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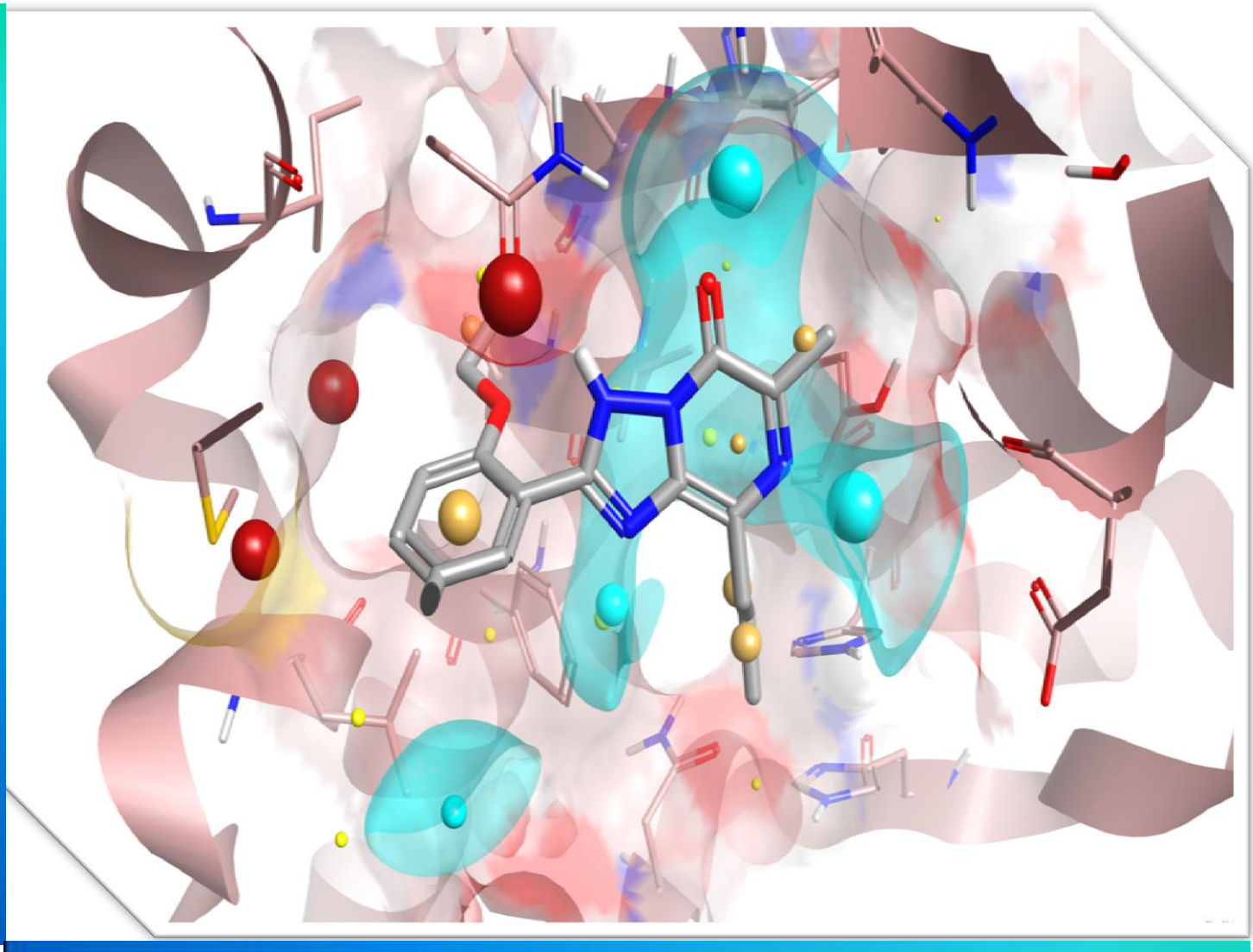
1st INTERNATIONAL CONFERENCE ON PHARMACY SCIENCE AND PRACTICE

"Recent Advancement on Natural Product for Drug Design"



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Optimizing Streptozotocin Dose for Inducing Type 1 Diabetes Mellitus in Male Wistar Rats

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ABSTRACT

The preclinical antidiabetic study generally uses animal models with cytotoxic agent induction, e.g., streptozotocin (STZ). However, a different STZ dose (35-80 mg/kg) and routes of administration (intraperitoneally or intravenously) were mentioned before. This study has the aim to determine the optimal STZ intravenous dose for diabetes induction in rats. Sixty male Wistar rats (150-200 g) were undergone oral glucose tolerance test to examine their glucose homeostasis. A week later all rats were fasted for 8 hours and divided into six groups: STZ 40, 50, 60, 70, 80 mg/kg and control. Fasting blood glucose (FBG) level was measured on day 3, 7, 14 and 21 after STZ administration and was analyzed using a Kruskal-Wallis test. At day 21 all rats were sacrificed, and their pancreas was taken for histopathology examination. STZ given at 40,50,60, 70, 80 mg/kg resulted in death of 0%, 10%, 60%, 80%, 90% rats from each group. Three days after STZ administration there was a significant FBG increase in all STZ dose groups, except in 40 mg/kg group ($p < 0.05$). On the 7th day, the increasing FBG were persisted in 50, 60 and 70 mg/kg groups. On the 14th and 21st day, all rats FBG have decreased to <200 mg/dL. Histology examination revealed that a higher dose of STZ has resulted in lower Langerhans island number and diameter. The optimal dose of STZ to induce diabetic in male Wistar rats is 50 mg/kg. At this dose, hyperglycemia was persistent for seven days, and the mortality was minimal.

Key Words: optimal dose, streptozotocin, diabetes, Wistar rats.

1. INTRODUCTION

It was estimated that in 2014 more than 422 million adults are living with diabetes (global prevalence 8.5%). The increasing number of the diabetic patient was in line with increasing diabetic risk factors, such as overweight or obese. High glucose level also a risk factor for cardiovascular and other diseases leading to 3.7 million death before 70 years of age in 2012 [1]. The high level of diabetics incidence has encouraged the researcher to find antidiabetic remedies from various resources, one of them

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is from medicinal plants. It is common to use experimental animals with human-like diabetic physiological abnormalities to study the potential antidiabetic effect of the plant; The most common and frequent method was chemically-induced diabetes in rats or mice. A cytotoxic agent such as streptozotocin or alloxan was on the top list of the most widely used for diabetic induction.

Streptozotocin (STZ), a hydrophilic glucose analog, was more favorable than alloxan for animal diabetic induction because of it caused specifical damage to pancreatic beta cells. One hour after STZ administration, glucose concentration is increased, and plasma insulin was decreased. In this first hyperglycemic phase beta cells, morphology was changed and eventually result in inhibition of insulin secretion. This phase is persisted for 2-4 h. The second phase, the hypoglycemic phase, caused by hyperinsulinemia starts 4-8 h after induction and lasts for several hours. In this phase, glucose supplementation was needed to prevent animal death. Pancreatic cell already pyknotic and it was an irreversible condition. The last phase was a permanent hyperglycemic phase or diabetic phase that was seen 12-48 h after induction. In this phase pancreatic beta cell, the structure was utterly damaged [2].

In the implementation of diabetic induction using diabetes, there were several limitations, such as many variations in administration route and STZ dose from the literature. An extensive range STZ dose (35-80 mg/kg) was mention in various paper with two different routes of administration, intraperitoneally or intravenously [3]. STZ could be given in multiple small doses (35-40 mg/kg for 3-5 days) or single moderate or single large dose (> 45 mg/kg). Frequently used a single high dose of STZ given intravenously is 40-60 mg/kg. A single large dose STZ administration could destroy almost all pancreatic beta cells that mimicking diabetes type 1 condition in human [4–6].

Rats sensitivity to STZ was influenced by several factors such as strain, gender, diet, circadian rhythm. Moreover, different sensitivity to STZ in a subgroup of strain was also found. It is likely that it cannot be assumed that a strain from the same background will react to STZ in the same manner. Little genetic variability within the same age and gender group using the same STZ dose could result in different success induction rate. Furthermore, animals from different suppliers, outbred strain, or animals from a different generation in a colony can contribute to those difference sensitivity to STZ. Unidentified environmental conditions and stimuli also contribute to the different outcome of STZ-induced diabetes [4]. Therefore, preliminary experiments to determine optimal STZ dose for diabetes induction in our laboratory Wistar rats need to be carried out.

2. METHODOLOGY

Materials

Streptozotocin (Sigma-Aldrich, China), Glucose (Merck, Germany), glucometer with glucose dehydrogenase method (Accu-Chek® Performa, Roche, Germany), citric acid (Weifang Ensign Industry Co. LTD., China), monosodium citrate (Weifang Ensign Industry Co. LTD., China), sucrose (PT. Mawar Jaya, Indonesia), microscope (Olympus CX-21, Japan), Optilab® Advanced Plus software (Indonesia), syringe (OneMed, Indonesia), needle 30G 1/2 (BD Precisionglide, United States), rats feeding needle 18G (Indonesia), microcentrifuge tube 1,5 ml (OneMed, Indonesia), standard laboratory diet (19,5-21,5%

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protein, 5% fat, 5% fiber, 0,9% calcium, 0,6% phosphate, 3125-3225 kcal/kg, BR2 CP512, PT. Charoen Phokphand, Indonesia), insulin detemir (Levemir® FlexPen®, Novo Nordisk Production SAS, France).

Methods

Animals

Sixty male Wistar rats (*Rattus norvegicus*) with 150-200 g body weight (b.w) were obtained from a veterinarian breeder. Rats were transferred into the animal laboratory in Widya Mandala Catholic University, Surabaya, Indonesia and acclimatized for seven days. The animals were housed under 12 hours light/dark cycle, fed with standard laboratory diet and water ad libitum. These procedures have requested approval by Animal Ethics Committee at Integrated Research and Testing Laboratories, Gadjah Mada University, Yogyakarta, Indonesia.

Oral Glucose Tolerance Test

A week before the experiment, all animals undergo the oral glucose tolerance test (OGTT) to asses glucose homeostasis based on the postprandial blood glucose level. Oral glucose tolerance test method was modified from the previous study [7]. All rats were fasted for 18 hours, then weighed to determine glucose (2 mg/kg) doses. Blood was collected from the tail vein and tested with a glucometer as basal fasting blood glucose (FBG). Hereafter, 40% glucose solution was administered intragastrically with a feeding needle. Blood glucose level was measured again after 15, 30, 60 and 120 min. Rats with high glucose level (≥200 mg/dl) at 120 minutes after glucose administration were excluded from the study. The rest was divided into six different group, namely STZ 40, STZ 50, STZ 60, STZ 70, STZ 80, and a control group.

STZ Preparation

Streptozotocin (STZ) was stored in the freezer (-20^{o}C) before used. STZ is weighed according to the dose (40 to 80 mg) then dissolved in one mL freshly prepared cold citrate buffer (pH 4.5) in a microcentrifuge tube. During the STZ preparation, the container was maintained cold and protected from the light.

DM Type 1 Induction using STZ

All animals fasted for 8 hours (7.00 a.m - 3.00 p.m.). Afterward, rats were divided into six groups depending on body weight (the variation in each group ≤ 10%). Blood was collected from the tail vein and tested as basal FBG. The treatment group was given STZ 40, 50, 60, 70, 80 mg/kg. Meanwhile, the control group was given citrate buffer pH 4.5. All substances were administered intravenously through the tail vein. After STZ induction, all animals were given 10% sucrose in the first 24 hours to avoid death due to severe hypoglycemia. FBG level measurement was repeated on day 3, 7, 14, and 21. Pancreas were taken if the rat was not undergone rigor mortis. In this study, all rats with diabetic symptoms (fasting blood sugar ≥400 mg / dL) were given 2 U insulin detemir to prevent death from crisis hyperglycemia.

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Histopathological examination of the pancreas

Rats were euthanized by giving ketamine 200 mg/kg and xylazine 35 mg/kg intraperitoneally. Pancreas were collected for histopathological analysis. All portions of the pancreas were fixed in 10% buffered formalin solution for 24 h and then trimmed. The pancreatic tissue was gradually dehydrated in 70% alcohol, 80% alcohol, 90% alcohol, 96% alcohol, xylol, and liquid paraffin. The following step was tissue vacuuming and embedding. Pancreatic tissue was sectioned using microtome at a thickness of 2-5 µm and then processed with hematoxylin and eosin staining. The observation was carried out under a microscope with 400 times magnification. The Langerhans island diameter was measured using calibrated Image Raster 3® software.

Statistical Analysis

All data were statistically analyzed using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., USA). All data were analyzed their normality of distribution using Shapiro-Wilk test before a specific test was accordingly performed. Difference analysis in blood glucose level and body weight were carried out using paired t-test or Wilcoxon signed rank test. Blood glucose level after STZ induction were analyzed using a Kruskal-Wallis test. A 0.05 level was adopted for any significant difference.

3. RESULT AND DISCUSSION

Oral Glucose Tolerance Test

Oral glucose tolerance test (OGTT) was the most common method to assess glucose homeostasis in rodents [7]. The ability of glucose tolerance can be maintained as long as the pancreatic beta cells can compensate by increasing insulin production. Hence the glucose tolerance test results can indirectly describe the function of pancreatic beta cells [8]. OGTT was performed one week before the series of this study was performed. All rats fasted for 18 hours before their basal fasting blood sugar levels were measured. After that glucose (2 g/kg) was administered and multiple blood glucose level measurements were performed at 15, 30, 60 and 120 minutes. There was a sharp increase in blood glucose level after 15 minutes. Sixty minutes after glucose administration, the blood glucose level was starting to decrease (Figure 1). There was a significant decrease in blood glucose level between 15 to 120 minutes ($p < 0,05$) and none of the rats have blood sugar level higher than 200 mg/dL. Consequently, all rats were included in this study.

STZ Dose and Mortality Rate

The administration of STZ in rats caused mortality in various number across the experimental group. Rats were mostly died (38,33%) within 0-5 days after STZ induction (Table 1). Two days after STZ administration, one rat from STZ 70 group was found dead. On day three, 14 rats from different experiment groups (five from STZ 60, four from STZ 70, and five from STZ 80 group) died at a different occasion. On day four, there were eight rats died (one from STZ 50 group, one from STZ 60, two from STZ 70 and four from STZ 80). The last recorded death was at day 20 when a rat from STZ 70 was found died. Overall, 24/60 (40%) rats were experienced death. From Table 2 it was known that the highest

mortality rate was found in the STZ 80 group (90%). A higher STZ dose may increase the risk of rats mortality [9]. It was already known that STZ given ≥75 mg/kg without insulin administration might cause natural ketosis and death within days after STZ administration [10].

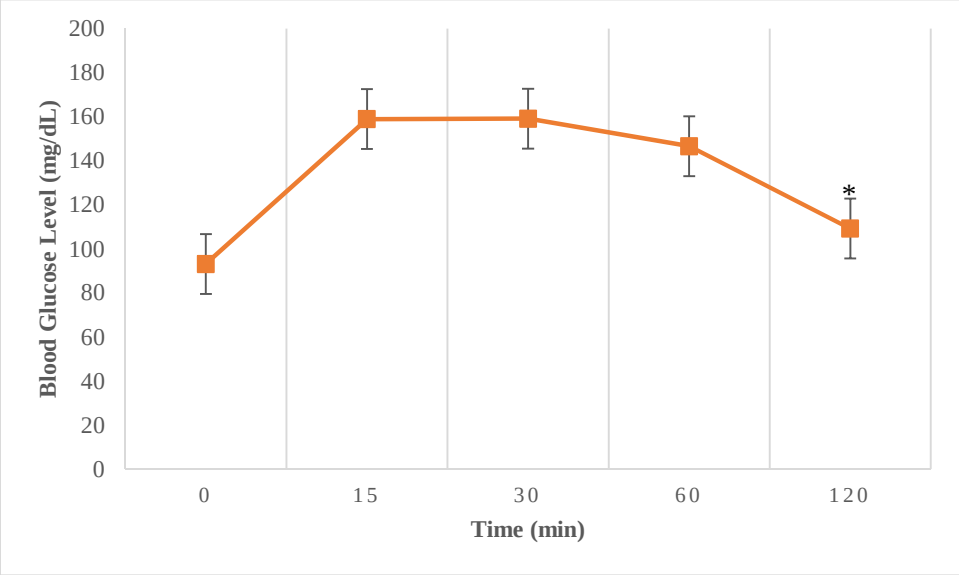

Figure 1. Blood glucose level during OGTT. Data express the mean ± S.E.M. for 60 rats. (*) show a significant different in FBG between 15 and 120 minutes (Wilcoxon signed rank test, $p < 0.05$).

Table 1. Rat mortality according to number of days after STZ administration

Days After STZ Induction	Number of Rats Found Dead	Number of Rats Left	% Mortality
0-5	23	37	38,3
6-10	0	37	0
>10	1	36	2,7

Table 2. Rat mortality according to STZ dose

Group	Number of Rats Found Dead	Number of Rats Left	% Mortality
Control	0	10	0
STZ 40	0	10	0
STZ 50	1	9	10%
STZ 60	6	4	60%
STZ 70	8	2	80%
STZ 80	9	1	90%

Body Weight after STZ Induction

Rats body weight was gradually decreasing after STZ administration (Figure 2). Significant decrease in body weight was found in STZ 50, 60, and 70 group at day 21 after STZ administration. Based on eye observation, physical activity and appetite in STZ-induced group were decreased.

Contrarily, their water consumption was increased. Those symptoms supposed to be a common phenomenon on day two and three after STZ administration. In that phase, rats may experience cachexia with decreased appetite, epistaxis, and hematuria it as well as hyperemia in the organs [11]. It was reported before that STZ administration can affect the body weight, particularly in Wistar and Sprague Dawley rats [12]. After STZ administration, male Wistar rats have more noticeable weight loss than female rats [4,12]. The body weight loss was proportional to the increase in STZ dose. Induction of T1DM with STZ causes glucose metabolism disorders with increased metabolism gluconeogenesis and glycogenolysis pathway [13]. Those metabolism change causes a decrease in bone and muscle mass after STZ induction, resulting in decreased body weight [5].

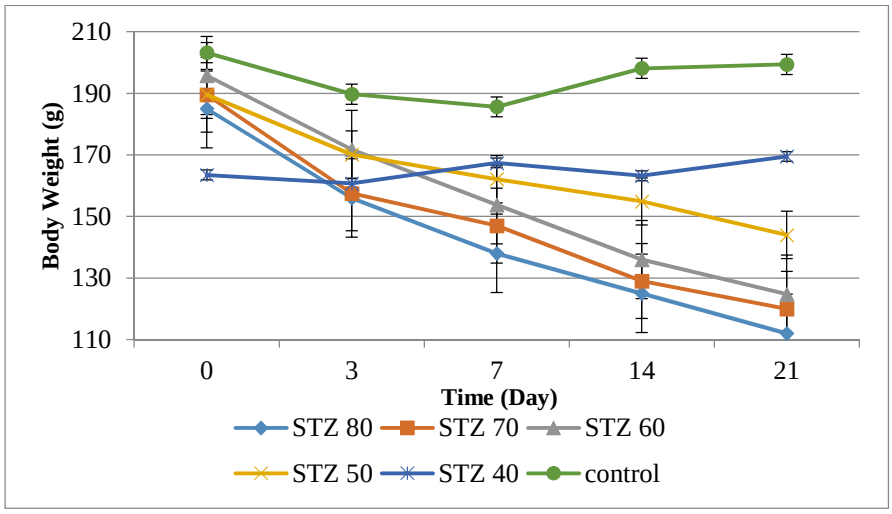

Figure 2. Body weight after STZ induction. Data express the mean ± S.E.M. Body weight was reducing significantly from day 0 to day 21 in STZ 50, 60, and 70 group (paired t-test or Wilcoxon test, $p < 0.05$). Only one rat in STZ 80 was survived on day 21 so the test result was not perfomed.

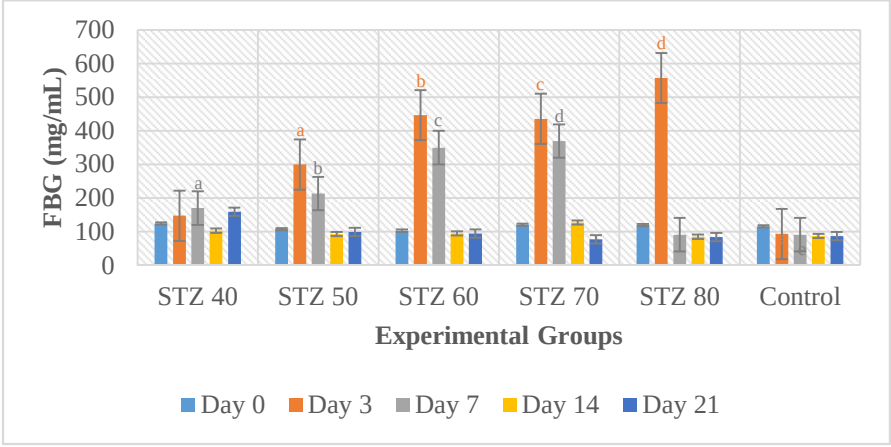

Figure 3. Fasting blood glucose level after STZ induction. Data express the mean ± S.E.M. Different letters indicate statistically significant different with control group on each day (Kruskal-Wallis, $p < 0.05$).

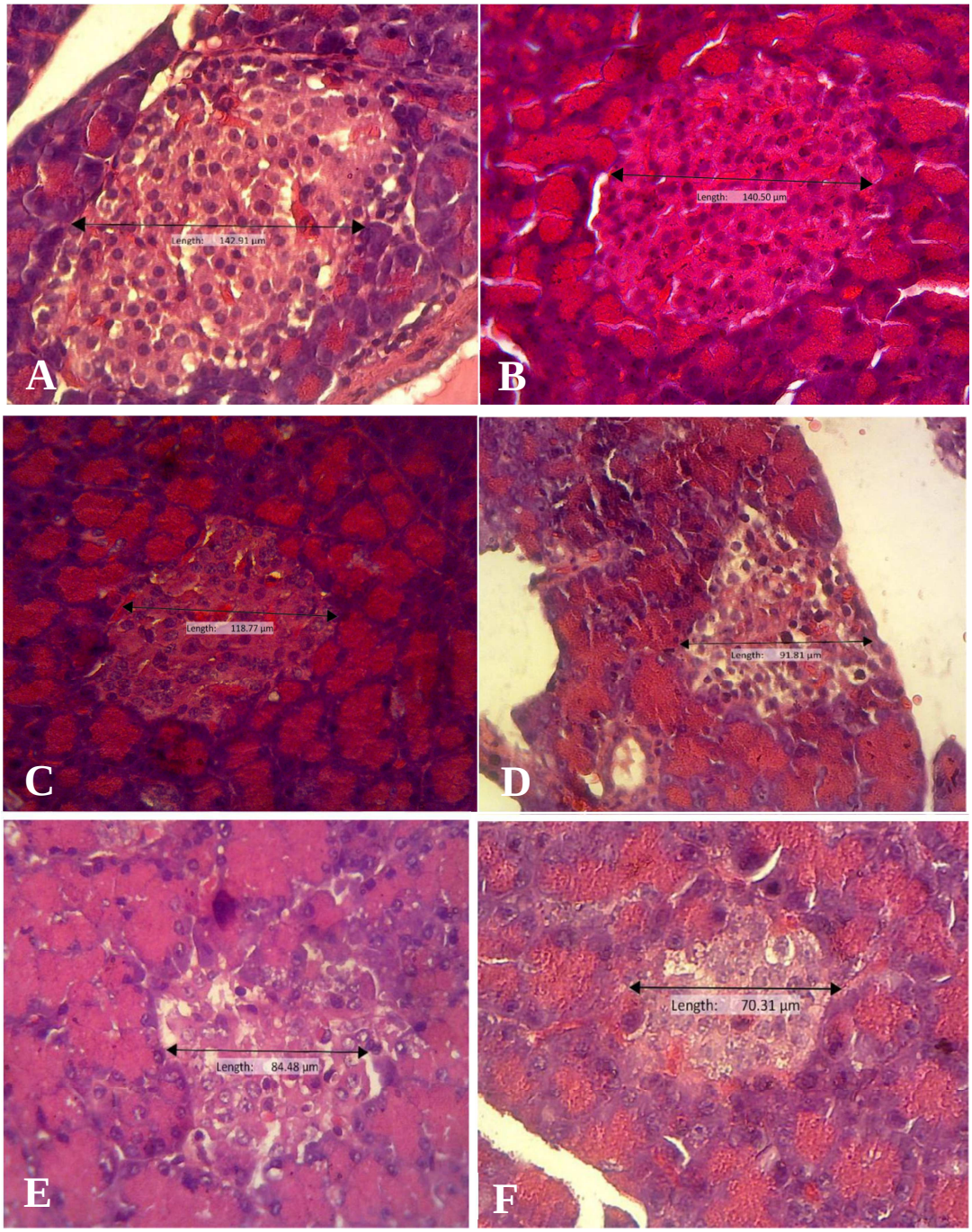

Figure 4. Histology of pancreatic rat cells in different experimental group: control (A), STZ 40 (B), STZ 50 (C), STZ 60 (D), STZ 70 (E), STZ 80 (F) (H&E staining, 400x magnification).

STZ Dose and Type 1 Diabetes Mellitus Induction Success Rate

Type 1 diabetes mellitus (T1DM) is a metabolic disorder related to glucose metabolism abnormalities. T1DM characterized by elevated blood sugar levels due to pancreatic beta cells damage. STZ may be used as a T1DM chemical inducing agent because of its specific action to pancreatic beta cells. STZ causes permanent damage to pancreatic beta cells resulting in permanent hyperglycemia as

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well. GLUT-2, an insulin receptor, helps STZ to enter the pancreatic beta cells. Also, pancreatic beta cells are more sensitive to glucose that facilitates STZ entry through GLUT-2. Therefore, STZ may cause toxicity in pancreatic beta cells. STZ is the material that causes DNA alkylation and increases oxidative stress. It also causes excessive glucose entry into beta pancreas cells that can cause cell damage [5].

FBG between groups at day 0 was found to be in normal range. Normal FBG usually lies between 95 to 100 mg/dL [14]. From day 2 -21, several rats of the various group were found dead (Table 1 and 2). Therefore, the number of rats examined for their FBG at each group was varied. Fig. Three showed that there was a sharp increase in blood glucose level in STZ 50, 60, 70, and 80 group on day three ($p < 0.05$). However, the increase was only survived until day seven. Their FBG returned to "normal" at say 14 and 21. Although the FBG level was decreased in all groups, our histopathology report showed a decrease in the number and diameter of the Langerhans island which proportionally to STZ dose (Figure 4). Those damage in Langerhans islands indicates a deterioration in insulin secretion by pancreatic beta cells [15]. However, pancreatic beta cells usually have a regeneration process to the cell damage, so that makes the hyperglycemia condition is less stable [13]. Second STZ induction may be needed to induce chronic diabetes mellitus in a longer antidiabetic experimental timeline.

At this present study, STZ 40 failed to induce diabetic state as their FBG always below 200 mg/dL (Figure 3). It was stated in a review that the administration of low dose STZ could not lead to persistent hyperglycemia [16]. The diameter of pancreatic islets of STZ 40 was also similar to the diameter of pancreatic islets of the control group (Figure 4). At low dose (20-40 mg/kg) STZ can be active at multiple administration regimentation [17].

4. CONCLUSION

STZ given at 40-80 mg/kg dose intravenously has a variable effect. STZ 40 has no diabetic effect while STZ 50 to 80 has actively induced T1DM. However, the mortality as a result from STZ administration was increased proportionally in according to STZ dose. Therefore, considering its effectivity and mortality rate, STZ 50 was the most effective and tolerable dose for inducing T1DM. At this dose, hyperglycemia was persistent for seven days and 10% mortality rate.

5. ACKNOWLEDGMENT

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