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Fwd: LoA_SEA-STEM

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Sulistyo Emantoko 61116 _
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May 25, 2021, 4:10 PM (6 days ago)

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From: **STEM Unsyiah** <stem@unsyiah.ac.id>
Date: Tue, Sep 29, 2020 at 12:09 PM
Subject: LoA_SEA-STEM

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PCR Method" with Paper ID#: 3685 has been accepted to be presented virtually at The South East Asia - Science Technology Engineering and Mathematics International Conferenc
by The STEM Research Center Universitas Syiah Kuala, Banda Aceh, Indonesia, on October 20-22, 2020.

Please find the signed invitation letter attached to this email.

Sincerely yours,
Rini Oktavia
Chief Editor SEA-STEM 2020

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From: **STEM Unsyiah** <stem@unsyiah.ac.id>
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Subject: LoA_SEA-STEM
To: emantoko@staff.ubaya.ac.id <emantoko@staff.ubaya.ac.id>

Dear Author Sulistyo Emantoko Dwi Putra,

We are pleased to inform you that your abstract entitled "Methylation Analysis of Igf2 / H19 Imprinting Control Region (ICR) in Type II Diabetes Mellitus Induced Mice using Methylatio
PCR Method" with Paper ID#: 3685 has been accepted to be presented virtually at The South East Asia - Science Technology Engineering and Mathematics International Conferenc
by The STEM Research Center Universitas Syiah Kuala, Banda Aceh, Indonesia, on October 20-22, 2020.

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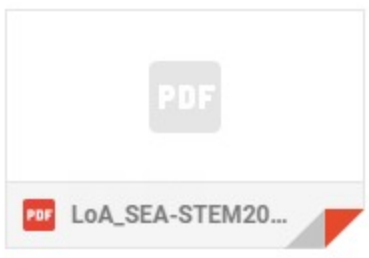

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To: STEM Unsyiah <stem@unsyiah.ac.id>

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Emantoko

On Tue, Sep 29, 2020 at 12:09 PM STEM Unsyiah <stem@unsyiah.ac.id> wrote:

> Dear Author Sulistyo Emantoko Dwi Putra,
>
> We are pleased to inform you that your abstract entitled "Methylation Analysis of Igf2 / H19 Imprinting Control Region (ICR) in Type II Diabetes Mellitus Induced Mice using Me
> Specific PCR Method" with Paper ID#: 3685 has been accepted to be presented virtually at The South East Asia - Science Technology Engineering and Mathematics Inte
> Conference, hosted by The STEM Research Center Universitas Syiah Kuala, Banda Aceh, Indonesia, on October 20-22, 2020.
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> Please find the signed invitation letter attached to this email.
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> Sincerely yours,
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Apakah paper Bapak dengan ID# 3685 sudah dikirim revisinya ke editor SEA-STEM. Record di sistem, paper Bapak yang sebetulnya hanya perlu format revisi belum kami terima revisi ter

Wassalam,
Rini Oktavia
Chief Editor

On Thu, Dec 31, 2020 at 6:07 PM STEM Unsyiah <stem@unsyiah.ac.id> wrote:

> Salam pak,
>
> Ini file review paper bapak.
>
> Wassalam,
> Rini

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Subject: Fwd: I am sharing '3685-8769-1-RV (2)' with you
To: Sulistyo Emantoko 61116 _ <emantoko@staff.ubaya.ac.id>

Semoga Bapak menerima email ini dengan baik.

Mohon revisi format papernya dikirimkan segera ke editor SEA-STEM sebelum tanggal 15 Februari 2021.

Terima kasih,
Rini
...

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Sulistyo Emantoko 61116 _ 11:21 AM (4 hours ago)
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To: STEM Unsyiah <stem@unsyiah.ac.id>

Yth. Bu Rini,

Di bawah ini adalah revisi saya. Terimakasih.

Salam Hormat,
Sulistyo Emantoko

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> Terima kasih,
> Rini
>
> ---------- Forwarded message ---------
> From: **STEM Unsyiah** <stem@unsyiah.ac.id>
> Date: Thu, Feb 11, 2021 at 4:29 PM
> Subject: Re: I am sharing '3685-8769-1-RV (2)' with you
> To: Sulistyo Emantoko 61116 _ <emantoko@staff.ubaya.ac.id>
>
> Salam pak Sulistyo,
>
> Apakah paper Bapak dengan ID# 3685 sudah dikirim revisinya ke editor SEA-STEM. Record di sistem, paper Bapak yang sebetulnya hanya perlu format revisi belum kami terima revisi terbarunya.
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> Wassalam,
> Rini Oktavia
> Chief Editor

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> Ini file review paper bapak.
>
> Wassalam,
> Rini

3685-8769-1-RV (...

About STEM Research Center, Unsyiah

DESCRIPTION, VISION, MISSION, AND OBJECTIVES OF STEM RESEARCH CENTER

A. Description

Starting with a Workshop on High School Physics Teachers with a theme Development of Science Interest and Empowerment of Physics Laboratories in Nanggroe Aceh High School Darussalam which was held in collaboration with Physics FMIPA Unsyiah with Fukui, Japan on March 20-21, 2006. Based on this experience, Irwandi, Ph.D (Current Chair of the STEM Research Center) proposes community service activities about the use of daily materials that can be used as making practical tools simple. Irwandi, Ph.D made 2 workshops related to the idea and was funded by Institute of Research and Community Service (LPPM) Unsyiah.

In 2012 Irwandi, Ph.D left for Italy to continue his S3 research at Trieste University. While undergoing his PhD research in Italy, he had the opportunity to participate Physware workshop in 2015 about learning physics through learning models ISLE (Investigative Science Learning Experiments) developed by Prof. Eugina Etkina from Rutgers University. After returning from Indonesia, the knowledge was implemented into in the form of activities to improve the ability of physics teachers at the secondary school level Banda Aceh and Aceh Besar by inviting Ms. Melvina, S.Pd., M.Ed who is a FKIP Physics lecturers as partners in these activities. The results of this cooperation were obtained some research results which eventually developed into the Pre-Proposal then submitted in the PEER Project Cycle 6 activity. Entering the full proposal stage, Irwandi, Ph.D invites Dr. Rini Oktavia, M.A (Mathematics Lecturer at FMIPA Unsyiah) and also Dr. Rajibussalim (Physics Lecturer of FMIPA) into the research until the proposal qualified and funded by USAID for 3 years. The title of the proposal submitted "Integrating ISLE in Integrated Science Instruction to Improve Science Teacher's Abilities on STEM Education. " After that, the chairperson of LPPM Unsyiah who was then chaired by Prof. Dr. Ir. Hasanuddin, M.S., proposed the establishment of a scientific research center based on its letter Decision of the Chancellor Number 1251 / UN11 / KPT / 2018 stipulated the decision to establish the Center Research in Science, Technology, Engineering, and Mathematics, hereinafter better known by the name of the STEM Research Center. At present the STEM Research Center has an organizational structure consisted of FMIPA, FT, FKIP and FKH research experts with various multidisciplinary science. The STEM Research Center is committed to improving the ability of Acehnese teachers and students in the STEM field through activities such as Focus Group Discussion (FGD), Workshop and training, Seminars, Expo, STEM Camp, Visit School, and good cooperation at the regional, national, regional and international level. At present the STEM Research Center is focused in the development of learning media based on ISLE Based STEM for the high school level and PAUD and will continue to grow over time.

B. VISION

Becoming a Research Center for STEM Development (Science, Technology, Engineering and Mathematics) which is innovative, independent and leading in the fields of education, research, and community service to prepare a tough generation to face the Industrial Revolution 4.0 in the 21st century.

C. MISSION

The mission of the Unsyiah STEM Research Center is as follows:

1. Create a STEM Research Center (Science, Technology, Engineering and Mathematics) as a center of superior study.
2. Developing science, technology, engineering and mathematics education integrated and creates a pleasant learning atmosphere with a motto "Learning is fun".
3. Establish and develop cooperation at the regional, national, regional and international in the field of STEM.
4. Producing ISLE-based STEM learning models that are able to create a generation that has the ability to think critically, creativity, communication, and Collaboration (4C) plus Character (1C) as noble moral scientists (5C = 4C + 1C).
5. Becoming a pioneer in developing ISLE-based STEM learning models at the national and international scope
6. Build and develop a STEM Center in Aceh that is a model STEM Development Center for other regions in Indonesia.

D. Strategic Objectives

1. Identifying STEM education problems in Indonesia.
2. Find the optimal solution from all STEM problems with the emphasis is on efforts to create a generation with scientific character and noble character (STEM-C).
3. Implement solutions to STEM education problems based on the concept of "learning is fun" at every level of education.
4. Generate integrated ISLE-based STEM curriculum recommendations at the regional, national, and regional levels.
5. Research the development of STEM education at the level of regional, national, regional, and international.
6. Producing various scientific works in the field of STEM education
7. Generate various collaboration programs in various STEM fields institutions at the regional, national, regional, and international levels.

See Documentation on the activities of the Unsyiah STEM Research Center

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Methylation analysis of Igf2 / H19 Imprinting Control Region (ICR) in Type II Diabetes Mellitus induced mice using Methylation-Specific PCR

To cite this article: V L I Tedjokusumo *et al* 2021 *J. Phys.: Conf. Ser.* **1882** 012098

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Methylation analysis of Igf2 / H19 Imprinting Control Region (ICR) in Type II Diabetes Mellitus induced mice using Methylation-Specific PCR

V L I Tedjokusumo[1], S P Chandra[1], E Surjadjaja[1], I B M Artadana[1], S E D Putra[1]*

[1] Faculty of Biotechnology, University of Surabaya, Indonesia

*Email: emantoko@staff.ubaya.ac.id

Abstract. The imprinting control region (ICR) is a region that controls imprinted expression of Igf2/H19 so that Igf2 will only be expressed from the paternal allele instead of the maternal allele. This research aimed to reveal if ICR methylation status' change contributes to the emergence of type II diabetes Mellitus (T2DM) throughout the induction in mice model. The methylation changes were detected by methylation-specific PCR (MSP). The Control group was given a normal diet and buffer vehicle injection while treatment group was given a high fat diet (HFD) and six times 60 mg/kg mice body weight streptozotocin injection. The induction process was conducted for 12 weeks. The insulin tolerance test (ITT) was conducted on week 0, week 9, and week 12. The treatment's mice group was successfully induced a significantly higher area under the curve (AUC) compared to the control mice (P<0,05). Moreover, ITT showed that diabetic induced mice had higher glucose levels at every time point of the test. Meanwhile, there were no evident ICR methylation changes through week 0 to week 12. Thus, ICR methylation is not proven to contribute to the emergence of T2DM.

Keyword: STZ, Animal, High-Fat, AUC, ITT

1. Introduction

The clinical symptom of diabetes mellitus type II (T2DM) includes when a person experiences an increase in blood sugar without a normal response to lower the levels back. This condition could be preceded by a decrease in insulin receptor sensitivity, which causes insulin resistance. This condition continues to cause pancreatic beta cells' failure to secrete insulin for a prolonged condition of hyperglycemia [1][2]. Pancreatic beta cells can then be damaged [3]. The mechanism of pancreatic beta cells' susceptibility to damage related to Igf2 gene expression [4].

Igf2 expression depends on the DNA methylation level in an imprinting regulatory region, namely the Imprinting Control Region (ICR). ICR regulates the expression of the Igf2 gene so that it is imprinted on the maternal allele and is expressed from the paternal allele only. Typically, Igf2 is expressed at low levels and plays a role in fetal growth and development [5]. The gene product, namely the IGF2 protein, also has the same affinity as insulin for insulin receptors. Igf2 overexpression could increase pancreatic beta cells to damage, leading to the pathophysiology of T2DM [4]. However, it is unknown whether these changes in T2DM progression cause or effect T2DM.

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This study aimed to determine whether there was a change in the CpG site methylation of ICR that correlate with T2DM. Another scientific question to be answered was whether the change is a cause or effect of the emergence of T2DM. Methylation pattern analysis was carried out using methylation-specific PCR (MSP) method to determine the differences in pre- and post-intervention treatment to make T2DM model mice.

2. Material and Method

2.1 Primers

The area selected region for MSP analysis was one CpG site on CpG island (CGI) 1 at the ICR. The Primers for MSP were designed with the help of the online software MethPrimer from Urogene. The inner primer was derived from MSP suggested primer by MethPrimer, while the outer primer was designed from modified bisulfite sequencing primer suggestion. PCR-MSP product specifications were checked using software from BiSearch (http://bisearch.enzim.hu). In silico analysis showed that specific primers produced one PCR product for both outer and inner primers. All primers in the experiment were ordered from Macrogen, South Korea.

Tabel 1. List of the primers used in this study

No	Primer Name	Sequence (5' → 3')	Tm (°C)	Amplicon size
1	F_Vel	GTTTTAAGGTAGAAGGGGATTATTT	59,2	449
2	R_Vel	TAAACCAAAAAACTTAACTCATTCC	57,6	
3	F_V_Met	ATATGTAGTATTGTATTGTTATTAC	54,3	122
4	F_V_Non	ATATGTAGTATTGTATTGTTATTAT	52,7	
5	R_V_MSP	TTAAATAACTCCTTCAATCTTA	56	

2.2 Animal Experiment

There were two groups of mice, namely 16 control mice and 16 treatment mice. The induction method refers to[6] with modifications. The treatment group was given a high-fat diet (HFD)[7] and injected with streptozotocin (STZ). The STZ injection was given at a dose of 60 mg/kg body weight of mice [8]. The injection is given six times with a frequency of once a week. The injection is carried out on the 5th to the 10th week of the experiment. The control group of mice was given a standard diet and injected with 0.1 M citrate buffer solution pH 4.5 as a vehicle of STZ. The STZ solution and buffer used for injection are freshly prepared. The blood sugar was measured at weeks 0, 3, 6, 9, and 12 weeks and the insulin tolerance test (UTI) following UTG's significant results [9]. Blood sugar in both tests was measured at 0, 15, 30, 60, and 120 minutes. Blood samples of mice were taken from retroorbital periodically every three weeks, starting from week 0, and used for DNA methylation analysis. The collected blood was stored in an EDTA tube at -20 ° C for further use.

2.3 Methyl Specific PCR Analysis

The blood DNA was isolated using the TIANamp Genomic DNA Kit (TianGen) kit with an elution volume of 70µL. The DNA was then bisulfite converted, according to [10] , with modifications. The concentration of Na-bisulfite 2.5 M were used. The bisulfite conversion result is in single-strand DNA, then firstly PCR first with outer primer. The first PCR procedure was performed with the following protocol: Five minutes of predenaturation step at 94 ° C, 40 cycles of denaturation step (94 ° C for 30 seconds, annealing of 53 ° C for 90 seconds, elongation of 72 ° C for 90 seconds), and final elongation of 72 ° C for 5 minutes. The result of the first PCR has used for the second step PCR as templates with inner primers. The second PCR was performed using the following step: Five minutes of

predenaturation at 94 ° C, 35 cycles of PCR (denaturation at 94 ° C for 30 seconds, annealing at 49.1 ° C for 90 seconds, and elongation at 72 ° C for 90 seconds), and final elongation at 72 ° C for five minutes.

The PCR results will then be electrophoresed using 2% agarose stained with ethidium bromide. Band visualization was performed using an ultraviolet illuminator with a 100 bp comparison of Promega's DNA ladder markers. Methylation analysis was carried out from the amplicon band visualization results with the help of AlphaEase software, which measures the density of the relative band thickness. The relative band density of the primary amplicon from analyzing methylated or non-methylated amplicon (FMET or FNON) interpreted in the form of integrated density value (IDV).

$$\text{IDV} = (\text{AVG pita} - \text{AVG } \textit{background}) \times \text{Area}$$

$$\%\ \text{Methylation} = \frac{\text{IDV of FMET Amplicon}}{\text{IDV of FMET Amplicon} + \text{IDV of FNON Amplicon}} \times 100\%$$

3. Statistical Analysis

Statistical analyses were performed using IBM SPSS 25.0. Statistical analysis was achieve using the independent t-test to examine differences between the control and treatment groups. The t-test used was two-tailed. Data are presented in mean ± SD. The results were statistically significant if the p-value was <0.05.

4. Results and Discussion

Figure 1 is the result of annealing temperature optimization for MSP settings. The temperature tested for annealing is set on the Techne TC-5000 thermocycler machine at a temperature of 50.2 ° C with a gradient of 23 ° C. PCR test tubes were reacted at block temperatures of 40.6 ° C (A), 43.6 ° C (B), 47.3 ° C (C), and 49, ° C (D). Each was compared with a negative control (-) without a template. At all of the above-mentioned temperature, a specific DNA band was obtained at a size of 122 bp for both (methylated) M and unmethylated (U) primers. In the reaction with an annealing temperature of 47.3 ° C, there was a faint band on the negative control primary M while the band on the negative control was not found. From these results, the optimum temperature used for the next MSP was 49 ° C.

The MSP primers were designed to successfully amplify the template and produce a specific band with the right size. The MSP optimization showed that the primer could be used in a temperature range of 40 ° C to 49 ° C. Negative control is used as a comparison while eliminating amplification interference. The temperature of 49 ° C was chosen for the next MSP of 49 ° C because the negative control results were cleaner than the impurity band.

Figure 2 shows the optimization results of the bisulfite conversion reaction time. The sample used was 18 μL of blood DNA. Incubation with 2.5 M Na-bisulfite reagent was adjusted for 12 hours (D) and 14 hours (E) to determine the best incubation time. The modified isolates were amplified with SS code PDK4 gene primers and with the expected amplicon size of 140 bp, compared to marker (M). The MSP results in these samples showed a specific band in the incubated isolates for 14 hours.

The bisulfite reaction was manually optimized for the incubation duration. The goal was to confirm and to know the conditions of the best conversion reaction results. From the PCR results with different optimization, it was found that the most optimum reaction in the manual method resulted in a specific amplicon band in samples incubated for 12 hours in pancreatic samples only, and 14 and 16 hours in blood samples. In blood samples, no amplicons were found at the 12 hour incubation period. For blood samples, an incubation time of 14 hours was selected.

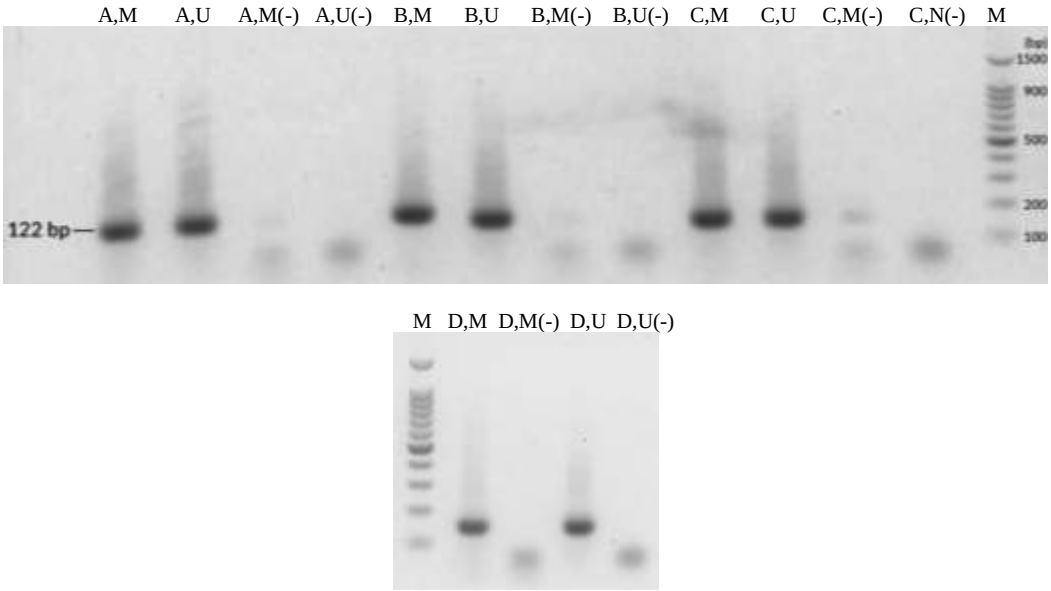
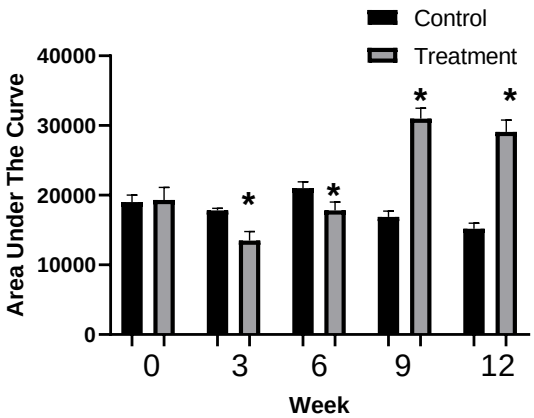


Figure 1. The visualization results of the annealing temperature optimization. The visualization used a 2% agarose gel with a marker of 100 bp (M). Negative control (-). The annealing temperature used was 40.6 ° C (A), 43.6 ° C (B), 47.3 ° C (C), and 49 ° C (D). AM and AU mean the PCR result of annealing temperature 40.6 ° C for methylated and methylated primer respectively. The same coding was used for different annealing temperatures.

Figure 2. The results of the concentration optimization of the bisulfite reaction. Incubation with 2.5 M sodium bisulfite reagent was adjusted at 12 hours (D) and 14 hours (E). The result of the modification reaction was amplified with SS code primer for the PDK4 gene with 140 bp amplicon size compared to marker (M).



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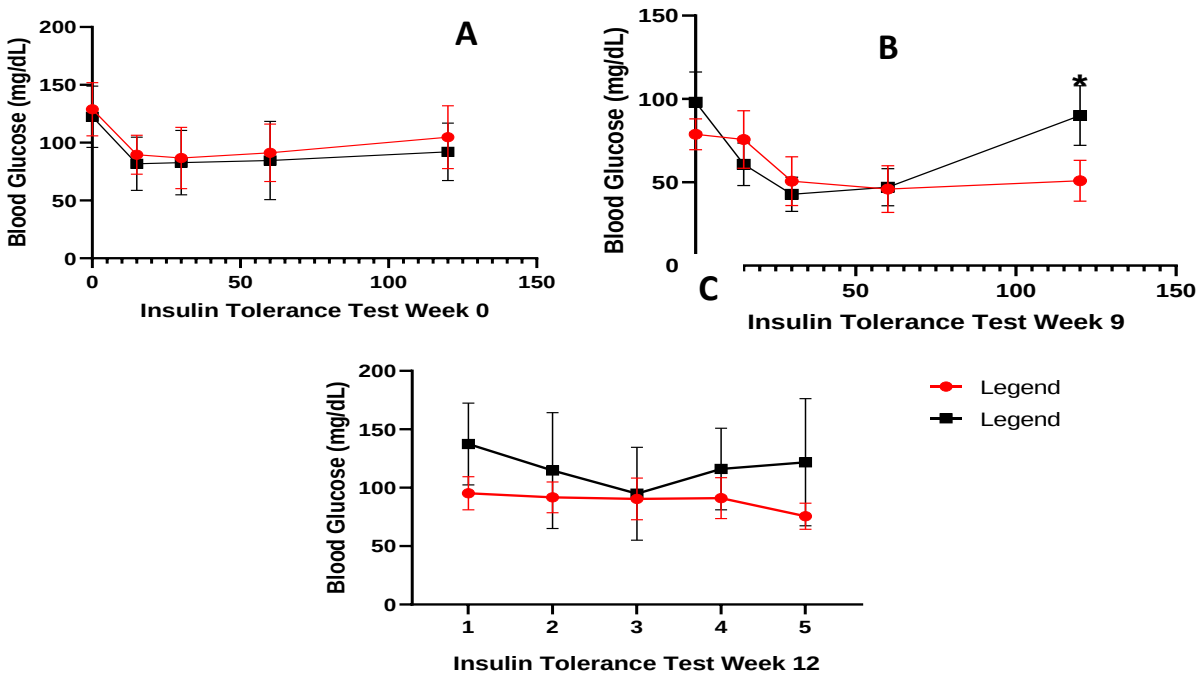

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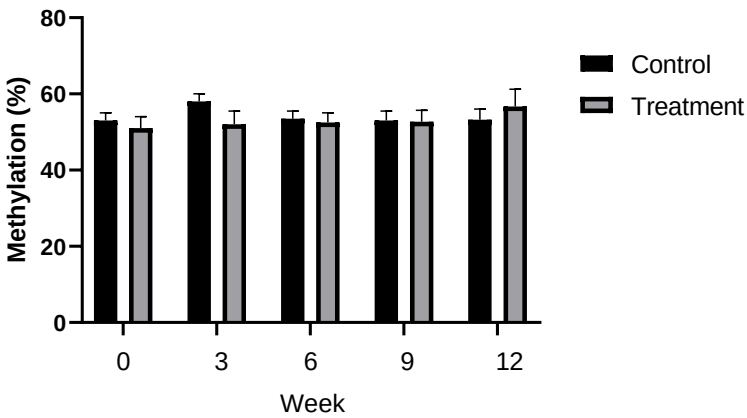

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Methylation analysis of Igf2 / H19 Imprinting Control Region (ICR) in Type II Diabetes Mellitus induced mice using Methylation-Specific PCR

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Abstract. The imprinting control region (ICR) is a region that controls imprinted expression of Igf2/H19 so that Igf2 will only be expressed from the paternal allele instead of the maternal allele. This research aimed to reveal if ICR methylation status' change contributes to the emergence of type II diabetes Mellitus (T2DM) throughout the induction in mice model. The methylation changes were detected by methylation-specific PCR (MSP). The Control group was given a normal diet and buffer vehicle injection while treatment group was given a high fat diet (HFD) and six times 60 mg/kg mice body weight streptozotocin injection. The induction process was conducted for 12 weeks. The insulin tolerance test (ITT) was conducted on week 0, week 9, and week 12. The treatment's mice group was successfully induced a significantly higher area under the curve (AUC) compared to the control mice (P<0,05). Moreover, ITT showed that diabetic induced mice had higher glucose levels at every time point of the test. Meanwhile, there were no evident ICR methylation changes through week 0 to week 12. Thus, ICR methylation is not proven to contribute to the emergence of T2DM.

Keyword: STZ, Animal, High-Fat, AUC, ITT

1. Introduction

The clinical symptom of diabetes mellitus type II (T2DM) includes when a person experiences an increase in blood sugar without a normal response to lower the levels back. This condition could be preceded by a decrease in insulin receptor sensitivity, which causes insulin resistance. This condition continues to cause pancreatic beta cells' failure to secrete insulin for a prolonged condition of hyperglycemia [1][2]. Pancreatic beta cells can then be damaged [3]. The mechanism of pancreatic beta cells' susceptibility to damage related to Igf2 gene expression [4].

Igf2 expression depends on the DNA methylation level in an imprinting regulatory region, namely the Imprinting Control Region (ICR). ICR regulates the expression of the Igf2 gene so that it is imprinted on the maternal allele and is expressed from the paternal allele only. Typically, Igf2 is expressed at low levels and plays a role in fetal growth and development [5]. The gene product, namely the IGF2 protein, also has the same affinity as insulin for insulin receptors. Igf2 overexpression could increase pancreatic beta cells to damage, leading to the pathophysiology of T2DM [4]. However, it is unknown whether these changes in T2DM progression cause or effect T2DM.

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This study aimed to determine whether there was a change in the CpG site methylation of ICR that correlate with T2DM. Another scientific question to be answered was whether the change is a cause or effect of the emergence of T2DM. Methylation pattern analysis was carried out using methylation-specific PCR (MSP) method to determine the differences in pre- and post-intervention treatment to make T2DM model mice.

2. Material and Method

2.1 Primers

The area selected region for MSP analysis was one CpG site on CpG island (CGI) 1 at the ICR. The Primers for MSP were designed with the help of the online software MethPrimer from Urogene. The inner primer was derived from MSP suggested primer by MethPrimer, while the outer primer was designed from modified bisulfite sequencing primer suggestion. PCR-MSP product specifications were checked using software from BiSearch (http://bisearch.enzim.hu). In silico analysis showed that specific primers produced one PCR product for both outer and inner primers. All primers in the experiment were ordered from Macrogen, South Korea.

Tabel 1. List of the primers used in this study

No	Primer Name	Sequence (5' → 3')	Tm (°C)	Amplicon size
1	F_Vel	GTTTTAAGGTAGAAGGGGATTATTT	59,2	449
2	R_Vel	TAAACCAAAAAACTTAACTCATTCC	57,6	
3	F_V_Met	ATATGTAGTATTGTATTGTTATTAC	54,3	122
4	F_V_Non	ATATGTAGTATTGTATTGTTATTAT	52,7	
5	R_V_MSP	TTAAATAACTCCTTCAATCTTA	56	

2.2 Animal Experiment

There were two groups of mice, namely 16 control mice and 16 treatment mice. The induction method refers to[6] with modifications. The treatment group was given a high-fat diet (HFD)[7] and injected with streptozotocin (STZ). The STZ injection was given at a dose of 60 mg/kg body weight of mice [8]. The injection is given six times with a frequency of once a week. The injection is carried out on the 5th to the 10th week of the experiment. The control group of mice was given a standard diet and injected with 0.1 M citrate buffer solution pH 4.5 as a vehicle of STZ. The STZ solution and buffer used for injection are freshly prepared. The blood sugar was measured at weeks 0, 3, 6, 9, and 12 weeks and the insulin tolerance test (UTI) following UTG's significant results [9]. Blood sugar in both tests was measured at 0, 15, 30, 60, and 120 minutes. Blood samples of mice were taken from retroorbital periodically every three weeks, starting from week 0, and used for DNA methylation analysis. The collected blood was stored in an EDTA tube at -20 ° C for further use.

2.3 Methyl Specific PCR Analysis

The blood DNA was isolated using the TIANamp Genomic DNA Kit (TianGen) kit with an elution volume of 70µL. The DNA was then bisulfite converted, according to [10] , with modifications. The concentration of Na-bisulfite 2.5 M were used. The bisulfite conversion result is in single-strand DNA, then firstly PCR first with outer primer. The first PCR procedure was performed with the following protocol: Five minutes of predenaturation step at 94 ° C, 40 cycles of denaturation step (94 ° C for 30 seconds, annealing of 53 ° C for 90 seconds, elongation of 72 ° C for 90 seconds), and final elongation of 72 ° C for 5 minutes. The result of the first PCR has used for the second step PCR as templates with inner primers. The second PCR was performed using the following step: Five minutes of

predenaturation at 94 ° C, 35 cycles of PCR (denaturation at 94 ° C for 30 seconds, annealing at 49.1 ° C for 90 seconds, and elongation at 72 ° C for 90 seconds), and final elongation at 72 ° C for five minutes.

The PCR results will then be electrophoresed using 2% agarose stained with ethidium bromide. Band visualization was performed using an ultraviolet illuminator with a 100 bp comparison of Promega's DNA ladder markers. Methylation analysis was carried out from the amplicon band visualization results with the help of AlphaEase software, which measures the density of the relative band thickness. The relative band density of the primary amplicon from analyzing methylated or non-methylated amplicon (FMET or FNON) interpreted in the form of integrated density value (IDV).

$$\text{IDV} = (\text{AVG pita} - \text{AVG } \textit{background}) \times \text{Area}$$

$$\%\ \text{Methylation} = \frac{\text{IDV of FMET Amplicon}}{\text{IDV of FMET Amplicon} + \text{IDV of FNON Amplicon}} \times 100\%$$

3. Statistical Analysis

Statistical analyses were performed using IBM SPSS 25.0. Statistical analysis was achieve using the independent t-test to examine differences between the control and treatment groups. The t-test used was two-tailed. Data are presented in mean ± SD. The results were statistically significant if the p-value was <0.05.

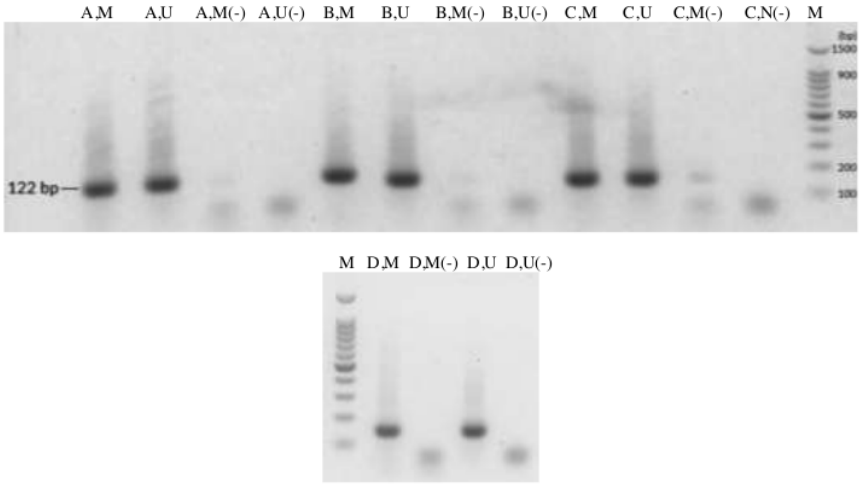
4. Results and Discussion

Figure 1 is the result of annealing temperature optimization for MSP settings. The temperature tested for annealing is set on the Techne TC-5000 thermocycler machine at a temperature of 50.2 ° C with a gradient of 23 ° C. PCR test tubes were reacted at block temperatures of 40.6 ° C (A), 43.6 ° C (B), 47.3 ° C (C), and 49, ° C (D). Each was compared with a negative control (-) without a template. At all of the above-mentioned temperature, a specific DNA band was obtained at a size of 122 bp for both (methylated) M and unmethylated (U) primers. In the reaction with an annealing temperature of 47.3 ° C, there was a faint band on the negative control primary M while the band on the negative control was not found. From these results, the optimum temperature used for the next MSP was 49 ° C.

The MSP primers were designed to successfully amplify the template and produce a specific band with the right size. The MSP optimization showed that the primer could be used in a temperature range of 40 ° C to 49 ° C. Negative control is used as a comparison while eliminating amplification interference. The temperature of 49 ° C was chosen for the next MSP of 49 ° C because the negative control results were cleaner than the impurity band.

Figure 2 shows the optimization results of the bisulfite conversion reaction time. The sample used was 18 µL of blood DNA. Incubation with 2.5 M Na-bisulfite reagent was adjusted for 12 hours (D) and 14 hours (E) to determine the best incubation time. The modified isolates were amplified with SS code PDK4 gene primers and with the expected amplicon size of 140 bp, compared to marker (M). The MSP results in these samples showed a specific band in the incubated isolates for 14 hours.

The bisulfite reaction was manually optimized for the incubation duration. The goal was to confirm and to know the conditions of the best conversion reaction results. From the PCR results with different optimization, it was found that the most optimum reaction in the manual method resulted in a specific amplicon band in samples incubated for 12 hours in pancreatic samples only, and 14 and 16 hours in blood samples. In blood samples, no amplicons were found at the 12 hour incubation period. For blood samples, an incubation time of 14 hours was selected.



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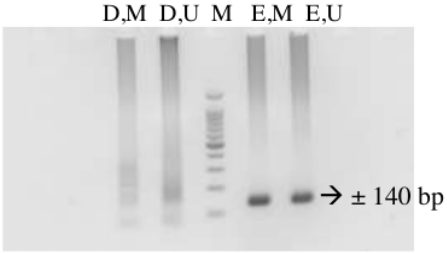

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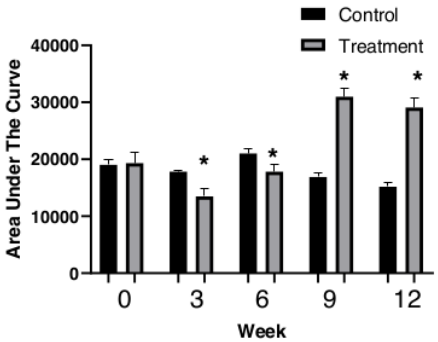

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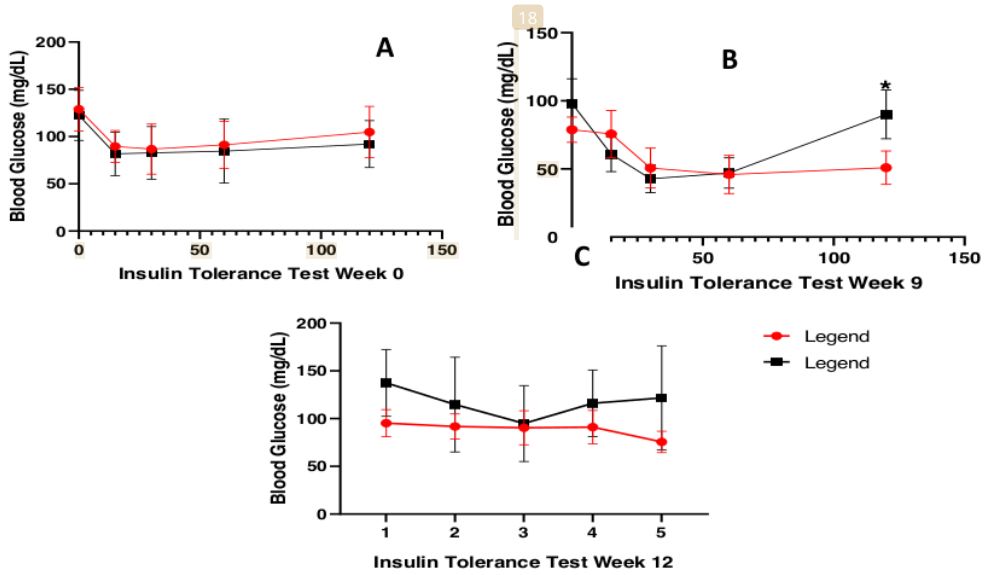

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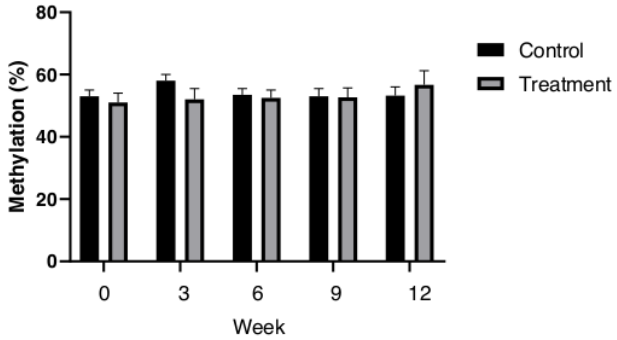

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