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Optimization of Mice Model of Painful Diabetic Neuropathy (PDN)

Fajrin FA[1,2*], Nurrochmad A[3], Nugroho AE[3], Susilowati R[4]
[1]Postgraduate Programme, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta [2]Department of Clinical and Community Pharmacy, Faculty of Pharmacy, Jember University, Jember, [3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia, [4]Department of Histology and Cell Biology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

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ABSTRACT

Painful diabetic neuropathy (PDN) is a complication of long-term diabetes mellitus (DM) characterized by hyperalgesia and allodynia. In streptozotocin (STZ)-induced diabetic mice, higher dose of STZ and lengthened hyperglycemic condition results in better model of PDN. However, higher dose of STZ tend to induce mortality. The aim of the study was to evaluate the doses of STZ that caused PDN with less mortality rate and the timing of pain behavior development in mice model of PDN. BALB/c mice were divided into non-diabetic and STZ-induced diabetic group. The doses of STZ were started from 180 mg/kg BW i.p. Serum glucose levels were measured 7 days (one week) after induction. Mice with glucose levels ≥ 200 mg/dL were considered as diabetic. Pain behaviour was determined by four method i.e. hot plate, tail flick, von Frey filament and Randall Selitto tests, measured on week-0 (baseline), 1, 2, 3, 4 and 5. Data were presented as mean ± SEM. The mean differences between weeks were evaluated by One-Way ANOVA and the mean differences between two groups by independent t-test. STZ doses 180, 150 and 120 mg/kg BW caused 100% death and STZ 90 mg/kg BW failed to induce diabetic condition. STZ 110 mg/kg BW resulted in 0% mortality while it induced diabetes in 100% of the mice. Latency time toward thermal stimulus decreased to 5.8 ± 0.4 sec at 1st week after the mice become diabetes ($p<0.05$) and it was continued to decrease until 4th week. The same result was also showed in tail flick and Randal Selitto tests. The pain sensitivity determined by von Frey filament decreased to 1.37 ± 0.12 g at 1st week ($p<0.05$) and continued to decrease until 5th week. In conclusion, optimum dose of STZ to induce PDN was 110 mg/kg BW. Pain behaviour of diabetic group was observed at 1st week after diabetes and continued until 5th week.

ABSTRAK

Nyeri neuropati diabetes (NND) adalah salah satu komplikasi diabetes melitus (DM) jangka panjang yang ditandai dengan hyperalgesia dan alodinia. Pada mencit diabetes yang diinduksi streptozotosin (STZ), dosis tinggi STZ dan kondisi hiperglikemia yang berkepanjangan menghasilkan model NND yang lebih baik. Akan tetapi dosis tinggi STZ cenderung menyebabkan kematian. Penelitian ini bertujuan untuk mengkaji dosis STZ yang menyebabkan NND dengan tingkat kematian rendah dan waktu timbulnya rasa nyeri pada model mencit NND. Mencit BALB/c dibagi menjadi kelompok tikus tidak DM dan tikus DM akibat induksi STZ. Dosis STZ dimulai dengan 180 mg/kg BB yang diberikan secara intraperitonial. Kadar gula darah diukur 7 hari (satu minggu) setelah induksi. Mencit dengan kadar gula darah ≥ 200 mg/dL ditetapkan sebagai mencit DM. Perilaku nyeri

Corresponding author: fiezz_15@yahoo.co.id

ditetapkan menggunakan 4 metode yaitu metode *hot plate, tail flick, von Frey filament* dan *Randall Selitto* yang diukur pada minggu ke 0 (data awal), 1, 2, 3, 4 dan 5. Data disajikan sebagai rerata ± SEM. Perbedaan rerata antara perilaku nyeri setiap minggu dievaluasi dengan ANOVA satu jalan dan perbedaan antara 2 kelompok dievaluasi dengan uji t. Dosis STZ 180, 150 dan 120 mg/kg BB menyebabkan 100% kematian dan dosis STZ 90 mg/kg BW gagal menginduksi diabetes. Dosis STZ 110 mg/kgBB menghasil 0% kematian dan menginduksi diabetes 100% mencit. Waktu latensi terhadap stimulus panas menurun 5,8 ± 0,4 detik pada minggu pertama setelah mencit diabetes ($p<0,05$) dan berlanjut menurun hingga minggu ke 4. Hasil yang sama juga ditunjukkan pada uji dengan *tail fick* dan *Randal Selitto*. Sensitivitas nyeri yang diukur dengan *von Frey filament* menurun menjadi 1,37 ± 0,12 g pada minggu pertama ($p<0,05$) dan berlanjut menurun hingga minggu ke 5. Dosis optimum STZ untuk menginduksi NND adalah 110 mg/kg BB. Perilaku nyeri kelompok diabetes teramati pada minggu pertama setelah diabetes dan berlanjut hingga minggu ke 5.

Keywords: Painful diabetic neuropathy, hot plate, tail flick test, von Frey filament, Randall Selitto

INTRODUCTION

Diabetes mellitus (DM) is the leading cause of degenerative diseases with the highest mortality rate.[1] Diabetes mellitus is caused by disturbance in insulin secretion, insulin action, or both that characterized by hyperglycemia.[2] Hyperglycemia and reduce cellular source of energy lead to many complication of diabetes. Painful Diabetic Neuropathy (PDN) is the most common complication of diabetes, occurs in 25-50% diabetic patients, as a result of abnormal pain signal system due to peripheral nerves damage.[3] Patient with PDN usually complains chronic pain with hyperalgesia and allodynia, which interfere their daily activities.[4] Therefore, the prevention of PDN development in diabetic patients are crucial in maintaining the patient's quality of life.[3,4]

For the purpose of drug development, animal model of PDN is needed. One of the most common animal model is Streptozotocin (STZ)-induced diabetic mice. From the literature, the doses of STZ used for diabetic induction in mice are varied.[5,6,7,8] Higher dose may induce lethal hyperglycemia that may prevent complete measurement and analysis. An appropriate dose of STZ is important, balancing the need to induce hyperglycemia and PDN, while at the same time reducing animal mortality rate.

PDN state occurs as early as 2-4 weeks after establishment of hyperglicemia. Prolonged hyperglicemia at more than six weeks caused hypoalgesia, related to loss of nerves fibers.[9] Several studies reported more than four weeks of hyperglicemia is needed to develop PDN in mice.[7,10,11] However, prolonged hyperglycemia increases diabetic mice mortality rate. With many animals died, the purpose of investigation won't be fulfilled. Therefore, it is important to evaluate the doses of STZ that induce PDN with less mortality rate in mice. The timing of pain behavior development in mice model of PDN also needs to be investigated.

MATERIALS AND METHODS

Materials

All the reagents and chemical used for this research were analytical grade.

Streptozotocin was purchased from Biolab (USA), Biochemical diagnostic such as glucose oxidase – glucose peroxidase (GOD-POD) kit was purchased from DyaSis (Germany) and citrate buffer pH 4.5 was from Sigma (Singapore). The pain behavior was determined using hot plate (UgoBasile, Italy), tail flick test (Columbus Instrument, USA), von Frey filament (Aesthesio, USA) and Randall Selitto (UgoBasile, Italy).

Preparation of STZ

Streptozotocin 1.1% was dissolved in freshly prepared ice cold citrate buffer (pH 4.5) before used.

Preparation of experimental animals

Fourty-three of male BALB/c strain mice (25-35 g, 8-10 w) were obtained from Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. Mice were placed in clean cages and maintained at temperature 25±1 °C with 12 hours light/dark cycle. Mice had free access on food and water *ad libitum*. Ethical clearance was obtained from the Medical and Health Research Ethic Committee, Faculty of Medicine, Universitas Gadjah Mada-Dr. Sardjito General Hospital, Yogyakarta number Ref. KE/FK/559/EC/2016.

Experimental protocol

Mice were fasted overnight for 12 hours, before the experiment. Each group of mice were given a single-high-dose of STZ (180, 150, 120, 110 or 90 mg/kg BW) i.p. The fasting-blood glucose levels were checked 7 days after STZ induction using GOD-POD kit. Mice with fasting-blood glucose levels ≥200 mg/dL were considered diabetes. Measurement of blood glucose levels were conducted once every week until week 5.

Pain behavioral assays

Pain behavior assays were measured before STZ induction and at week 1, 2, 3, 4 and 5 after diabetes. All experiments were taken in three replications.

Hot plate test

Each mouse was placed individually on a hot plate that adjusted at 50±0.5°C. The latency time toward thermal stimulus was evaluated when the mice showed pain responses such as licking, jumping, tapping, rearing, flattering and frizzing (whatever which came first). The cut off time was 30 sec in order to avoid damage of the nerves.[12,13]

Tail flick test

The tail of each mouse was exposed to nichrome-radiant heat. The intensity of the radiant heat was adjusted to give a basal latency of 6-8 sec in both diabetic and non-diabetic groups. Tail flick latency was time interval that taken by mice to flick its tail after the exposure of radiant heat. The maximum cut-off time was 15 sec to prevent tissue damage.[14,15]

von Frey filament test

The sensitivity of mechanical stimulus was measured by von Frey filament. Mice were placed in an individual glass-caged that completed by wire-mesh-bottom. A von Frey filament was applied 10 times for 5 sec to the foot pads of the hind paw and the number of positive response (such as jumping, scratching or scraping) was recorded. The increasing of sensitivity was interpreted as more than 5 positive responses from 10 times test.[12,13]

Randall Selitto test

Randall Selitto test applied pressure to the dorsal surface of the hind paws via conical

probe with 250 g cut-off. The pressure was increased until the mouse withdrew the paw, squeaked or struggled.[12,13]

Statistical analysis

Data was statistically analyzed using SPSS version 20. All values were described as mean ± SEM. The mean differences of pain behaviour measurement between weeks were compared using One-Way ANOVA and between two groups (diabetic and non-diabetic) using independent t-test. The significance level were set at 95%.

RESULTS

The result of the diabetic induction and subsequent survival of the mice after administration different dose of STZ was presented in TABLE 1. At day 7 after STZ administration, doses 180, 150, 120 and 110 mg/kg BW resulted in 100% diabetic induction. Lower STZ dose at 90 mg/kg BW failed to induce diabetic condition in any mice. Although STZ dose 180, 150 and 120 mg/kg BW were successful in inducing diabetes, the mice survival was a problem. Most of the mice were died before week 3 and all of the mice from 180 and 120 mg/kg BW groups were died at week 7. The mean blood glucose level of the died mice at week 1 were 378.77±74.45 mg/dL, higher than the mean of blood glucose level of the surviving mice (281.64±66.84 mg/dL). However, it was not significantly different ($p > 0.05$).

TABLE 1. The percentage of diabetic mice and mortality in various doses of STZ induction until 5 weeks

Dose (mg/kg BW)	n	DM+		Number of mortality at week						Total mortality	
		n	%	0	1	2	3	4	5	n	%
180*	6	3	50	3	1	0	0	1	0	5	83.30
150	7	7	100	0	2	1	0	0	0	3	42.86
120	4	4	100	0	1	1	1	0	0	3	75
110	8	8	100	0	0	0	0	0	0	0	0
90	10	0	0	0	0	0	0	0	0	0	0

* 3 mice died before the first examination of blood glucose level

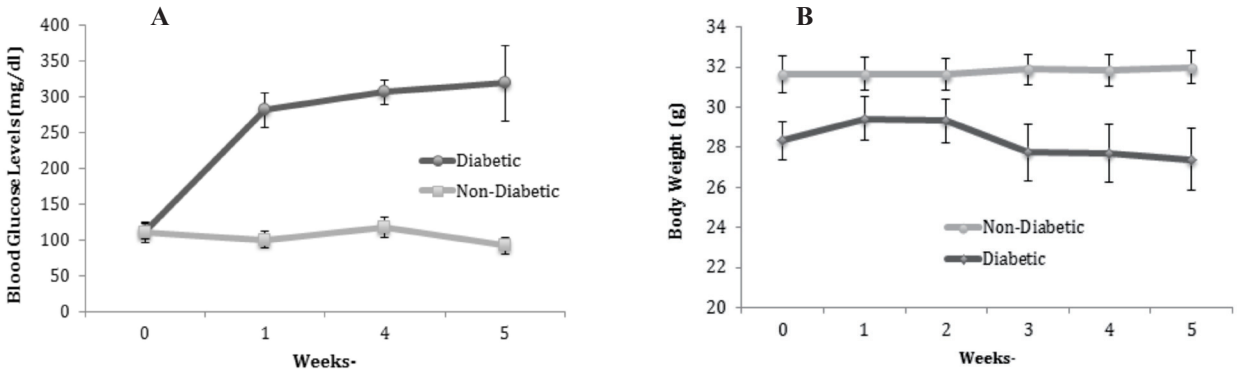

FIGURE 1. A. Blood glucose levels between diabetic and non-diabetic groups at weeks-0 (baseline), 1, 4 and 5; # indicated significant differences compared to non-diabetic group. B. The body weight of diabetic and non-diabetic groups at weeks-0 (baseline), 1, 2, 3, 4 and 5

TABLE 2. Pain behaviour assessment in non-diabetic and diabetic groups after STZ induction dose 110 mg/kg BW.

Test	Groups (n=8)	Week-					
		0	1	2	3	4	5
Hot plate (sec±SEM)	Non-diabetic	10.5± 0.2^{a}	10.6± 0.4$^{a\#}$	11.6± 0.4$^{a\#}$	10.3± 0.2$^{a\#}$	11.0± 0.2$^{a\#}$	10.0± 0.2$^{a\#}$
	Diabetic	10.3± 0.3^{a}	5.8± 0.4$^{b\#\#}$	4.9± 0.3$^{b\#\#}$	3.9± 0.3$^{b\#\#}$	3.3± 0.3$^{b\#\#}$	4.6± 0.4$^{b\#\#}$
Tail flick (sec±SEM)	Non-diabetic	8.11± 0.12^{a}	7.88± 0.22$^{a\#}$	8.02± 0.07$^{a\#}$	8.19± 0.18$^{a\#}$	8.36± 0.24$^{a\#}$	7.97± 0.25$^{a\#}$
	Diabetic	7.95± 0.18^{a}	5.04± 0.25$^{b\#\#}$	3.68± 0.24$^{b\#\#}$	2.66± 0.23$^{b\#\#}$	2.04± 0.08$^{b\#\#}$	3.06± 0.14$^{b\#\#}$
Randall selitto (g±SEM)	Non-diabetic	94.16± 4.21^{a}	88.96± 2.25$^{a\#}$	95.63± 0.83$^{a\#}$	91.88± 1.62$^{a\#}$	90.21± 2.62$^{a\#}$	92.5± 2.52$^{a\#}$
	Diabetic	97.29± 3.55^{a}	53.75± 4.66$^{b\#\#}$	24.58± 2.50$^{b\#\#}$	15.83± 2.29$^{b\#\#}$	11.04± 1.41$^{b\#\#}$	17.08± 1.43$^{b\#\#}$
von Frey filament (g±SEM)	Non-diabetic	8.79± 0.48^{a}	8.58± 0.29$^{a\#}$	8.91± 0.31$^{a\#}$	8.71± 0.52$^{a\#}$	8.50± 0.33$^{a\#}$	9.08± 0.72$^{a\#}$
	Diabetic	9.33± 0.62^{a}	1.37± 0.12$^{b\#\#}$	0.67± 0.05$^{b\#\#}$	0.36± 0.04$^{b\#\#}$	0.10± 0.01$^{b\#\#}$	0.10± 0.02$^{b\#\#}$

Data analysis between groups using independent t test.

a indicated significant differences ($p<0.05$) of pain behaviour between diabetic and non-diabetic groups of each day of measurement.

\# indicated significant differences ($p<0.05$) compared to baseline (week-0).

Pain behaviour in mice were evaluated using four different methods. Hyperalgesia was determined by hot plate, tail flick and Randall Selitto tests Allodynia was determined by von Frey filament. The pain behaviour of diabetic mice and control mice were described in TABLE 2. Diabetic mice shortened latency time toward thermal stimulus using hot plate from 10.3 sec at baseline to 5.8 sec in the first week after STZ induction and lasted up fourth week with consecutive latency time 4.9, 3.9, and 3.3 sec. This result was significantly different with non-diabetic group, which maintained the latency time arround 10 sec ($p<0.05$). At week-5, latency time toward thermal stimulus become 4.6 sec, longer than previous week but still significantly lower ($p<0.05$) than non-diabetic group and not returned yet to baseline.

The pain behavior evaluation using other tests such as tail flick and Randall Selitto gave similar results. Tail latency time using tail flick test shortened from 7.95 s to 5.04 at week-1 after STZ induction. This condition lasted up 4th week. Decreasing of withdrawal load threshold using Randall Selitto test was observed from 97.29 g at baseline to 53.75 g at week-1 after STZ induction and had been reducing until week-4. At the end of the study (week-5), the pain responses were less severe than the previous week.

Measurement of pain behaviour using von Frey filament showed slightly different outcome. The tactile sensitivity towards mechanical stimulus was continuously decreased until 5th week from 9.33±0.62 g to 1.37 ± 0.12 g, 0.67 ± 0.05 g, 0.36 ± 0.04 g, 0.10 ± 0.01 g and 0.10 ± 0.02 g and significantly different with non-diabetic group ($p<0.05$).

DISCUSSION

In this study, the dose of STZ 110 mg/kg BW was found to be the most effective in inducing diabetic state while preventing mortality of the mice. The STZ dose was lower than previous report i.e. 150 mg/kg, 180 mg/kg and 200 mg/kg.[5,6,8] Administration of higher dose of STZ induced higher level of blood glucose more than 450 mg/dl. In our study, mouse with blood glucose levels up to 450 mg/dl was more vulnerable to die. When we used STZ dose 110 mg/kg BW, the blood glucose level was found in the range of 231.89 until 413.96 mg/dL (mean 281.64±66.84 mg/dL). Our result was slightly similar to the report by Hayashi et al. (2006).[16] Their study showed that dose 100 and 125 mg/kg BW of STZ induced non-insulin dependent diabetes in BALB/c mice model and showed a slowly progressive diabetic state until 12 weeks.[16] However, our result showed significantly increasing of high glucose level in the beginning of the study. The glucose levels continued to increase until week 5, more than the threshold (200 mg/dL).

Animal gender and strain may influence the response after STZ induction. Male pancreatic islet ß-cells are more prone than female cells to STZ-induced toxicity, that is why male rodent is more popular for diabetic study. Different strain of animals also display different sensitivity to STZ. Based on Cardinal et al (1998),[17] the action of STZ in ß-cells varies in different strain due to differences in the uptake or metabolism of the drug, the rate of DNA repair, the activity of Poly (ADP-ribose) polymerase (PARP) or damage to Nicotinamide adenine dinucleotide (NAD^+)-generating mechanism.[17] CD-1 and C57BL/6 mice were reliably sensitive to STZ, while, there was a report that BALB/c mice are resistant to multiple low doses of STZ-

induced diabetes mellitus.[18] In this study, we showed that BALB/c mice could be used as STZ-induce diabetic animal model.

At week-0 (baseline), there were no pain behavior found in all groups. One week after STZ induction, the latency time toward thermal stimulus using hot plate and tail flick test declined in the hyperglycemia group compared to the baseline and the control group, supporting previous studies.[13,14,19] According to Saikh and Somani (2010),[9] mechanical hyperalgesia and tactile allodynia occur within 1-8 weeks after STZ induction.[9] In this study, both of mechanical hyperalgesia tested by Randall Selitto and tactile allodynia tested by von Frey filament were developed one week after STZ induction. This result was faster compared to other report that showed the occurance of mechanical hyperalgesia and tactile allodynia in mice model of PDN varies within 1-8 weeks after STZ induction.[15,19,23-26] The report from Sugimoto et al. (2013)[15] showed that mechanical hyperalgesia was started to be observed at 6th week after diabetic state and this condition continued until 10-11 weeks.[15]

Based on our result, hyperalgesia and allodynia in diabetic condition occured at the same time (Figure 2). Thermal stimulus-induced hyperalgesia started in the early phases of diabetic condition until week 4. There was a trend of reducing hyperalgesia in week 5, suggested failure to report the stimulus to the central nervous system due to severe nerve fibers damage. Mechanical stimulus-induced allodynia also occurred at the early phases of diabetic condition and continues until week 5. Hyperalgesia usually implicates the damage of C and A∂ nerve fiber, but in allodynia, Aß nerve fibers are also involved.[21] The smaller diameter and non-myelinated nature of C-fibers require higher energy consumption, hence more prone to the diabetic condition.[21] Furthermore, C and A∂ nerve fibers contain a lot of TRPV1 molecules that are responsible to early hyperalgesia and advanced hypoalgesia.[22]

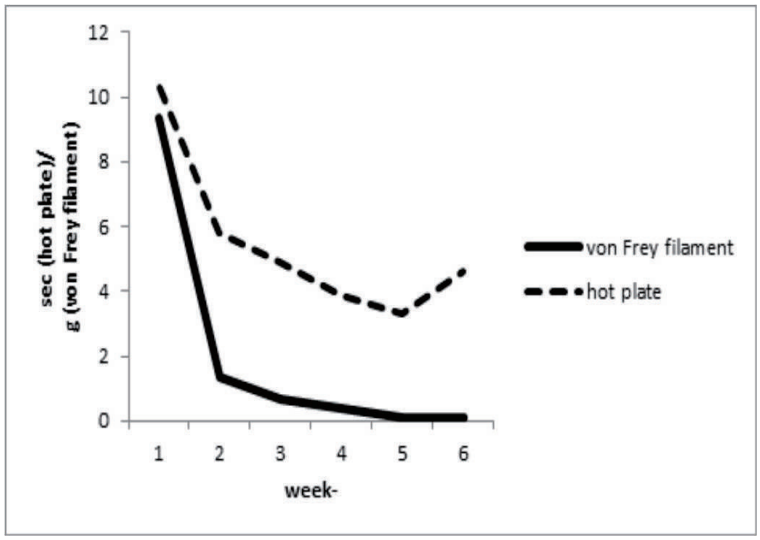

FIGURE 2. Development of hyperalgesia, tested with hot plate in secon units (dotted line) and B. allodynia, tested by von Frey filaments in g units (straight line) in BALB/c Mice after a singe-dose 110 mg/kgBW of STZ-induced PDN.

CONCLUSION

Optimum dose of STZ to induce PDN is 110 mg/kg BW. Pain behaviour of diabetic group is reached at 1st week after diabetic and continued until 5th week.

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