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Research Article
Development of Diabetic Animal Model via Insulin Glargine Induction

[1]Tyas Rini Saraswati, [2]I. Gusti Ayu Agung Suartini and [1]Silvana Tana

[1]Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro Jl. Prof. H. Sudarto SH. Tembalang Campus, Semarang 50275, Indonesia
[2]Fakultas Kedokteran Hewan Udayana, Jl. P.B. Sudirman, Denpasar, Bali, Indonesia

Abstract

Background and Objective: Diabetic patients depend on insulin, however a long-term insulin treatment caused insulin resistance which is caused by Insulin Receptor substrate (IRs) damage. This study aimed to get animal diabetic model caused by insulin resistance induced by insulin glargine. **Materials and Methods:** The experiment was performed with fully random design. Male *Rattus novergicus* Wistar is induced by glargine insulin 1.80 IU kg^{-1} weight per day, every day for 14 and 28 days and compared with control mice. Injection used as subcutaneous on the abdomen by using xiulin pen. At the end of the treatment, the samples have fasted for 10 hrs. The blood was taken directly from the heart after dissection. The glucose level was compared between treatments. Side effect parameters were observed to the consumption of food, drink, lipid profile, body weight, liver function and liver histology description. **Results:** The results showed that glargine insulin induction at the dosage of 1.80 IU kg^{-1} weight per day for 28 days caused hyperglycemic with 117,33 mg dL^{-1} fasting glucose level in blood followed by the increase of food and drink consumption, liver weight and the change in lipid profile. **Conclusion:** Glargine insulin induction at 1.80 IU kg^{-1} weight per day for 28 days caused pre-diabetic in male *Rattus novergicus*.

Key words: Diabetic, glargine insulin, fasting glucose hyperglycemic, insulin resistance

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Corresponding Author: Tyas Rini Saraswati, Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro Jl. Prof. H. Sudarto SH. Tembalang Campus, Semarang 50275, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetic causes chronic diseases. In 2015, diabetic patients in Indonesia reached to 9.1 million people. Diabetic is caused by the high level of glucose in blood around 110-130 mg dL^{-1} [1] and glucose level during fasting for more than 126 mg dL^{-1} [2]. When the glucose from food is absorbed into the bloodstream, beta cells in the pancreas will secrete insulin[3]. The presence of insulin allows glucose in blood to enter cells as energy source. Insulin reacts with the targeted cell through bounds with insulin receptor which consisted of 2 glycoprotein sub-units (α dan β sub-units). Insulin creates a bound with β sub-unit and activates tyrosine-specific kinase autophosphorylation from α sub-unit[4], leading to intracellular response, which cause glucose transport protein units (GLUTs) translocation from Golgi apparatus to plasma membrane allowing for glucose taking by cell. There are 4 types of glucose transporters (GLUT) working in different cells[5].

Glucose transporter 2 (GLUT-2) is protein transporter of glucose to liver cell[6]. Glucose can transfer to the cells through diffusion with the help of insulin receptor[7]. Some parts of body cells have insulin receptors which have a role in insulin signal transduction. Insulin Receptor Substrate (IRS) is needed to phosphorylate insulin[8]. If the presence of insulin in the blood is too high, the numbers of insulin receptors decrease and there will be insulin resistance[9] and insulin can't bind with cells, causing glucose not to be able to enter into cells[10,11], leading to hyperglycemic.

Human insulin treatment at the dosage of 1.80 IU kg^{-1} weight per day for 14 day increased glucose level in male mice blood galur Wistar and increased insulin resistance. Insulin resistance is achieved by induction of human insulin for 14 days with the dosage of 1.80 IU kg^{-1} weight per day in male mice and increased glucose level in blood. Glargine insulin is human insulin analog designed to be soluble at neutral OH. Glargine insulin is soluble at acid from glargine insulin injection (pH 4). Glargine insulin is similar to human insulin regarding the kinetics of insulin receptor attachment. In pharmacologist clinical study, glargine insulin and human insulin have the same potential at the same dosage. Animal model with diabetic mice is highly needed for hyperglycemic experiment. Normal glucose level within fasting mice is around 80-100 mg dL^{-1}. Insulin resistance in diabetic patients is responsible to blood glucose change during fasting but the cellular mechanism is not clear and hyperglycemia animal model is needed. Hyperglycemic leads to long term damages in vital organs such as liver, kidney and blood vessel. Diabetic patients tend to have dyslipidemia caused by lipid profile change[12].

Research on the making of diabetic animal models is important to do in an effort to find the right treatment strategy in preventing and overcoming diabetes and its complications. This study was designed to find animal models of diabetes due to insulin resistance through insulin glargine induction.

MATERIALS AND METHODS

Study area: This research was conducted at the Animal Structure and Function Laboratory of the Department of Biology, Diponegoro University, Indonesia, in April-October, 2020.

Ethical approval: This study has approval from the commission of health research ethic at the Department of Health, Universitas Diponegoro No.96/EC/H/FK-UNDIP/IX/2020.

Specimen collection: This research used male *Rattus norvegicus* strain Wistar of 2 months year old with the weight of 175-200 g, which came from a breeder in Ngaliyan, Semarang, Central Java. The mice were acclimated in individual cages at the size of 30×30×40 cm within a week. The floor is made from husk and replaced once in 3 days. The environmental temperature around 25-28°C with humidity of 60-80% with bright and dark cycle of 12 h each. The mice were fed with a diet standard (merk HI-PRO-VITE 594) containing 19.5% of protein, 3% of fat, 8% of fibre, 7% of ash, 0.9% of calcium and 0.6% of phosphor and were given drink by ad libitum during the experiment.

Chemicals and reagents: Glargine insulin with Ezelin brand, Glargine insulin was produced by DNA recombinant of Escherichia coli which was produced by Gan and Lee Pharmaceutical Ltd. No.8 Nanfeng West 1st Road, Huoxian Town, Tongzhou District, Beijing, China.

Experiment procedure: Experiment through fully random design. 21 *Rattus norvegicus* were divided into 3 groups. P_0: *R. norvegicus* control group, P_1: *R. norvegicus* group which was induced with glargine insulin 1.80 IU kg^{-1} weight per day for 14 days. P_2: *R. norvegicus group* which was induced with glargine insulin 1.80 IU kg^{-1} weight per day for 28 days.

Glargine insulin induction in *Rattus norvegicus*: Injection area on subcutaneous abdomen contains more fat and less

nerves. The needle was attached on xiulin pen. The smallest tip was inserted from the insulin cartridge to the cartridge saving place in the pen. The pen was rotated and reversed upside down 10 times, to uniformly disperse the insulin. Insulin dosage of 1.80 IU can be controlled by injection button. The glargine insulin was then injected into the mice by subcutaneous, to the abdomen. Injection was done for 14 days for P_1 group and 28 days for P_2.

Blood collection: At the end of the treatments, the mice have fasted for 10 hrs. Before dissection, the mice were anesthetized by chloroform. The blood was taken through the heart and kept in a vacutainer containing EDTA. Some of the blood was saved in an Eppendorf and centrifuged with a mini diagnostic with a speed of 3000 rpm for 20 min to get the serum.

Blood glucose level measurement: Blood glucose level is gotten enzymatically through GOD-PAP method[13]. The principle of this method is the reaction between glucose with GOD-PAP reagent producing complex compound with the color of kuinonimin which can be calculated with a visible spectrophotometer. This serum was then taken as much as 20,0 µL from the sample and mixed with 10 mL of TCA. The mixture was then reacted enzymatically with 2.0 mL of GOD-PAP reagent and vortexed for ±10 sec. It was then incubated at 37°C for 105 min and the absorbance is read at maximum λ of 505 nm.

Blood lipid profile measurement: Triglyceride level, High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT) and Triglyceride levels of the collected fractions were determined using a commercially available kit (Roche Diagnostics, Mannheim, Germany)[14]. The LDL/HDL analysis was conducted by using KIT from DiaSys (Diagnostic System) and CHO-PAP method[15]. The SGPT analysis was done by IFCC kinetic method. (SGOT IFCC UV kinetic method)[16].

Measurement of food and drink consumption and body weight: Measurement of food and drink consumption was observed every day. Body weight scaling was done by Salter Stainless Steel Digital Animal Weighing Scale (3 kg Capacity). Data collection was done at the end of treatment after fasting for 10 hrs. The mice is determined as diabetic if the fasting glucose in its blood >126 mg dL^{-2}.

Preparation of liver histology: After surgery, the liver was taken and measured before inserted into fiksatif BNF 10% for 2 weeks. The liver histology preparation was done in Balai Besar Veteriner Yogyakarta by using paraffin method and Haematoxylin and Eosin staining[17]. Hepatocytes cell measurement was done by using photomicrograph Olympus BX51 and liver histology description was done.

Statistic analysis: The result is determined as mean±deviation standard. The data is analyzed by using analysis of Varian (ANOVA)[18]. The difference between each of the treatments is determined by using Duncan ($p<0.05$ test through SPSS version 23 for windows).

RESULTS

Food metabolism: The results of the analysis of insulin glargine induction 1.80 IU kg^{-1} day on fasting blood glucose levels showed significant results ($p<0.05$). The lowest fasting blood glucose level was in the control group (72.4±11.6) mg dL^{-1}. The P_1 (induced insulin glargine 1.80 IU kg^{-1} day for 14 days) had to fast blood glucose levels with a mean value of 82.63± 5.5 mg dL^{-1}. There was no significant difference between P_0 and P_1. The P_2 had the highest fasting blood glucose levels with an average value of 117.33±8.8 mg dL^{-1}. Statistically, P_2 has a significant difference with P_0 and P_1.

The results of the analysis of insulin glargine induction 1.80 IU kg^{-1} day on body weight measurement showed significant results ($p<0.05$). There is a significant difference in body weight between P_0 and P_1 and P_0 and P_2 but there is no significant difference between P_1 and P_2. The lowest mean body weight was P_0 (15.60±1.2 g), followed by P_1 (17.34±0.8 g) and P_2 (17.36±1.2 g).

The results of the analysis of insulin glargine induction 1.80 IU kg^{-1} day on daily feed consumption showed significant results ($p<0.05$). There is a significant difference between P_0 and P_1 and P_0 and P_2 but there is no significant difference between P_1 and P_2. The lowest feed consumption was at P_0 with a mean value (24.9±2.2 g), followed by P_1 (28.09±2.1 g). The highest daily feed consumption was at P_2 (29.16±2.8 g). The results of the analysis of insulin glargine induction 1.80 IU kg^{-1} day on daily drinking consumption showed significant results ($p<0.05$). There is a significant difference between P_0 and P_1 and P_0 and P_2 but there is no significant difference between P_1 and P_2. The lowest drinking consumption was at P_0 with a re value of 246.43±8.3 average

Table 1: Analysis result of glargine insulin induction influence to blood glucose level, food consumption, daily drink consumption and body weight of male *Rattus norvegicus*

Parameters	P_0	P_1	P_2
Fasting Blood glucose (mg dL^{-1})	72.4±11.6[b]	82.63±15.5[b]	117.33±8.8[a]
Body weight (g)	15.60±1.2[b]	17.348±0.8[a]	17.36±1.2[a]
Food consumption/day(g)	24.9±2.2.[b]	28.09±2.1[a]	29.16±2.8[a]
Consumption of drinking water/day (mL)	227.41±5.7[b]	242.01±10.2[a]	246.43±8.3[a]

[a,b]Values bearing similar, superscript between rows do not differ at $p<0.05$, P_0: *R. Norvegicus* control group, P_1: *R. norvegicus* group which was induced with glargine insulin 1.80 IU kg^{-1} weight per day for 14 days. P_2: *R. norvegicus group* which was induced with glargine insulin 1.80 IU kg^{-1} weight per day for 28 days

Table 2: Analysis result of glargine insulin induction to liver function of male Rattus norvegicus

Parameters	P_0	P_1	P_2
Liver weight (g)	6.74±0.14[b]	6.87±0.18[b]	7.23±0.31[a]
Hepatocyte diameter (μm)	19.69±0.8[b]	19.23±0.6[b]	22.89±1.2[a]
SGPT (U l^{-1})	72.4±8.2[a]	75.06±6.6[a]	66.4±9.1[a]
SGOT (U l^{-1})	224.6±11.6[a]	232.23±20.5[a]	214.6±25.6[a]

[a,b]Values bearing similar, superscript between rows do not differ at $p<0.05$, SGPT: Serum glutamic pyruvic Transaminase, SGOT: Serum glutamic-oxaloacetic transaminase

(227.41±5.7) mL, followed by P_1 (242.01±10.2 mL). The highest daily feed consumption was P_2 (29.16±2.8 mL) (Table 1).

Liver function: The results of the analysis of insulin glargine induction 1.80 IU kg^{-1} day on liver weight showed significant results ($p<0.05$). There is a significant difference between P_0 and P_2 and P_1 and P_2 but there is no significant difference between P_0 and P_1. The lowest liver weight was at P_0 (6.74±0.14 g), followed by P_1 (6.87±0.18 g). The highest liver weight pad P_2 (7.23±0.31 g).

The effect of insulin glargine induction 1.80 IU kg^{-1} day on the diameter of the hepatocytes showed significant results. There is a significant difference between P_0 and P_2, P_1 and P_2 but there is no significant difference between P_0 and P_1. The smallest diameter of hepatocytes was P_0 (19.69±0.8 μm), P_1 (19.23±0.6 μm), P_2 (22.89±1.2 μm). The results of the analysis of insulin glargine induction 1.80 IU kg^{-1} day on SGPT and SGOT levels showed insignificant results ($p>0.05$) (Table 2).

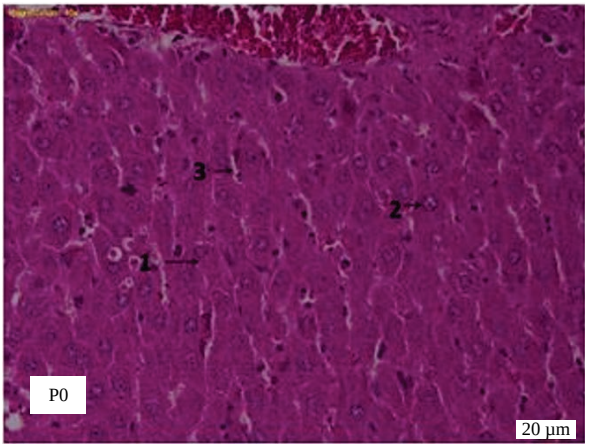

Fig. 1: Liver histology structure in rattus norvegicus P_0 (not induced by insulin glargine) showing normal hepatocyte cells, arranged regularly, central nucleus (Stain H and E, 40×10 magnification)
1: Hepatocyte, 2: Nucleus, 3: Sinusoid

Liver histology structure: Histological photo-micrographs of *Rattus norvegicus* were made by the paraffin method and Haematoxylin and Eosin staining. In the P_0 treatment (control), hepatocytes arranged radially around the central vein, the nucleus in the middle was clear, the cytoplasm was homogeneous and the sinusoids between hepatocytes had the same width. In P_1 (*Rattus norvegicus* induced by insulin glargine 1.80 IU kg^{-1} day for 14 days), the histological structure of the hepatocytes was partly swollen, the cell arrangement was irregular, some of the core hepatocytes were absent (undergo karyolysis), the cytoplasm of some hepatocytes was not homogeneous, there was infiltration of erythrocytes in the sinusoids, the sinusoids in some parts experienced enlargement. P_2 (*Rattus norvegicus* induced by insulin glargine 1.80 IU kg^{-1} day for 28 days) shows that the histological structure of the hepatocytes was mostly swollen, cell arrangement was irregular, most of the core hepatocytes were absent, pale cytoplasm and there was erythrocyte infiltration in the sinusoids (Fig. 1-3).

Lipid profile: Study on the effect analysis of insulin glargine induction 1.80 IU^{-1} kg^{-1} day towards triglyceride levels showed significant results ($p<0.05$). There is a significant difference between P_0 and P_2 and P_1 and P_2 but there is no significant difference between P_0 and P_1. The lowest triglyceride levels were P_0 (25.8±1.54 mg dL^{-1}), followed by P_1

Table 3: Analysis result of glargine insulin induction to lipid profile of male *rattus norvegicus*

Parameters (mg dL^{-1})	P_0	P_1	P_2
Triglyceride	25.8±1.54[b]	24.4±1.9[b]	27.8±1.1[a]
Total Cholesterol	59.8±6.9[a]	57.97±8.7[a]	54.4±6.8[a]
HDL	21.0±3.4[a]	20.04±4.7[a]	20.4±2.9[a]
LDL	14.2±3.2[b]	13.17±4.2[b]	25.8±5.4[a]

[a-b]Values bearing similar, superscript between rows do not differ at p<0.05, HDL: high-density lipoproteins, LDL: low-density lipoproteins

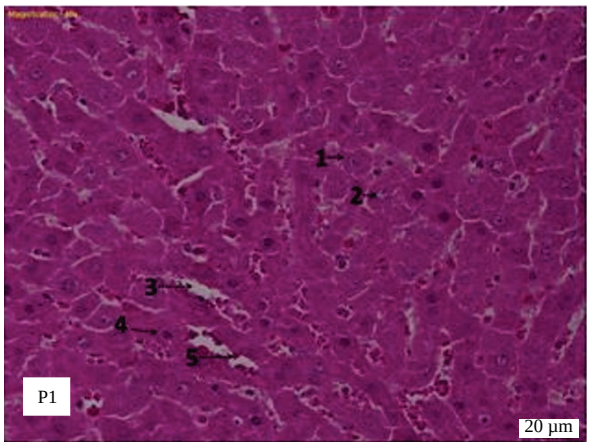

Fig. 2: Liver histology structure in Rattus norvegicus P_1 (induced with Glargine 1.80 IU kg^{-1} day for 14 days) shows hepatocyte cells arranged irregularly, some swollen hepatocytes, some cariolisi nuclei, some sinusoid parts are enlarged, infiltration erythrocytes (Stain H and E, 40×10 magnification)
1: Hepatocyte, 2: Nucleus, 3: Sinusoid, 4: Blood cell, 5: Infiltrasi eritrosit

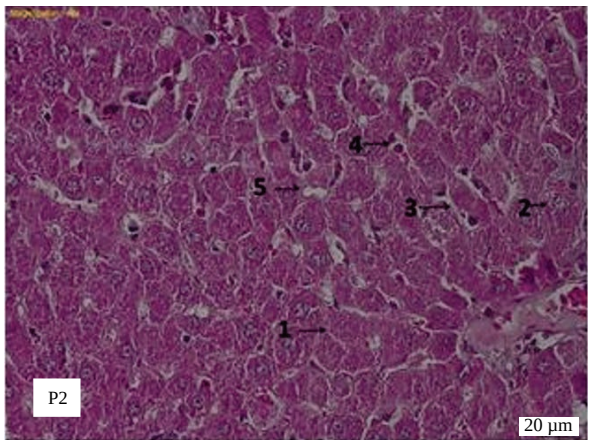

Fig. 3: Liver histology structure in Rattus norvegicus P_2 (induced with Glargine 1.80 IU kg^{-1} day for 28 days) shows hepatocyte cells arranged irregularly, most hepatocytes swell, cariolisi nuclei, dilated sinusoids (Stain H and E, 40×10 magnification)
1: Hepatocyte,2: Nucleus, 3: Sinusoid, 4: Blood cell, 5: Infiltrasi eritrosit

(24.4±1.9 mg dL^{-1}). The highest triglyceride level was P_2 (27.8±1.1 mg dL^{-1}). However, the results of the analysis on total cholesterol, HDL levels and LDL levels showed insignificant results (p>0.05) (Table 3).

DISCUSSION

Result showed long term hyper insulinemia with glargine insulin induction of 1.80 IU kg^{-1} weight per day to blood glucose during fasting of male Rattus norvegicus shows significant difference (p<0.05). There was an increase of blood glucose during fasting in both glargine insulin induction of 1.80 IU kg^{-1} weight per day for 14 and 28 days. The average blood glucose during fasting in mice that was glargine insulin inducted for1.80 IU kg^{-1} weight per day for 14 days is 82.63 mg dL^{-1}. The blood glucose is still in normal level. Blood glucose level for normal mice is 80-100 mg dL^{-1} [19]. Blood glucose of fasting mice which was glargine insulin-induced with 1.80 IU kg^{-1} weight per day for 28 days on average is 117.33 mg dL^{-1}, shows increase of 44.93 mg dL^{-1}. This result shows that the mice are in prediabetic state. According to World Health Organization (WHO) standard, prediabetic or disturbed glucose tolerance, is a condition where blood glucose is lower than diabetic but higher than normal (100-125 mg dL^{-1})[20,21]. Glycemic prediabetic will develop into diabetic[22,23]. Pre diabetic usually happens after insulin resistance[24].

Insulin resistance is a condition where body cells can't use blood glucose because the body cells response to insulin is disturbed[25,26]. When there's insulin resistance, body cells can't absorb glucose as it should be. This condition causes the piling up of glucose in the bloodstream, leading to the high level of glucose compared to normal state.

This analysis of glargine insulin induction of 1.80 IU kg^{-1} weight per day showed a significant difference to food and drink consumption (p<0.05). There was an increase in feed consumption. Current findings are contrast with previous studies which reported that insulin induction in rat decreased feed intake[27]. The observation to food and drink consumption can be used as an indicator to diabetic mellitus development[28]. According to other study[29,30], 3 diabetic symptoms are polyphagia (increase of food consumption),

polydipsia (increase of water consumption) and polyuria (increase of urine secretion). An individual with those 3 symptoms has the probability of diabetes as much as 47.06%. The increase of food consumption shows insulin resistance[31]. It is caused by the insensitivity of cell to insulin, causing the decrease of glucose ability to enter cells. This condition causes the lacking of glucose in cells, leading to hunger and increasing food consumption.

Drink consumption increase is caused by hyperglycemia causing an increase of body liquid osmolarity[32,33]. The increase of body liquid osmolarity will trigger thirst nerve, inducing the need to drink[34,35].

Alongside the increase of food intake, there is an increase in body weight. According to analysis, glargine insulin induction analysis for 1.80 IU kg^{-1} day affects body weight ($p<0.05$). This is because the mice are in prediabetic condition. Prediabetic is not yet categorized as Diabetic Mellitus (DM) but the blood glucose is higher than normal[36,37]. One of the signs of a diabetic is the presence of leptin[38]. Leptin is the hormone to produce fat network. Piled-up fat network will secrete leptin hormone and circulate in blood, bounding leptin receptor in hypothalamus, causing the feeling of fullness[39,40]. The hunger feeling will be delayed when fat reached to some level. Leptin also regulates appetite and body weight[41].

Diabetic and prediabetic patients have a high level of leptin[42] which leads to insulin resistance[43], removing the hunger control. Insulin resistance blocks leptin's work to send the fullness signal to brain, causing the body to ask for energy and by the feeling of hunger, which followed by increase of food intake, increasing the body weight. Leptin resistance causes obesity and hiperfagia[44]. Increase of body weight is parallel to the increase of insulin resistance.

Analysis result of glargine insulin induction of 1.80 IU kg^{-1} day shows the significant difference to liver weight and hepatocytes diameter ($p<0.05$) but not to SGPT and SGOT level in blood. Liver histology (Fig. 1) in P_0 is arranged normally, creates cell plates with an average of hepatocyte size by 19.69 µm, round nucleus in the middle and bright cytoplasm. In P_1 the hepatocytes arrangement is irregular, some cells are bigger and some are smaller, with an average of 19.23 µm. Some cell nucleus looks more picnotic, there is infiltration, cell is inflamed in sinusoid, sinusoid looks bigger. In P_2, hepatocyte size is 22.89 µm, the arrangement is irregular, cell size is bigger than other treatments, some cells had karioreksis, with missing nucleus. Vacuole was found in some hepatocytes.

Glucose level isn't stable because of insulin resistance leads to the damage in hepatocyte membrane, causing disruption in liquid transportation and damage cells metabolism. Further damage happens in cell organelle. If there is a damage in lysosome, there will be autophagocytosis by proteolytic enzymes from lysosome. There is pyknosis in nucleus. In P_1, some hepatocyte undergo cell swelling, while some other undergo cell shrinking, causing irregular arrangement of hepatocyte. The average size of hepatocyte is the same with P_0 hepatocyte. In some hepatocyte cells, pyknosis nucleus was found, which has a darker color. The damage in cell cause the presence of inflamed cells transported in inflamed area through sinusoid, to fix damaged network. Inflammation is vascular reaction by sending inflamed cells to inflamed cells[45]. Sinusoid in P_1 looks bigger than in P_0. In P_2, hepatocytes undergoes swelling, with the average hepatocyte of 22.89 µm, cytoplasm looks unclear, this is a sign of degeneration in cytoplasm organelle, some cell nucleus disappeared or karioreksis. Karioreksis happened because there is damage in nucleus membrane, caused lyses in some elements in nucleus. In P_2, there is fat addition in liver, shown by vacuole presence in hepatocyte cytoplasm. Liver is an important organ in glucose metabolism. Insulin resistance patients have the risk of fatty liver damage[46]. Fatty liver is a buildup of fat in hepatocytes. Accumulation of fat and swelling of hepatocytes causes liver weight to increase.

Fatty liver is caused by an imbalance in the making and renovation in triglyceride[47]. Insulin resistance affects the initial formation of fatty acid, because with insulin resistance there will be an increase in the synthesis of triglyceride transport into liver and there is an increase of lipolysis in adipose in the central of body, where fatty acid (FFA) as the result of lipolysis will be brought through vena porta to the liver to be processed and caused the high level of free fatty acid in liver. A high level of lipogenesis and triglyceride synthesis in liver causes the formation of hepatic fat in syndrome metabolik[48].

Analysis result of glargine insulin of 1.80 IU kg^{-1} weight per day shows the significant result to triglyceride and LDL level ($p<0.05$). There was triglyceride and LDL level increase in mice treated with glargine insulin 1.80 IU kg^{-1} weight per day for 28 days but no significant difference in total cholesterol and HDL ($p<0.05$). Insulin function decrease caused hormone-sensitive lipase[49], which cause lipolysis and in the end cause the release of fatty acid and glycerol into the bloodstream which cause an increase in free fatty acid and if too much amount is delivered into the liver to fat metabolism which will be converted into phospholipid, cholesterol and triglycerides, which will cause triglycerides to increase. Then, it will be sent into circulation through lipoprotein and LDL level in blood also increases. This phenomenon is in accordance with previous basic theory and the same result was found in the previous study[50], in which dyslipidemia caused LDL and triglycerides level increase. There's no significant difference between cholesterol levels in blood presumably because the

cholesterol inside of liver was used for other metabolism process to repair damaged network structure. There is no HDL decrease presumably because the mice is still in prediabetic state and thus is still able to maintain HDL level in normal state.

CONCLUSION

Glargine insulin induction of 1.80 IU kg^{-1} day for 28 days caused pre diabetic in male *Rattus norvegicus* with blood glucose level during fasting of 117, 33 mg dL^{-1}. Pre diabetic is followed by symptoms of polyphagia, polydipsia, body weight increase, hepatocytes damage in blood lipid profile.

SIGNIFICANCE STATEMENT

This study found that pre-diabetes animal models can be induced with insulin Glargine 1.80 IU kg^{-1} day for 28 days, which can be useful for the development of diabetes pathology research. The diabetic mouse model is believed to play an important role in explaining the pathogenesis of diabetes in humans. In addition, the diabetic mouse model is essential for investigating and developing new drugs for diabetes and its complications.

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