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Original article

Four-week induction of type 2 diabetes mellitus in rats by streptozotocin and high-fat diet

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Background: Current rodent models for diabetes mellitus type 2 could be divided into genetic-engineered and environmental-induced. Since genetic-modified rodents are difficult to acquire and costly for Thai researchers, we tried to develop a protocol for food-induced diabetic rats.
Objectives: To develop a convenient and economical type 2 diabetic mellitus rats with a short course (4 - week) of induction for research purpose.
Methods: Eight-week-old male Wistar rats were divided into two groups: control group and streptozotocin with high-fat diet (STZ-HFD) group (n = 4 each). The control group received normal chow (c.p.082 diet with 20.0% energy from fat). For the STZ-HFD group, STZ (30 mg/kg) was intraperitoneal injected at week 0 of experiment with HFD feeding (58.0% energy from fat). Rats were fed *ad libitum*. Blood samples were collected for diabetic evaluation weekly for 4 weeks.
Results: The body weight of the STZ-HFD group was significantly higher than that of the control group. Blood glucose, plasma insulin, homeostasis model assessment of insulin resistance (HOMA-IR) index and HbA1C were increased in the STZ-HFD group. Moreover, STZ-HFD rats suffered with a non-alcoholic fatty liver disease.
Conclusion: Low dose STZ injection (30 mg/kg) with HFD feeding (58.0% energy from fat) could be used to induced type 2 diabetes in rats within 4 weeks.

Keywords: Type 2 diabetes, animal model, high-fat diet, streptozotocin.

Diabetes mellitus (DM) is a chronic disease characterized by elevated blood glucose level due to abnormal secretion or response of insulin, a hormone that plays a vital role in regulation of blood glucose levels. Insulin is synthesized and released by beta cells of islets of Langerhans in the pancreas. Insulin facilitates glucose uptake of adipocytes and muscle cells as well as enhances glucose utilization in several tissues. Therefore, decreased insulin secretion or function leads to hyperglycemia. Furthermore, long-term hyperglycemia causes several complications such as atherosclerosis, fatty liver, diabetic nephropathy, and diabetic retinopathy.

Diabetes mellitus is categorized into several types, however, two major etiologies were identified: insulin deficiency (such as, type 1 DM, mature-onset diabetes in the young or MODY) and insulin resistance (type 2 DM, gestational DM, etc.) In the present study, we mainly discuss about type 1 and type 2 DM.

Type 1 DM is characterized by insulin deficiency due to the destruction of beta cell, which is commonly caused by autoimmune disease, or toxins such as alcohol and drugs (streptozotocin, etc.) Type 1 DM is usually diagnosed in childhood period and required extrinsic insulin injection to maintain normal serum

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glucose. While type 2 DM is the most common type of DM found in global population, accounted for more than 90.0% of all diabetic patients. Type 2 DM is caused by insulin resistance and relative insulin deficiency. Insulin resistance is a condition that normal insulin level could not lower serum glucose to the appropriate level, thus beta cell is stimulated to secrete higher insulin amount to keep normoglycemia or slightly hyperglycemia. Type 2 DM is characterized by hyperinsulinemia with hyperglycemia. This disease is genetic predisposing with multifactorial risk factors, such as obesity, aging, and low physical activity. [1]

Although the typical presentations of type 1 and type 2 DM are similar, but their pathogenesis, complication and treatment are different. For example, ketoacidosis is more common in type 1 DM, while hyperinsulinemia in type 2 DM is associated with obesity, non-alcoholic steatohepatitis, and abnormal urinary acidification. Regarding this, studies in pathogenetic and therapeutic aspects of diabetes must be concerned about this discrepancy. In diabetic research in Thailand, rodents are commonly used for a few decades. Type 1 diabetes is conveniently achieved by drug-induced destruction of beta-cells. Streptozotocin, an antibiotic that induces beta cells death, is widely used to induce type 1 diabetes in animal models. [2] This drug enters the pancreatic beta cell through glucose transporter 2 (GLUT2) and causes alkylation of DNA leading to beta cell death. Thus, insulin production is reduced. [3-5]

For type 2 diabetes model, currently genetically-engineered such as $Lep^{ob/ob}$ mice, $Lepr^{db/db}$ mice were generally used, because these mice were genetically-induced severe obesity, subsequently developed type 2 DM without further procedure is required. The diabetogenesis of these type 2 diabetic mice due to the dysfunction of leptin hormone, leading to severe hyperphagia, morbid obesity and insulin resistance. However, these mice are not available in Thailand and the cost is very high (more than 10,000 baht each or about 10 - 20 times of conventional bred rodents. [6]) Many Thai researchers preferred to use external stimulation to induce type 2 diabetes, such as high fat diet feeding with or without drug induction. High-fat feeding induces weight gain and fat accumulation, obesity and insulin resistance, similar to genetic-modified mice.[7] However, The protocols were vastly different in age and strain of rodents, composition of high fat diet and induction time. In addition, many models preferred to utilize both drug and diet to induce diabetes, which were also various in dosage and route of administration.[8] The precaution of these models were that the longer the induction time, the higher rate of degenerative disease including diabetes itself could be detected in control animals, and high dose of drug used to cause partial beta cell destruction associated with high chance of type 1 DM development in older animals. According this, the age of rodents, induction time and dosage of the drug were the main factors contributed to the accomplishment of the experiment.

Although there were several protocols for type 2 diabetes rodent induction in international reports,[2, 8] but very limited in Thai studies. This study targeted to establish a budget and convenient type 2 diabetes rats using both high-fat diet and optimal dose of streptozotocin (STZ-HFD) to yield the type 2 diabetic rats within 4-week period.

Materials and methods

Animals and treatment

The research has been approved by Chulalongkorn University Animal Care and Use Committee (approved certificate no. 022/2562) and were carried out in accordance with the guidelines for the care and use of laboratory animals. Six-week-old male Wistar (BrlHan:Wist@Jcl) rats were purchased from Nomura Siam International, Co.Ltd, (n = 8, average weight 200 - 240 grams) and acclimatized for two weeks. The rats were housed in two per cage in an environmentally controlled room (12 hr. light/dark cycle, 23 ± 1°C temperature) and had free access to normal chow (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) and water all the time. Then rats were divided into two groups (n = 4 each): control group which was continued normal chow (20.0% energy from fat) and STZ-HFD group which was fed with high fat diet (HFD, 58.0% energy from fat) for 1 week before induction by Streptozotocin. The composition of 082 chow diet and HFD is described in Table 1.

STZ-HFD induced type 2 diabetes

High-fat diet is prepared in-house by mixing of 082 chow, lard and casein to have energy from fat about 58.0 - 60.0%. The diet was formed and stored in 4°C refrigerator until use, but not over than 10 days. For the STZ-HFD group, after 1-week HFD-fed, rats were starved for 8 hours before intraperitoneal STZ injection at week-0 of experiment. Fresh STZ solution was prepared by dissolving in 0.1M citrate buffer and injected at a dose of 30 mg/kg without wasting time (~2 min). Experimental design is shown in Figure 1.

Table 1. The composition of normal chow and high fat diet.

Composition (%energy)	Normal chow diet (c.p.082) (%)	High fat diet (HFD) (%)
Carbohydrate	56.6	26.9
Protein	23.8	14.9
Fat	19.6	58.2

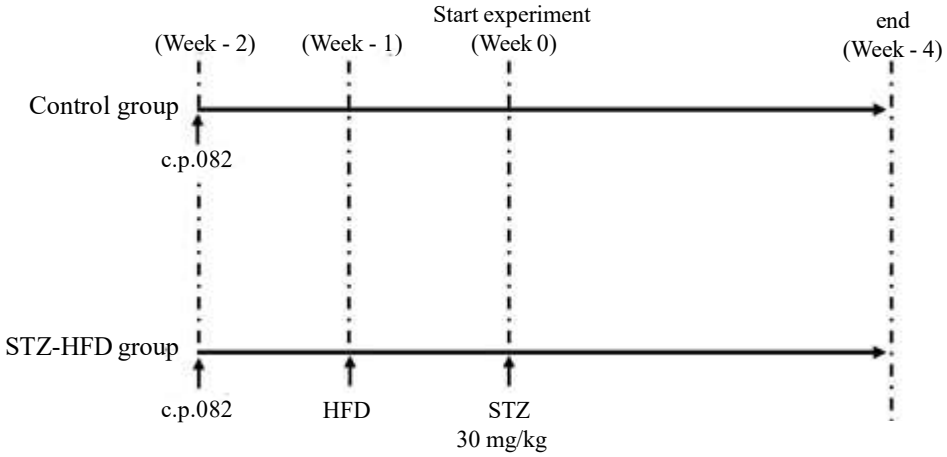

Figure 1. Experimental design.

Sample collection

The body weight and food intake of each rat were recorded once a week. The rats were fasted for 12 hr before blood collection. Blood samples were collected from the tail vein each week in different tubes for whole blood (Ethylene diamine tetraacetic acid (EDTA) tube) and plasma (heparinized tube). Plasma was separated by centrifugation. Urine samples were collected in metabolic cages. In addition, liver and kidney tissues were harvested from a STZ-HFD rat that accidentally perished after the end of the experiment (week 5).

Biochemical analyses

Blood sugar was measured by glucose strip test (EasyMax®, EnMax, Canada). Blood and urine samples were sent to Laboratory Medicine, King Chulalongkorn Memorial Hospital for enzymatic calorimetric measurement by an automated clinical chemistry analyzer. Insulin was measured by Insulin (INS) ELISA Kit (Cat.No.MBS2602037, Mybiosource Inc, San Diego, USA). Then The homeostatic model assessment for insulin resistance index (HOMA-IR) was calculated according to Roza NA, *et al*. [9]

Histology

Liver and kidney tissues were obtained from a rat whom unintentionally succumbed during the anesthesia 1 week after the end of the experiment. Liver and kidneys were fixed in 10.0% buffered formalin and embedded in paraffin. The sections of these tissues were stained with hematoxylin and eosin for light microscopy to evaluate liver and kidney pathology related to diabetes mellitus.

Statistical analysis

The results are expressed in terms of the mean ± standard deviation (SD) of the mean. A Student's *t* - test was used to evaluate the difference between groups for independent samples. The differences were considered significant at $P < 0.05$. GraphPad Prism (GraphPad Software, USA) version 7 was used for statistical analysis in the present study.

Results

Characteristics of STZ-HFD rats

STZ-HFD rats gained more weight than the control rats, with significantly higher weight at week 1 and later, although no difference in energy intake was observed during this period. There was no correlation between weight gain and food intake each week (Pearson correlation $r = - 0.005$, $P = 0.964$) These results suggested that STZ-HFD induced body weight gain which was independent to total energy consumption (Figure 2).

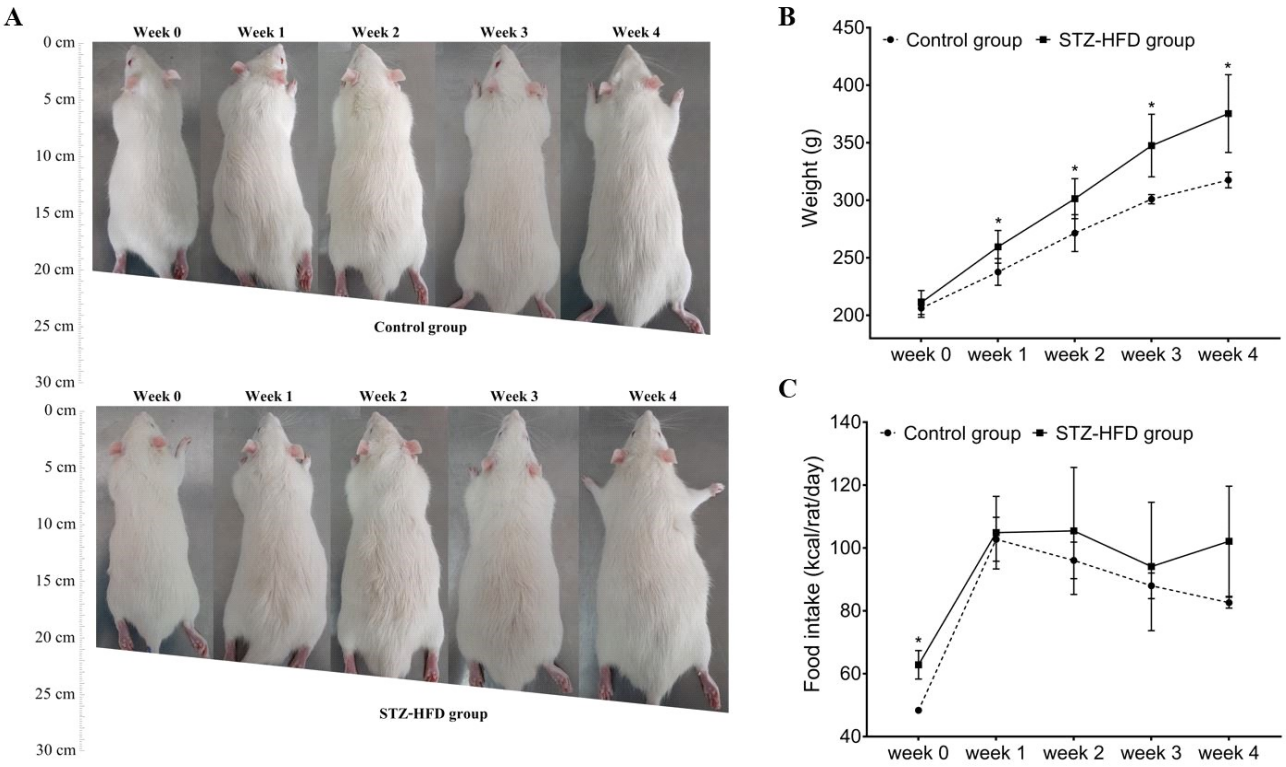

Figure 2. **(A)** Morphology and size of experimental rats from each group; **(B)** The average body weight of each group (P = 0.459, 0.042, 0.033, 0.017, and 0.017, respectively); **(C)** Food intake (P = 0.016, 0.571, 0.786, 0.786, and 0.057, respectively). Data are shown as means ± SD (n = 4). *P < 0.05 versus control group.

STZ-HFD rats developed insulin resistance

As shown in Figure 3, blood sugar, insulin and HOMA-IR levels trended to be elevated in STZ-HFD group since week 0. In week 3, the STZ-HFD group had significantly higher plasma insulin and HOMA-IR than the control group. We implied that STZ-HFD protocol successfully induced type 2 DM in rats at week 3 of the experiment. HbA1C was not statistically different between these group because its long half-life, although STZ-HFD rats appeared to have slightly elevated HbA1c level compared with the control.

Effects of STZ-HFD on renal and liver functions

Renal function

Urine creatinine, urine microalbumin, microalbumin / creatinine ratio and plasma creatinine levels are shown as Figure 4. Although no statistical significance was obtained, the STZ-HFD trended to have a slightly elevated plasma creatinine compared with the control group. Renal pathology of a single STZ-HFD rat revealed a large glomerulus size that was compatible with the early pathological change detected in diabetic nephropathy.

Liver function

Liver histology of a single STZ-HFD rat demonstrated fat droplets accumulated in hepatocytes accompanied with tortuous bile canaliculi, suggested subacute liver injury (Figure 5). This may indicate that STZ-HFD induction induce liver injury and fatty change in rats. However, additional number of animals with longer period of experiment are required for validation of these findings.

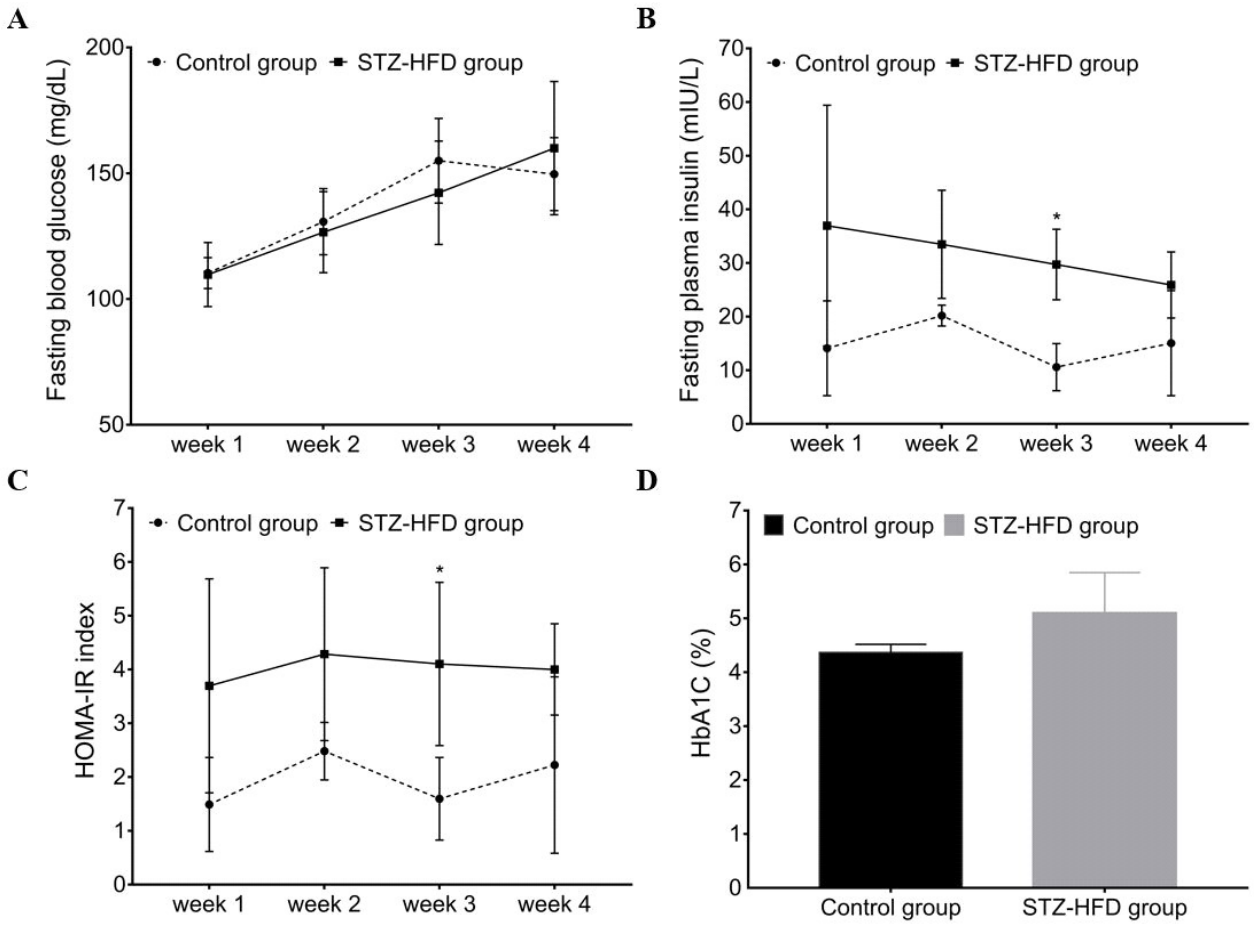

Figure 3. (A) Fasting blood glucose (P = 0.945, 0.690, 0.229, and 0.573, respectively); **(B)** Fasting plasma insulin (P = 0.150, 0.057, 0.008, and 0.268, respectively); **(C)** HOMA-IR index (P = 0.133, 0.107, 0.049, and 0.265, respectively); **(D)** HbA1C at week 4 (P = 0.265). Data are shown as means ± SD (n = 4). *P < 0.05 versus control group.

Discussion

Since the pathogenesis and consequences of type 1 and type 2 DM are distinctive, all research required a clear definition and validation for the classification of diabetes. The most distinct characteristic between type 1 and type 2 DM was the level of serum insulin, which was very low in type 1 and typically high in type 2. In general, the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) which is the multiplied factor between serum glucose and insulin has been extensively used to determine the type of DM. The low HOMA-IR with high serum glucose indicated the insulin deficiency, while high HOMA-IR with high glucose represented insulin resistance. [10-12] However, a universal cut-off point has not been established because the variation between animal species. Therefore, type 2 DM was commonly identified as a high HOMA-IR with high blood glucose. Regarding this, the present study also used HOMA-IR to announce insulin resistance.

According to the previous literatures in both mice and rats revealed the preferable age of rats was between 5 - 12 weeks, average weight of 140 - 200 g, with HFD-induction phase was from 2 - 8 weeks, and doses of STZ injection range between 25 - 50 mg/kg, intravenously or intraperitoneally. [13, 14] A few studies administered STZ twice during their experiments. The results showed that using low dose STZ (20 - 30 mg/kg i.p.) was commonly used with a long period of HFD induction (4 - 8 weeks). While high dose STZ (35 - 50 mg/kg i.p.) administration, especially intravenous route, frequently caused hypoinsulinemia in the late stage. In addition, not all protocol could induce abnormal plasma lipid profiles or hepatic enzymes. [15, 16]

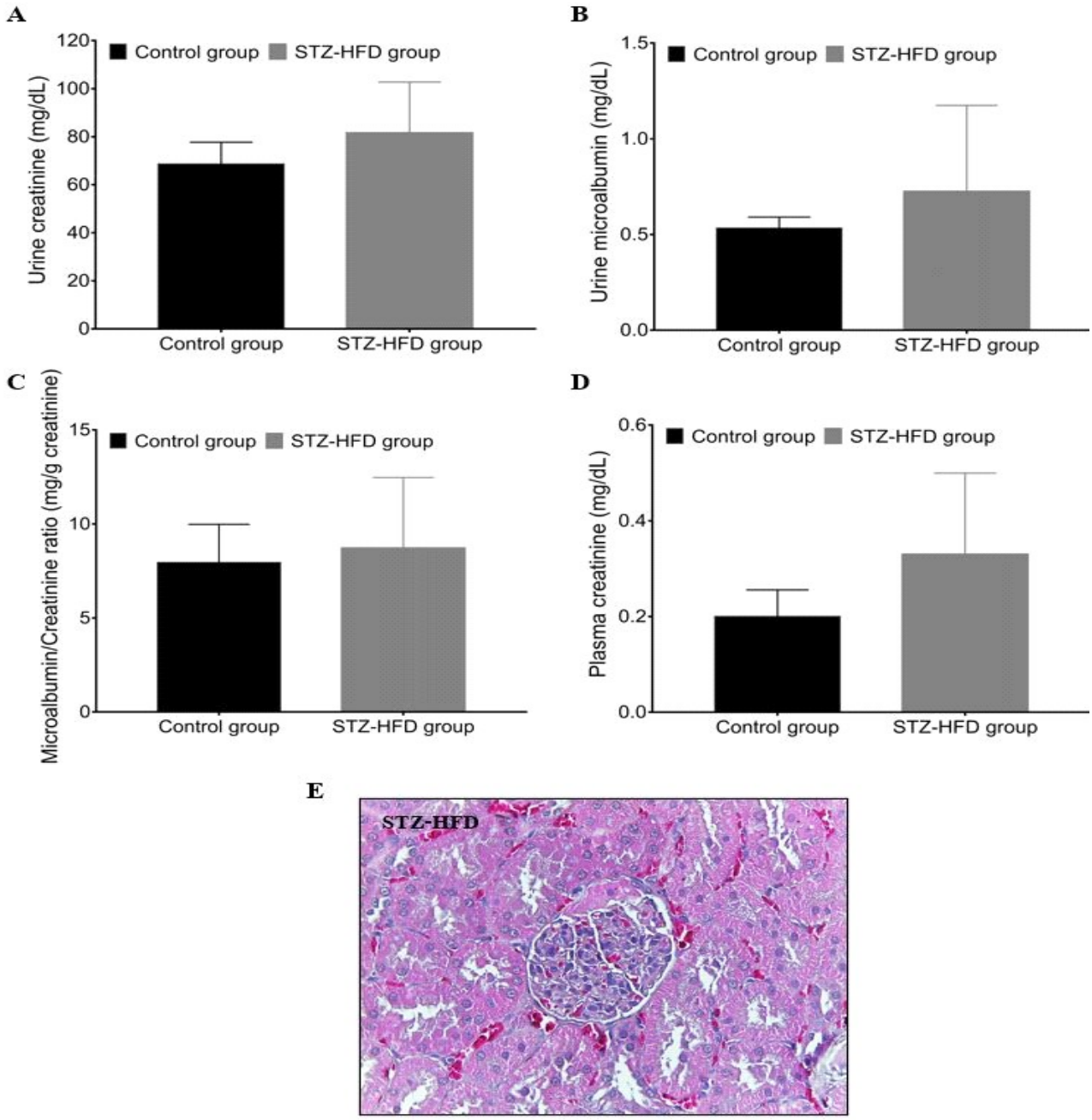

Figure 4. Renal function: Creatinine and albumin levels at week 4. **(A)** Urine creatinine ($P = 0.374$); **(B)** Urine microalbumin ($P = 0.506$); **(C)** Microalbumin/creatinine ratio ($P = 0.766$, **D)** Plasma creatinine ($P = 0.467$). Data are shown as means ± SD (n = 4**); (E)** Kidney sections were stained with H&E and representative images (magnification: 40×) of STZ-HFD group are shown.

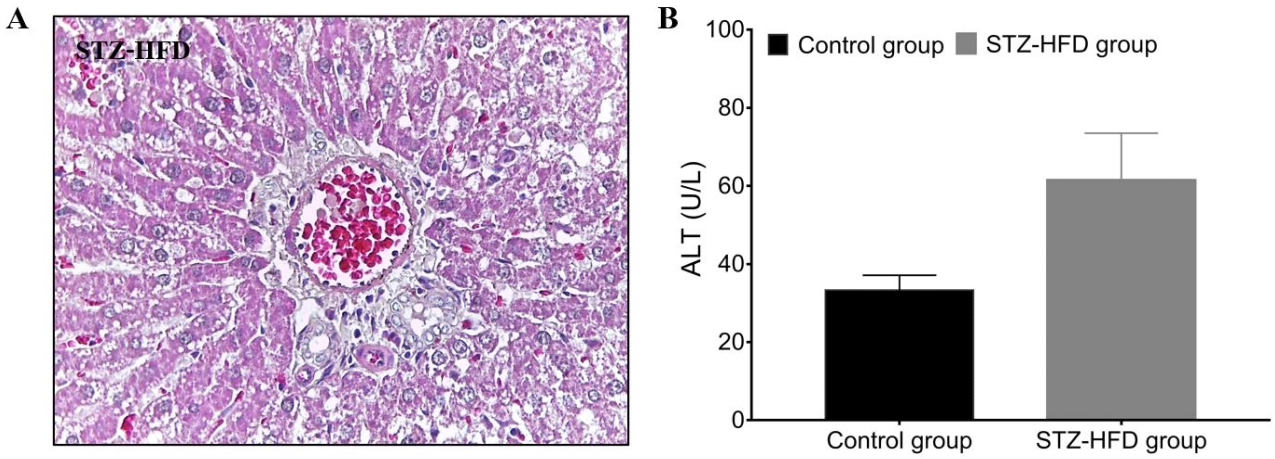

Figure 5. (A) Liver sections were stained with H&E and representative images (magnification: 40×) of STZ-HFD group are shown; **(B)** Alanine aminotransferase (ALT) level (P = 0.168). Data are shown as means ± SD (n = 4).

The aim of this study was to establish a protocol to produce type 2 DM rats with reasonable budget and uncomplicated. The induction period was likewise considered to shorten the caring time and cost. Our study adapted the former protocols, aimed to produce type 2 diabetic rats with hyperinsulinemia in a short period of experiment. Initially, we designed our in-house HFD with targeted fat energy more than 50.0% of total calorie, mixing with standard rat chow. Pre-treated HFD feeding was a vital step to activate beta cell proliferation and effective response to low - medium dose of STZ. We decided to feed the rats with HFD for 1 week since there was an evidence that 1-week HFD introduction was adequate to induce the elevation of plasma insulin. A 30 mg/kg of STZ for intraperitoneal injection single dose was used in our study to prevent extensive destruction of beta cell. STZ preparation was another critical step since STZ was easily degenerated after dissolving in citrate buffer. We recommended the instant preparation and injection of STZ solution within 3 minutes to minimize the ineffectiveness of the drug.

Even though our protocol could validate the type 2 DM disease after 3 weeks of the experiment, the drawback of our short-induction time, low dose STZ-HFD was that we could not obtain very high blood sugar (> 200 mg/dL) in the first few weeks after STZ injection as previous reports. [2, 7, 8] Insulin measurement, which was not a routine laboratory test, was used in our study to confirm high HOMA-IR level. In case that the researcher does not intent to measure serum insulin, we suggested a protocol of 2-week HFD feeding and high dose STZ (40 mg/kg) intraperitoneal injection, which high blood sugar [13 - 15] could be obtained in the first 2 weeks after STZ administration. [17 - 19] However, researchers should take precaution of pervasive destruction of beta cell and late stage hypoinsulinemia development.

Regarding to HFD, since the commercial HFD is expensive (more than 1,000 baht per kilogram), we develop an in-house HFD recipe using normal chow, lard, and casein. Lard was a regular lipid source used in standard HFD recipe due to the high content of saturated fatty acid and cholesterol. Basically, HFD contains energy from fat over than 50.0% (preferable 55.0 – 70.0%); however, our previous pilot study found that increasing the fat content to 65.0% of energy by lard had some disadvantages in the forming and rancidity of the diet, as well as the perishability. In addition, the content of protein lower than 13.0% of total energy might relate to the failure-to-thrive and poor weight gain of the rats. Thus, we recommend the composition of fat about 55.0 - 65.0%, protein 13.5 - 15.0% and carbohydrate about 20.0 - 30.0% of total energy. The HFD should be stored in 4°C refrigerator for up to 7 - 10 days.

In summary, we proposed a rat model using STZ-HFD induction for type 2 diabetic animal study. This technique was convenient, economical, and short duration of induction. The downside of our model was that the blood sugar of rats was not high at day 7 - 10 after STZ injection, which could not be used to monitor the diabetic progression. We considered that this was due to the limited beta cell destruction from low dose STZ causing compensatory hyperinsulinemia and normoglycemia. We suggested to prolong the pre-treatment HFD period or slightly increased the dosage of STZ to 35 - 40 mg/kg if the researcher requires the early phase of hyperglycemia.

Conclusion

This study demonstrates that a combination of low dose STZ injection (30 mg/kg) with HFD feeding (58.0% energy from fat) can induce a type 2 diabetes mellitus in rat within 4 weeks.

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Conflict of interest

We declare, hereby, that we have no conflict of interest.

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