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The effect of warfarin on tail bleeding time of male wistar rats model type 2 diabetes mellitus

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Abstract---Diabetes mellitus (DM) is a chronic metabolic disease that characterized by elevated blood glucose levels. Warfarin is an anticoagulant drug that is often used to treat and prevent microvascular and macrovascular complications. This study wanted to find out the effect of warfarin on the tail bleeding time of male Wistar rats with type 2 diabetes mellitus model. This study used quasi-experimental with posttest research design with an in vivo control group with male Wistar rats model DMT2 induced by nicotinamide and streptozotocin, the experimental group was given warfarin at a dose of 2 mg/Kg of body weight. The number of samples as many as 24 tails. The samples were divided into 4 groups, namely DM + Warfarin, DM + Aquades, Normal + Warfarin, Normal + Aquades. Data were analyzed by Kruskal-Wallis and Mann-Whitney tests. The results of the average duration of tail bleeding time for each group is DM + Warfarin for 1200 ± 0.00 seconds, DM + Aquades for 913 ± 194.84 seconds, Normal group + Warfarin for 1173.3 ± 65.31 seconds, Normal + Aquades for 619.3 ± 319.64 seconds. The Kruskal-Wallis test showed that there was an effect of warfarin on tail bleeding time. The Mann-Whitney test showed a significant difference between DM + Warfarin and DM + Aquades but the difference was not significant with Normal + Warfarin. There is no significant difference between DM + Aquades with Normal + Warfarin and Normal + Aquades. There is a significant difference between Normal + Warfarin and Normal + Aquades. In this study, it can be concluded that there is an effect of

giving warfarin on the tail bleeding time of male Wistar rats with type 2 diabetes mellitus model.

Keywords--diabetes mellitus, rattus norvegicus, warfarin, tail bleeding time.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by elevated blood glucose (or blood sugar) levels, which from time to time continue to increase and cause serious damage to the heart, blood vessels, eyes, kidneys, and nerves. The most common type of Diabetes Mellitus is type 2 Diabetes Mellitus (T2DM), which generally occurs in adults, occurs when the body becomes resistant to insulin or does not make enough insulin. In the last three decades the prevalence of T2DM has increased from 4.5% in 1990 to 7% in 2019 (IDF, 2019). The results of Basic Health Research 2018 show that the prevalence of DM in Indonesia based on doctor's diagnosis is 2%. The prevalence of T2DM cases is 85-95% of all DM cases (Basic Of Health Research, Indonesian Ministry Of Health, 2018).

Uncontrolled diabetes mellitus will cause acute and chronic complications. Chronic complications in DM patients are generally macrovascular and microvascular complications. The prevalence of macrovascular complications is 34% while the prevalence of microvascular complications is 10.4-30.1% (Zhaolan L., Chaowei F. & X., 2018). Macrovascular complications are more common in T2DM patients. Macrovascular complications that commonly occur are brain platelets (blood clots in part of the brain), coronary heart disease (CHD), congestive heart failure, and stroke. Microvascular complications mainly occur in patients with type 1 DM (DMT1) such as nephropathy, diabetic retinopathy (blindness), neuropathy, and amputation (Fatimah, 2016).

Prevention and management of macrovascular complications in DM patients is carried out by administering anticoagulant drugs to prevent blood clots. Anticoagulants are drugs that function to prevent blood clots that work by inhibiting the work of proteins involved in the blood clotting process. Based on the mechanism of action, anticoagulants are divided into four groups. The four groups are warfarin, namely coumarin anticoagulant drugs, factor Xa inhibitors, thrombin inhibitors, and heparin (Harter et al., 2015). Warfarin is an anticoagulant that works by reducing vitamin K stores in the liver thereby inhibiting the production of coagulation factors II, VII, IX, and X. Warfarin is indicated for the prevention and management of various thromboembolic cases, including thrombotic stroke. The usual dose of warfarin for stroke prevention in patients with atrial fibrillation is 2–10 mg/day. The dose given to patients without DM is the same as the dose given to patients with DM (Culebras et al., 2014).

Recent in vivo and in vitro studies conducted by Qiu et al found that glycated human serum albumin (HSA) both in humans and in mice tested had an increased affinity for the anticoagulant drugs warfarin and heparin, resulting in the levels of free anticoagulant drugs, especially warfarin in the blood. blood plasma is reduced. This study concluded that glycated HAS in DM patients had a

significant effect in influencing the pharmacokinetics of the anticoagulant drug warfarin and reducing the effectiveness of warfarin in patients with DM (Qiu et al., 2020). This latest study contradicts the guidelines for the management of macrovascular complications in DM patients, which previously stated that the dose of anticoagulant in DM patients was the same as the dose in patients without DM. This should be of concern because the dose of warfarin can affect the safety of DM patients who have macrovascular complications such as stroke. With the right dose of warfarin can reduce the risk of stroke by 64% compared to antiplatelet drug therapy which only reduces the risk of stroke by 22% (Amin, 2015).

Based on the Decree of the Indonesia Ministry Of Health Number 328/Menkes/SK/VIII/2013. Warfarin is included in the drugs covered by the National Health Insurance (JKN) program but is only available at level 2 health care facilities. Warfarin is still used as anticoagulant drugs because they have an affordable price compared to other anticoagulant drugs (Safrina, 2013). This shows that research on warfarin is still relevant because it is still often used in Indonesia as the anticoagulant drug of choice. Coupled with the limited research related to the effects of warfarin in rat model DMT2. Therefore, researchers are interested in testing the effectiveness of warfarin on the tail bleeding time of DMT2 rats. *Tail bleeding time* will describe the bleeding since it begins until there is a blockage and formation of fibrin in wound area, this test can assess whether the blood clotting system works within normal limits or not.(Liu, 2012).

Method

Types of research in this study was a quasi-experimental with a posttest research design with a control group (Posttest only with Control Group Design) in an in vivo laboratory with diabetes mellitus model animals, namely male Wistar (*Rattus Norvegicus*) rats with type diabetes mellitus. 2 induced by nicotinamide and streptozotocin which aims to determine the difference in the effect of warfarin on the tail bleeding time of male wistar rats with T2DM and without Diabetes Mellitus. This research was conducted at the Integrated Laboratory 1 of Al-Azhar Islamic University. The research was conducted from November to December 2021.

The measurement of the sample size in this study was carried out using the Federer formula. The Federer formula is a formula that is generally used to determine the number of samples in experimental research (Widiyatno & Muniroh, 2018). In this study, there were 4 groups that were grouped based on the treatment given, namely the male wistar rats with DM who were given warfarin and the male wistar rats without DM who were given warfarin as an experimental group, while the male wistar rats with DM were not given warfarin and the rat group Wistar males without DM who were not given warfarin as a control group. In this study, a sample of 24 male wistar rats was used, each group consisted of 6 rats. The following criteria were used in determining the sample: Age of male wistar rats: 8 – 12 weeks. Weight of male wistar rats: 150-250 grams. DMT2 rats with fasting blood sugar: 126 mg/dL. Non-DM rats with fasting blood sugar: 100 mg/dL.

The collected data will be processed and analyzed with computer software namely the Computer Software Statistical Package For The Social Sciences (SPSS) Version 23. Analysis in the form of normality test and hypothesis testing using univariate and bivariate analysis.

Results

Table 1. Weight Data for DM + Warfarin Group

Sample Group	Number of Rats	Average Weight I (grams)	BodyAverage Weight II (grams)	BodyAverage Weight (grams)
DM group + warfarin	6	196.16 ±8.15	163.33 ±8.89	32.83
DM group + Aquades	6	206.83 ±9.53	190.00 ±10.21	16.83
Normal Group + Warfarin	6	198.66 ± 18.28	198.83 ± 18.38	0.17
Normal Group + Aquades	6	185.83 ± 35.21	188.00 ± 39.13	2.17

Tabel 2. Data GDP sampel sebelum & setelah induksi Na-STZ

Data GDP I			Jumlah Tikus	Rata-Rata GDP I (mg/dL)	Rata-Rata GDP II (mg/dL)	Rata-Rata GDP Δ (mg/dL)	Rata-Rata GDP
Kelompok Warfarin	DM	+	6	69.6 ± 7.52	396 ± 92.62	326.4	
Kelompok Aquades	DM	+	6	74.3 ± 4.03	140 ± 35.62	63.7	

Tabel 3. Data Uji *Tail Bleeding Time*

Kelompok	Jumlah Tikus	Rata-Rata <i>Bleeding Time</i> (detik)	<i>Tail</i> 95% CI	Asymp.Sig.
Kelompok DM + Warfarin	6	1200 ± 0.00 ^a	1200	0.001
Kelompok DM + Aquades	6	913 ± 194.84 ^b	718.16 - 1,107.84	
Kelompok Normal + Warfarin	6	1173.3 ± 65.31 ^{ab}	1,107.99 - 1,238.61	
Kelompok Normal + Aquades	6	619.3 ± 319.64 ^b	299.66 - 938.94	



Image 1. Tail Bleeding Time Test for DM Group + Warafarin (Left) Normal Group + Warfarin (Right)

Discussion

The research sample consisted of 24 rats that had gone through the acclimatization process. The samples of wistar rats that had been obtained were then divided into 4 groups, namely the male wistar rats with DM who were given warfarin and the male wistar rats without DM given warfarin as an experimental group, while the male wistar rats with DM were not given warfarin and the wistar rats group. DM-free males who were not given warfarin as a control group. In this study, the data taken were body weight, fasting blood glucose levels before and after Na-STZ induction, tail bleeding time after administration of warfarin. The data obtained from this study will be tested for normality using the Shapiro-Wilk test. The results of Shapiro-Wilk test showed that the distribution of the data in this research was not normal, so it was continued with the Kruskal-Wallis non-parametric test to determine the significance of the differences data for each group of sample.

Based on the results of weighing for all groups before and after induction of Na-STZ, it was found that the DM + Wafarin group had an average weight loss of 32.83 grams. Furthermore, the DM + Aquades group had an average weight loss of 16.83 grams. Then the Normal + Warfarin group had an average weight gain of 0.17 grams. Meanwhile, the Normal + Aquades group had an average weight gain of 2.17 grams. The DM + Warfarin group was the group with the largest weight loss. Data on body weight of rats showed significant weight loss in the group of DM rats induced with Na-STZ. According to Ewenighi this is common in DM conditions, This is due to the body's inability to use glucose as fuel for metabolism and cellular respiration, resulting in a process of reshuffling adipose cells into fatty acids and ketone bodies and reshuffling proteins into amino acids which will become a source of energy for cells apart from glucose. Both of these things cause a decrease in body mass in DM patients (Ewenighi et al., 2015). The weight loss of rats that are induced by Na-STZ in the DM + Warfarin group with an average weight loss of 32.83 grams and DM + Aquades with an average weight loss of 16.83 grams. (Nørgaard et al., 2018; Obasi et al., 2019)

Based on the tail bleeding time data, the results showed that the DM + Warfarin group had an average tail bleeding time duration of 1200 ± 0.00 seconds, the DM + Aquades group had an average tail bleeding time of 913 ± 194.84 seconds, then the Normal + Warfarin group had an average tail bleeding time of 1173.3 ± 65.31 seconds, then the Normal + Aquades group had an average tail bleeding time of 619.3 ± 319.64 seconds. The results of the tail bleeding time data analysis with the Shapiro-Wilk normality test showed that the data distribution was not normal. Then continued with the Kruskal-Wallis non-parametric test, the Asymp value was obtained. Sig. (P value) of 0.001 indicates that there is a significant difference between the groups given warfarin and those not given warfarin on the duration of the tail bleeding time. Then continued with the Mann-Whitney non-parametric test which showed that the duration of the tail bleeding time in the DM + Warfarin group did not have a significant difference with the duration of the tail bleeding time in the Normal + Warfarin group (Widiyanto, 2018). but had a significant difference with the duration of the tail bleeding time in the DM+Aquades group and the Normal+Aquades group. The duration of tail bleeding time in the DM + Aquades group did not have a significant difference with the duration of the tail bleeding time in the Normal + Warfarin group and the Normal + Aquades group. The duration of the tail bleeding time of the Normal + Warfarin group had a significant difference with the duration of the tail bleeding time of the Normal + Aquades group.

The results of the study of the tail bleeding time tested using the Kruskal-Wallis test showed that there was an effect between the administration of warfarin and the tail bleeding time in male Wistar rats with DM model. The anticoagulants warfarin or coumarin block γ -carboxylation of several glutamate residues in prothrombin and factors VII, IX, and X as well as endogenous anticoagulants proteins C and S. Blockade results in incomplete coagulation factor molecules that are biologically inactive (Widiyanto, 2022). The mechanism of warfarin causes disruption of the blood coagulation process in the intrinsic and extrinsic pathways. Disruption of this pathway causes the formation of fibrin threads that function to close wounds and capture blood cells to help blood clot. An increase in bleeding duration, prothrombin time (PT) and activated partial thromboplastin time (APTT) is one indication of the active mechanism of warfarin in the body (Cheung et al., 2021).

The results of the study of tail bleeding time tested using the Mann-Whitney test showed that the duration of the tail bleeding time in the DM + Warfarin group did not have a significant difference with the duration of the tail bleeding time in the Normal + Warfarin group, but had a significant difference with the duration of the tail bleeding time in the DM + Aquades group. and the Normal + Aquades group. The duration of the tail bleeding time in the DM + Aquades group did not have a significant difference with the duration of the tail bleeding time in the Normal + Warfarin group and the Normal + Aquades group. The duration of the tail bleeding time of the Normal + Warfarin group had a significant difference with the duration of the tail bleeding time of the Normal + Aquades group.

The tail bleeding time of the DM + Warfarin group had a significant difference with the DM + Aquades group. This occurs due to the pharmacodynamic mechanism of warfarin which dilutes the blood in the DM + Warfarin group with

Warfarin being the most commonly used oral anticoagulant in the world. Warfarin produces an anticoagulant effect by interfering with the cyclical interconversion of vitamin K and the 2,3-epoxide group leading to impaired production of coagulant factors (Yang et al., 2014). In the comparison of these two groups, it was found that warfarin had an effect on DM rats. This shows that in DM conditions, warfarin is still a good choice as anticoagulant as the pharmacotherapy to prevent or to cure macrovascular complication and microvascular complication on Diabetics..

The duration of tail bleeding time in the DM + Warfarin group did not have a significant difference with the Normal + Warfarin group. This can be caused by the dose of warfarin given to these two groups, which is 2 mg/Kg of body weight which is the standard dose of warfarin (Qiu et al., 2020). Apart from the same dose, the tail bleeding time examination also has a weakness, namely the recommended maximum examination duration is 20 minutes or 1200 seconds, so it is not able to calculate bleeding if it occurs more than 20 minutes (Liu, 2012). Although the duration is not different, there is a difference in the picture of bleeding that comes out of the tail bleeding time test performed.

There is a difference in the description of blood bleeding that comes out of the tail bleeding time test based on the picture of the bleeding, but unfortunately there is no data on the details of the volume of blood released when this test is carried out. The picture of bleeding with a higher amount can be seen in the Normal + Warfarin group when compared to the DM + Warfarin group. According to Parasuraman et al, the blood volume of rats is 64 mL/Kg of body weight (Parasuraman et al., 2010). Previously, it was known that the weight of the rats from the DM group decreased and was lower than the normal group, this could cause differences in the picture of bleeding in the picture. In addition, there are factors that affect the distribution of warfarin in the body of rats, namely glycated albumin, according to Qiu et al, Glycated albumin has a higher affinity for warfarin than normal albumin (Qiu et al., 2020 which may lead to lower effectiveness of warfarin in DM rats compared to normal rats. This can occur due to several factors, based on research Desouza et al stated that changes in glycated albumin levels in DM patients who changed their diet to a low-sugar diet for 12 weeks, there was a decrease in the amount of glycated albumin by 20-30% in the first week (Desouza et al. al., 2015) so it is estimated that the amount of glycated albumin is not yet at optimal levels because a low-sugar diet is only given for 3 days after being declared DM. The same thing was also stated by those who stated that changes in glycated albumin levels in the human body occurred at least for a duration of 21 days (Giglio et al., 2020). Unfortunately, there has been no study of glycated albumin in male wistar rats as a comparison.

The duration of tail bleeding time in the DM + Warfarin group had a significant difference with the Normal + Aquades group. This of course occurs due to the warfarin mechanism that occurs in the DM + Warfarin group. In addition, under normal circumstances, the body is able to produce coagulant factors and carry out the blood clotting process through the intrinsic and extrinsic pathways. The intrinsic pathway involves activation of the prekallikrein contact factor, HMKW, factor XII, and factor XI. These factors interact on the surface to activate factor IX to factor IXa. Factor IXa reacts with factor VIII, PF3, calcium to produce factor X

to Xa. Together with factor V, factor Xa activates prothrombin (Factor II) to thrombin. which in turn converts fibrinogen to fibrin, whereas the extrinsic pathway is triggered by thromboplastin and involves factor VII and calcium ions. The two pathways combine to form a common pathway involving factors X, V, platelets, factor III, prothrombin, and fibrinogen. The extrinsic pathway is a pathway initiated by the entry of tissue thromboplastin into the blood circulation (Hall, 2016).

The duration of tail bleeding time in the DM + Aquades group did not have a significant difference with the Normal + Warfarin group. There are many factors that can cause prolongation of bleeding time, one of which is the administration of warfarin which occurs in the Normal + Warfarin group, but there is no significant difference with the DM + Aquades group, it can occur due to hyperglycemia. As stated by Li et al, that in DM patients, several abnormalities in the blood clotting process can be triggered, including platelet hypersensitivity, coagulation disorders, and hypofibrinolysis. These changes interfere with the prothrombotic state, thereby prolonging the duration required for blood clotting (Li et al., 2021). the results show a disturbance in the platelet aggregation process in DM patients (Asrat et al., 2019; Dhule & Gawali, 2014).

The duration of tail bleeding time in the DM + Aquades group did not have a significant difference with the Normal + Aquades group. This can occur due to vitamin K deficiency and vitamin D deficiency. Vitamin K deficiency can be caused by the diet given to wistar rats, but in this study AIN 93 feed was used as the standard feed for all sample groups, AIN 93 had an average vitamin K level 51.97 ± 21.88 mg/kg, the feed was given according to the body weight of the rats (Ariningtyas, 2017). The minimum requirement of vitamin K in rat feed is 0.64 ± 0.03 g/g or 0.64 ± 0.03 mg/kg (Mi et al., 2017). So that vitamin K deficiency was not the cause of the absence of a difference in the duration of the tail bleeding time between the groups. Vitamin D deficiency can occur due to lack of sun exposure. 80-90% of vitamin D is obtained from the synthesis of vitamin D in the skin which requires sunlight (Baggerly et al., 2015). In this study the rats were indoors and were never taken out of the room to sunbathe, this could lead to vitamin D deficiency. rats given vitamin D supplements for 10 days, showed that rats exposed to sunlight had vitamin D levels within normal limits so that they were able to maintain body homeostasis in calcium and phosphate transport, while the rats given vitamin D supplementation experienced an increase in vitamin D levels above normal so that it interfered with the production of parathyroid hormone and calcium transport in the blood, the normal reference value for vitamin D levels in the blood was 20-50 g/mL, vitamin D levels were less than 12 g/mL can be expressed as a state of vitamin D deficiency (Abulmeaty, 2017). Vitamin D deficiency can cause thrombosis in blood vessels, vitamin D has a role in the production of thrombomodulin which prevents the occurrence of thrombosis in blood vessels. Excessive thrombosis will cause thrombocytopenia which will interfere with the blood coagulation process. Thrombocytopenia will prolong the time of the prothrombin phase and can prolong the bleeding time (Mohd, 2021) (Mohammad et al., 2019).

The duration of tail bleeding time in the Normal + Warafarin group had a significant difference with the Normal + Aquades group. This of course occurs due

to the administration of warfarin at a dose of 2 mg/Kg of body weight. Under normal circumstances, warfarin acts by inhibiting the cyclic interconversion of vitamin K in the liver. The reduced form of vitamin K is required for the carboxylation of factors II, VII, IX, and X so that these coagulation factors become active forms. Thus, without reduced vitamin K, the above factors cannot function as coagulant factors. Warfarin intervenes in the conversion of vitamin K to its reduced form, so that warfarin indirectly reduces the amount of these coagulation factors. Therapeutic doses of warfarin reduce the amount of vitamin K dependent active coagulant factors produced by the liver by up to 30%-50% (Limdi & Veenstra, 2012). It is necessary to check the levels of coagulation factors and liver function to determine the cause of the prolongation of the tail bleeding time.

Conclusion

In this study, it can be concluded that there is an effect of giving warfarin on the tail bleeding time of male Wistar rats with type 2 diabetes mellitus model. The effect is in the form of lengthening the duration of the tail bleeding time. The duration of tail bleeding time in the DM + Warfarin group had a significant difference with the DM + Aquades and Normal + Aquades groups, but did not have a significant difference with the Normal + Warfarin group. The duration of tail bleeding time in the DM + Aquades group did not have a significant difference with the Normal + Warfarin and Normal + Aquades groups. The duration of tail bleeding time in the Normal + Warfarin group had a significant difference with the Normal + Aquades group.

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