4^{b} \pm 75.0$	$311.2^{b} \pm 69.9$	$329.6^{b} \pm 90.2$	$297.2^{b} \pm 125.0$	$212.4^{b} \pm 125.0$

Different superscript letters in different columns symbolize significantly different on the blood glucose level using *t* test at $P < 0.05$ (control group, $n = 8$; STZ group, $n = 7$)

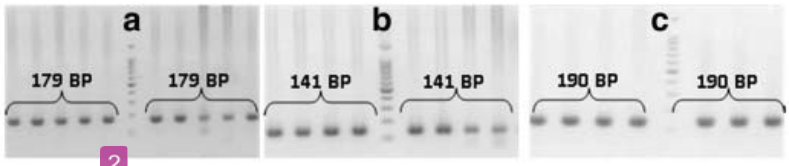

Fig. 4 MSP result of CpG site 1, CpG site 6, and CpG site 7 of *PDK4* area promoter (A, B, and C, respectively)

increased damage in their pancreatic beta cells over time. Another method to achieve hyperglycemia is by using single high-dosed STZ injection. A single STZ injection in large doses may generate adequate levels of pancreatic beta cell damage at once and lead to hyperglycemia. Large doses of a single STZ injection are not applicable here since this study requires only a slight increment in glucose concentration during the experimental time to achieve the purpose of the study.

In this study, CpG site 6 methylation level at the *PDK4* promoter area was significantly higher in the STZ group than that in the control group. This difference was initially observed during the 6th week of the experiment—3 weeks prior to the 9th week which was the initial development of hyperglycemia. There was no significant difference on CpG site 1 and CpG site 7 methylation levels between the STZ group and the control group. However, the methylation level on CpG site 6 was increased until the 12th week of the experiment. de la Rocha et al. used 5 sites of CpG of 5′ UTR of *PDK4* from blood samples of overweight patients as a target of interest [13]. In contrast, Kulkarni et al. analyzed the muscle sample of *PDK4* promoter region +160 to +446 from T2D patients [15] and reported a reduction in the methylation level. Another study also showed that only one out four loci in *PDK4* gene was

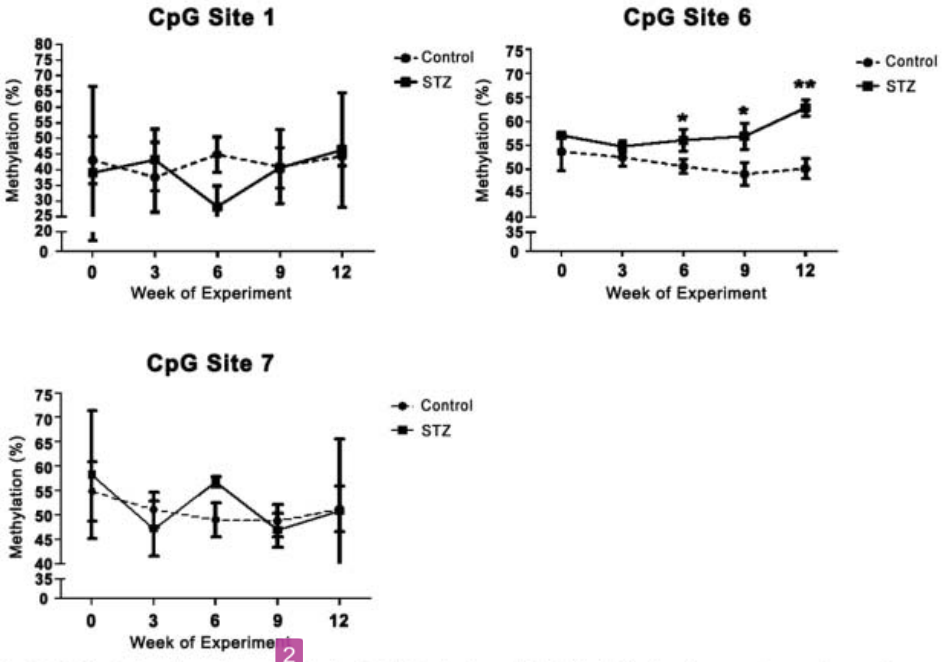

Fig. 5 Methylation dynamics of CpG site 1, CpG site 6, and CpG site 7 during the experiment (control group, $n=8$; STZ group, $n=7$). Comparison between the averages of the methylation level for each week of the experiment was analyzed using *t* test. $^{*}P<0.05$, $^{**}P<0.01$

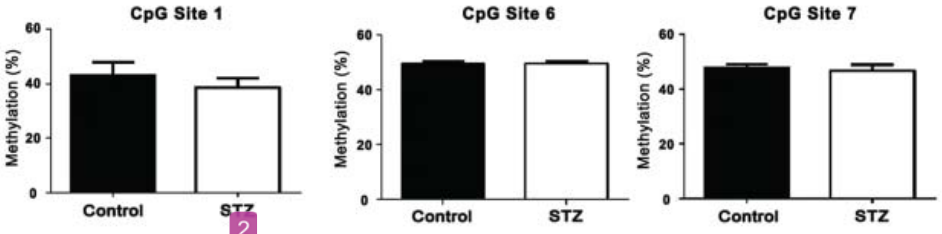

Fig. 6 Methylation level of CpG site 1, CpG site 6, and CpG site 7 of *PDK4* promoter of the muscle cell at week 12 of the experiment (control group, $n = 8$; STZ group, $n = 7$). Comparison between the averages of the methylation level was analyzed using t test. $P < 0.05$

significantly reduced [23]. The different trend compared with some previous studies could be due to the difference between the analyzed region and the cell sample [24].

Nevertheless, since the significant difference of the blood glucose level between the STZ group and the control group was observed during the 9th week of the experiment, this result gives insight to the use of CpG site 6 methylation level as a molecular marker of hyperglycemia. The previous study had reported candidate gene methylation or global methylation that could be used as a molecular marker for diabetes. The aberrant methylation of *TCF7L2*, *KCNQ1*, *ABCG1*, *TXNIP*, *PHOSPHO1*, *SREBF1*, *SLC30A8*, and *FTO* in the blood cells was reproducibly associated with diabetes [25]. Global methylation of pancreatic cells was also shown to be associated with diabetes [4]. However, most of the studies were only performed in the correlation analysis method. The report in this study which shows that aberrant methylation of *PDK4* came before hyperglycemia can be very useful to anticipate the upcoming unfavorable events. This experiment result benefits the impacted individuals through the management of their diets or lifestyles in order to avert hyperglycemia.

PDK4 is a gene that is expressed in pancreatic cells slightly and in several other cells such as the muscles, brain, liver, lungs, and kidneys. Mostly, the active site of *PDK4* is the muscle cell. *PDK4* activated the β-oxidation pathway and could be repressed by insulin. However, *PDK4* is used to maintain homeostasis between glucose and fat levels while in short-term fat diet, increase of *PDK4* expression helped to protect muscle cells from fatty acid–induced oxidative stress [26]. *PDK4* works effectively in muscle cells to remove glucose from the bloodstream. Methylation is specifically considered for use in different cells, and the methylation analysis was conducted to

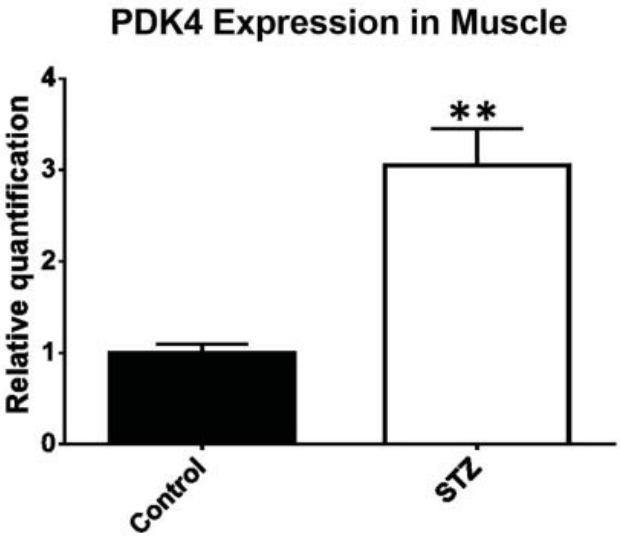

Fig. 7 *PDK4* expression in the muscle organ at week 12 of the experiment analyzed with RT-qPCR (control group, $n = 4$; STZ group, $n = 5$). Comparison between the averages of the *PDK4* expression was analyzed using t test. $^{**}P < 0.01$

analyze muscle cells. There was no significant reduction in the methylation level at the *PDK4* promoter area between the STZ group and the control group. Regulatory damage to *PDK4* expression in the muscle cells is associated with insulin resistance [27]. Although the reduction in the methylation level was not observed in this study, *PDK4* expression in the muscle sample was repressed in the STZ group compared with the control group. This difference can be explained since our study employed the specific-site PCR of bisulfite conversion, in contrast to bisulfite sequencing which could read whole regulatory sequence [28]. Only three CpG sites were analyzed through numerous CpG sites in the *PDK4* promoter.

The investigation of metabolic-related gene expression and methylation such as *PDK4* in blood can potentially result in a production of marker for early detection of metabolic disorder. The blood *PDK4* promoter methylation level and *PDK4* expression in the muscle sample were not coherent. However, this might be because one protein behaves differently in different organs. *Scd1* overexpression in the liver was associated with insulin resistance [29]. In contrast, activation of *Scd1* in the skeletal muscle cell induced fatty acid oxidation and increased cell metabolism, thus protecting mice from getting obese [30]. Previous study stated that methylation at a specific locus of *ABCG1* positively correlated with the future risk of diabetes mellitus. This result is in contrast with the methylation that negatively correlated at specific CpG locus of *PHOSPHO1* [23]. One of the CpG loci in the *PPARγ* and two CpG loci FTO from peripheral blood cells showed almost a significant methylation increase in T2D-diagnosed people [24].

CpG site 6 of *PDK4* methylation level in blood cell and the blood glucose level observed in this study correlate positively. Furthermore, the methylation had appeared before the hyperglycemia period occurred. This occurrence indicated the mechanistic link between the methylation level of CpG site 6 at the *PDK4* promoter of blood cell and the blood glucose concentration. Further studies, however, are needed to determine the mechanistic link between *PDK4* methylation level of blood cell and hyperglycemia.

Conclusion

Changes in the methylation at the *PDK4* promoter area of blood cells successfully occurred before the observation of hyperglycemia in the STZ group compared with those in the control group. No methylation level difference was observed between the STZ group and the control group in muscle cells.

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Compliance with Ethical Standards The experimental procedures have passed the guidelines of institutional ethical committee, University of Surabaya, Indonesia (No. 012/KE/III/2018).

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Cho, N. H., Shaw, J. E., Karuranga, S., Huang, Y., da Rocha Fernandes, J. D., Ohlrogge, A. W., & Malanda, B. (2018). IDF diabetes atlas: global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice, 138*, 271–281.

2. Das, S. K., & Elbein, S. C. (2006). The genetic basis of type 2 diabetes. *Cellscience., 2*(4), 100–131.
3. Kolb, H., & Martin, S. (2017). Environmental/lifestyle factors in the pathogenesis and prevention of type 2 diabetes. *BMC Medicine, 15*(1), 131.
4. Reichetzeder, C., Dwi Putra, S. E., Li, J., & Hocher, B. (2016). Developmental origins of disease - crisis precipitates change.*Cellular Physiology and Biochemistry, 39*(3), 919–938.
5. Mamrut, S., Harony, H., Sood, R., Shahar-Gold, H., Gainer, H., Shi, Y.-J., et al. (2013). DNA methylation of specific CpG sites in the promoter region regulates the transcription of the mouse oxytocin receptor. *PLoS One, 8*(2), e56869.
6. Chen, X., Liu, L., Mims, J., Punska, E. C., Williams, K. E., Zhao, W., et al. (2015). Analysis of DNA methylation and gene expression in radiation-resistant head and neck tumors. *Epigenetics., 10*(6), 545–561.
7. Shao, W.-J., Tao, L.-Y., Gao, C., Xie, J.-Y., & Zhao, R.-Q. (2008). Alterations in methylation and expression levels of imprinted genes H19 and Igf2 in the fetuses of diabetic mice. *Comparative Medicine, 58*(4), 341–346.
8. Pang, J., Xi, C., Huang, X., Cui, J., Gong, H., & Zhang, T. (2016). Effects of excess energy intake on glucose and lipid metabolism in C57BL/6 mice. *PLoS One, 11*(1), e0146675.
9. Galgani, J. E., Moro, C., & Ravussin, E. (2008). Metabolic flexibility and insulin resistance. *The American Journal of Physiology - Endocrinology and Metabolism, 295*(5), E1009–E1017.
10. Tareen, S. H., Kutmon, M., Adriaens, M. E., Mariman, E. C., de Kok, T. M., Arts, I. C., & Evelo, C. T. (2018). Exploring the cellular network of metabolic flexibility in the adipose tissue. *Genes & Nutrition, 13*(1), 17.
11. Zhang, S., Hulver, M. W., McMillan, R. P., Cline, M. A., & Gilbert, E. R. (2014). The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutrition and Metabolism, 11*, 10.
12. Jeoung, N. H., & Harris, R. A. (2010). Role of pyruvate dehydrogenase kinase 4 in regulation of blood glucose levels. *Korean Diabetes Journal, 34*(5), 274–283.
13. de la Rocha, C., Pérez-Mojica, J. E., León, S. Z.-D., Cervantes-Paz, B., Tristán-Flores, F. E., Rodríguez-Ríos, D., et al. (2016). Associations between whole peripheral blood fatty acids and DNA methylation in humans. *Scientific Reports, 6*:25867.
14. Kirchner, H., Nylen, C., Laber, S., Barrès, R., Yan, J., Krook, A., et al. (2014). Altered promoter methylation of *PDK4*, IL1 B, IL6, and TNF after Roux-en Y gastric bypass. *Surgery for Obesity and Related Diseases, (4)*, 671–678.
15. Kulkarni, S. S., Salehzadeh, F., Fritz, T., Zierath, J. R., Krook, A., & Osler, M. E. (2012). Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. *Metabolism., 61*(2), 175–185.
16. Wang, K., Tang, Z., Zheng, Z., Cao, P., Shui, W., Li, Q., & Zhang, Y. (2016). Protective effects of Angelica sinensis polysaccharide against hyperglycemia and liver injury in multiple low-dose streptozotocin-induced type 2 diabetic BALB/c mice. *Food & Function, 7*(12), 4889–4897.
17. Andrikopoulos, S., Blair, A. R., Deluca, N., Fam, B. C., & Proietto, J. (2008). Evaluating the glucose tolerance test in mice. *American Journal of Physiology. Endocrinology and Metabolism, 295*(6), E1323–E1332.
18. Yang, A. S., Estécio, M. R., Doshi, K., Kondo, Y., Tajara, E. H., & Issa, J. P. J. (2004). A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Research, 32*(3), e38–e38.
19. Chaudhry, Z. Z., Morris, D. L., Moss, D. R., Sims, E. K., Chiong, Y., Kono, T., & Evans-Molina, C. (2013). Streptozotocin is equally diabetogenic whether administered to fed or fasted mice. *Laboratory Animals, 47*(4), 257–265.
20. Furman, B. L. (2015). Streptozotocin-induced diabetic models in mice and rats. *Current Protocols in Pharmacology, 70*(1), 5–47.
21. Vatandoust, N., Rami, F., Salehi, A. R., Khosravi, S., Dashti, G., Eslami, G., Momenzadeh, S., & Salehi, R. (2018). Novel high-fat diet formulation and streptozotocin treatment for induction of prediabetes and type 2 diabetes in rats. *Advanced Biomedical Research, 7*, 107.
22. Barrière, D. A., Noll, C., Roussy, G., Lizotte, F., Kessai, A., Kirby, K., Belleville, K., Beaudet, N., Longpré, J. M., Carpentier, A. C., Geraldes, P., & Sarret, P. (2018). Combination of high-fat/high-fructose diet and low-dose streptozotocin to model long-term type-2 diabetes complications. *Scientific Reports, 8*(1), 424.
23. Dayeh, T., Tuomi, T., Almgren, P., Perfilyev, A., Jansson, P. A., de Mello, V. D., Pihlajamaki, J., Vaag, A., Groop, L., Nilsson, E., & Ling, C. (2016). DNA methylation of loci within ABCG1 and PHOSPHO1 in blood DNA is associated with future type 2 diabetes risk. *Epigenetics., 11*(7), 482–488.
24. van Otterdijk, S. D., Binder, A. M., vel Szic, K. S., Schwald, J., & Michels, K. B. (2017). DNA methylation of candidate genes in peripheral blood from patients with type 2 diabetes or the metabolic syndrome. *PLoS One, 12*(7), e0180955.

25. Willmer, T., Johnson, R., Louw, J., & Pheiffer, C. (2018). Blood-Based DNA Methylation biomarkers for type 2 diabetes: potential for clinical applications. *Frontiers in Endocrinology, 9*, 744..
26. Chokkalingam, K., Jewell, K., Norton, L., Littlewood, J., Van Loon, L. J. C., Mansell, P., MacDonald, I. A., & Tsintzas, K. (2007). High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: an important role for skeletal muscle pyruvate dehydrogenase kinase 4. *The Journal of Clinical Endocrinology and Metabolism, 92*(1), 284–292.
27. Zhou, Z., Sun, B., Li, X., & Zhu, C. (2018). DNA methylation landscapes in the pathogenesis of type 2 diabetes mellitus. *Nutrition & Metabolism (London), 15*, 47.
28. Kurdyukov, S., & Bullock, M. (2016). DNA methylation analysis: choosing the right method. *Biology., 5*(1), 3.
29. Drąg, J., Goździalska, A., Knapik-Czajka, M., Gawędzka, A., Gawlik, K., & Jaśkiewicz, J. (2017). Effect of high carbohydrate diet on elongase and desaturase activity and accompanying gene expression in rat's liver. *Genes & Nutrition, 12*(1), 2.
30. Rogowski, M. P., Flowers, M. T., Stamatikos, A. D., Ntambi, J. M., & Paton, C. M. (2013). SCD1 activity in muscle increases triglyceride PUFA content, exercise capacity, and PPARδ expression in mice. *Journal of Lipid Research, 54*(10), 2636–2646.

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