Volume 5 Number 1 (2024)
July-December 2024
Page:1705-1712

E-ISSN: 2745-4584
https://ejournal.insuriponorogo.ac.id
DOI:https://doi.org/10.37680/almikraj.v5i01.6436

AL MIKRAJ
Jurnal Studi Islam dan Humaniora

Effect of Breadfruit Leaf (*Artocarpus altilis*) Extract on Histological Features of the Seminiferous Tubules in Diabetic Wistar Rats

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Submitted: Revised: 2024/09/01; Accepted: 2024/10/11; Published: 2024/11/05

Abstract

Hyperglycemia can lead to increased free radicals formation that can potentially cause oxidative stress as a trigger for male reproductive organ damage, damaging the histological structure of seminiferous tubules as a place for sperm cell formation. Flavonoids in breadfruit (Artocarpus altilis) are known to be able to counteract free radicals caused by diabetes. The research was conducted to observe the effect of breadfruit leaf extract on the histological features of seminiferous tubules in diabetic Wistar rats. This true-experimental research with post-test-only control group design and purposive sampling technique to obtain 30 Wistar white male rats 8-12 weeks old weighing 150-200 g were grouped into 5 groups. The negative control group was fed standard food; the positive control was induced with STZ, and treatments 1, 2, and 3 were given the extract at doses of 200 mg/kgBW, 400 mg/kgBW, and 800 mg/kgBW consecutively for 30 days. Assessment of spermatogenesis in the histology of seminiferous tubule using Johnsen score criteria. Data were analyzed by the Kruskal-Wallis and the Mann-Whitney U posthoc test, which revealed significance with a p-value of <0.001 (<0.05). This study showed that breadfruit leaf extract can improve the histology of seminiferous tubules. Despite not being as good as the histology of seminiferous tubules under normal conditions, it has a therapeutic effect.

Keywords

Breadfruit Leaf Extract, Diabetes Mellitus, Seminiferous Tubules, Spermatogenesis, Johnsen score

INTRODUCTION

The international diabetes federation estimated that diabetes cases worldwide in 2019 were 463 million people, with 9% of prevalence in women and 9.65% of prevalence in men. This number has been projected to reach 578 million in 2030 and 700 million in 2045. More than that, the occurrence of diabetes is higher in men than in women[1]. Hyperglycemia in diabetes causes high production of free radicals that can lead to oxidative stress[2]. Cellular damage from oxidative stress can trigger various complications, such as cardiovascular disorder, kidney failure, diabetic retinopathy, diabetic ulcers, hyperosmolar coma, and even reproductive disorders[2–4]. Recent studies have discovered that diabetes can affect sperm development and androgen production, which leads

to male infertility[1]. Low insulin levels cause reproductive disorders in men with diabetes by the decreased reproductive hormone secretion that plays a vital role in spermatogenesis and may interfere with spermatogenesis[5,6]. Besides that, disruption in spermatogenesis could also be affected by the alteration in the histological structure of seminiferous tubules[7].

The effect of diabetes on male fertility and testicular function has not been much explored[8]. Impaired testicular function due to diabetes condition can impede spermatogenesis, which leads to a decline in sperm quality as well as declining semen volume, sperm count, motility, and morphology abnormality[9]. Folk medicine is often used as a substitute for synthetic drugs to cure various diseases with minimal side effects[10]. Research proves one of them is the breadfruit leaf (*Artocarpus altilis*), which has anti-diabetic properties[11]. This is due to essential metabolites, such as flavonoids, tannins, saponins, and phenols[12]. Previous studies revealed that ethanol leaf extract of breadfruit at a dose of 400 mg/kg BW has been shown to have an anti-diabetic effect for lowering blood glucose up to 77.07% and also given the improvement of pancreatic islets of Langerhans in hypercholesterolemia-diabetic rats[12,13].

The streptozotocin-induced diabetic rat model is one of the most commonly used to study the effect of diabetes on fertility. Testicular dysfunction was observed under the condition of the experimentally diabetes-induced animal prototype[1]. The administration of breadfruit leaf extract has been shown to have a repairing activity against diabetogenic agent-induced pancreas damage. However, there have been limited studies on the effect of breadfruit leaf extract on the histological features of seminiferous tubules. This study aimed to find out the effective dose to give the repairing effect on histological features of seminiferous tubules in STZ-induced diabetic Wistar rats.

METHOD

The research was a post-test-only control group designed and conducted in the Department of Pharmacology and Therapy Laboratory, Faculty of Medicine, Universitas Padjadjaran. The ethical clearance was obtained from the Universitas Pembangunan Nasional "Veteran" Jakarta ethics committee under ethic number 402/XI/2023/KEP.

A total of 30 healthy adult male Wistar rats, weighing between 150 g and 200 g, were housed under standard environment conditions of temperature and humidity, and 12-hour light and dark cycles were used in this study. The animals were fed with a regular diet and water ad libitum. Rats were randomly divided into five groups with six rats in each group, such as negative control group

(K1), positive control group (K2), treatment 1 group (K3), treatment 2 group (K4), and treatment 3 group (K5).

The leaves used in this experiment are the old dark green-colored leaves purchased from the traditional medicine market in Bandung. The leaves were cleaned from dirt, then chopped into pieces, and dried in the open air with no direct sunlight exposure. The dry leaves were then blended and sieved. The maceration process is used to make breadfruit leaf extract. The simplicia was soaked in 70% ethanol for three days and filtered with filter paper. The filtrate was concentrated in a vacuum rotary evaporator and evaporated in a water bath at 60°C, forming a thick extract used in this experiment.

A total of 30 male Wistar rats adapted for seven days before 30 days of treatment. Fasting blood glucose levels were measured after overnight fasting following the acclimatization. Excluding the negative control group, diabetes induction was performed in four groups by giving them a single STZ intraperitoneal injection at a dose of 40 mg/kg BW using a sterile needle and syringe. Fasting blood glucose levels were measured again 72 hours after injection from the lateral vein in the tail. Animals with fasting blood glucose levels >200 mg/dl were considered diabetic and were included in this study. All groups were given the breadfruit leaf extract on the third day after STZ injection at a dose of 200 mg/kg BW (K3), 400 mg/kg BW (K4), and 800 mg/kg BW (K5). All rats were sacrificed on day 31. The testicular samples were dissected and fixed in 10% buffer-neutral formalin for histological examinations.

The fixed tissues were processed with paraffin embedding, and sections were cut to a 5-7 μm thickness. The sections were given to the hematoxylin-eosin staining to assess the testicular histological features. The examination was conducted under the trinocular microscope at 400x magnification in five fields of view. The veterinarian expert read the preparations to determine the score of spermatogenesis. Assessment of spermatogenesis according to the Johnsen score criteria of 1-10[5].

Table 1. Johnsen Score Assessment Criteria

Score	Criteria
10	Complete spermatogenesis with many spermatozoa, normal germinal epithelium, and open lumen of tubules
9	Incomplete spermatogenesis with many spermatozoa, damaged germinal epithelium, and blocked lumen of tubules
8	Some spermatozoa (<5-10)
7	No spermatozoa but many spermatids

Score	Criteria
6	No spermatozoa but some spermatids (<5-10)
5	No spermatozoa and spermatids but many spermatocytes
4	Some spermatocytes (<5), no spermatids or spermatozoa
3	Only spermatogonial cells
2	No spermatogonial cells but there are Sertoli cells
1	No spermatogonial cells or Sertoli cells

The descriptive data were the average of the Johnsen score of five different microscopic visual fields. The data obtained were then tested for normality using the Saphiro-Wilk test, and the significance of the data was tested by the non-parametric Kruskal-Wallis test, which was continued with the post hoc Mann-Whitney U test to analyze the differences between groups. The degree of significance is if $p<0.05$ with a confidence interval of 95%.

RESULTS AND DISCUSSION

All groups were acclimatized for 7 days. Rats were starved for 12 hours, and then STZ injection was given according to the dose except for the negative control group. About 72 hours after STZ administration, the blood glucose levels of all samples measured >200 mg/dl, indicating that the samples had undergone diabetes. The negative control group had the highest average spermatogenesis score compared to other groups, with a value of 8.6. On the other hand, the positive control group had the lowest mean spermatogenesis score compared to other groups, with a value of 6.96.

The figure shows this study's lowest to highest spermatogenesis score. Figure A is the seminiferous tubules of a positive control group that was only injected with STZ. The group had the poorest features of spermatogenesis due to oxidative stress caused by diabetes. Figures B and D showed a picture of the seminiferous tubules of the group that received breadfruit leaf extract at a dose of 200 mg/kg BW, while Figure C is from the group that received breadfruit leaf extract at 800 mg/kg BW. Figures E and F showed a picture of seminiferous tubules with the best score from the group that got a dose of breadfruit leaf extract 400 mg/kg BW.

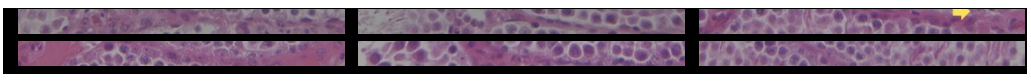

Figure 1. Examination of the histological features of seminiferous tubules with hematoxylin-eosin staining and assessed at a magnification of 400x using the Johnsen score

criteria. Score 5 (A), Score 6 (B), Score 7 (C), Score 8 (D), Score 9 (E), Score 10 (F), Sertoli cells (marked by yellow arrow), spermatogonia (marked by red arrow), spermatocytes (marked by blue arrow), spermatids (marked by green arrow), and spermatozoa (marked by black arrow).

The normality test used in this study is the Saphiro-Wilk test because the sample size was no more than fifty. The data is normally distributed if the p-value is >0.05. The control and treatment groups have normally distributed data (p>0.05) except for the treatment group 1 (K3) because it has 0.046 of value (p<0.05). The homogeneity test results produced a significance value of 0.147, and it can be concluded that the data is homogenous. Furthermore, the non-parametric test was carried out with Kruskal-Wallis because the data was not normally distributed and homogenous.

Table 2. Outcomes of the Kruskal-Wallis Test

Groups	Mean ± SD	P-value
K1	8.6 ± 0.28	<0.001 *
K2	6.96 ± 0.38	
K3	7.48 ± 0.18	
K4	7.96 ± 0.17	
K5	7.04 ± 0.46	

* Significant (p<0.05)

The data is stated to have a significant difference if the p-value <0.05. The test revealed a p-value of <0.05, indicating significant differences between the five groups. After that, it is necessary to conduct the post hoc test to compare the differences between groups.

Table 3. Outcomes from the Posthoc Test

Groups	K2	K3	K4	K5
K1	0.008 *	0.007 *	0.010 *	0.008 *
K2	-	0.025 *	0.009 *	0.752 *
K3		-	0.008 *	0.104 *
K4			-	0.008 *

* Significant (p<0.05)

Table 3 shows the result of the post hoc Mann-Whitney U test. There were significant differences (p<0.05) between the negative control group (K1) and positive control group (K2), treatment group 1 (K3), treatment group 2 (K4), and treatment group 3 (K5). There were also significant differences (p<0.05) between the positive control group (K2) and treatment group 1 (K3) and treatment group 2 (K4), between treatment group 1 (K3) and treatment group 2 (K4), and between treatment group 2 (K4) and treatment group 3 (K5). Meanwhile, there was no significant difference (p>0.05) between the positive control group (K2) and treatment group 3 (K5) and between

treatment group 1 (K3) and treatment group 3 (K5).

This study showed that the microscopic image score of the seminiferous tubules of the positive control group was lower than the other groups. Based on the score, the average value of the positive control group is 6.96, where this value is lower than the negative control groups, namely 8.6, treatment group 1 is 7.48, treatment group 2 is 7.96, and treatment group 3 is 7.04. This shows that streptozotocin injection to induce diabetes in rats damages spermatogenesis, as stated by the previous study[5].

Volpe et al. stated that hyperglycemia triggers the activation of several signaling pathways and causes cells to become more susceptible to necroptosis, apoptosis, and necrosis. Hyperglycemia arises due to reactive oxygen species' excess production and causes oxidative stress and activation of apoptosis, which results in cellular death[14]. Reactive oxygen species (ROS) can form bonds with molecules in cells and cause damage to nucleic acids, proteins, and fats[15]. Cell damage caused by oxidative stress due to diabetes in the form of vasculopathy can cause microvascular complications. Inadequate blood supply is also a cause of testicular dysfunction in diabetic patients[16]. Another study in an STZ-induced diabetes model rats revealed a decrease in testosterone, FSH, and LH levels[17]. Diabetes affects testicular tissue and causes a reduction in FSH and LH levels due to decreased function of Leydig cells and Sertoli cells[18,19]. Ding et al. state that the loss of the insulin stimulatory effect reduces Leydig cell function and then causes inadequate testosterone production, subsequently affecting the secretion of FSH and LH from the pituitary gland, and this condition disrupts spermatogenesis[3].

The statistical result of the non-parametric Kruskal-Wallis test shows that it has a p-value of <0.001 ($p<0.05$), so there was a significant difference in all groups, which means there was an effect of giving the breadfruit leaf extract. It was followed by a post hoc Mann-Whitney U test to determine the difference between groups. Post hoc results revealed that the K2 group has no significant difference against the K5 group but significant differences against the K1, K3, and K4 groups. Various physiological conditions of each rat may cause the differences in responding to the extract given[12]. This shows that breadfruit leaf extract at a dose of 200 mg/kgBW and 400 mg/kgBW has the ability to increase spermatogenesis scores in diabetic rats. However, it has a higher average of the Johnsen score at a dose of 400 mg/kgBW. Administration of breadfruit leaf extract at a dose of 400 mg/kgBW was found to be the most effective because it can provide an effect on changing the histological features of the seminiferous tubules, which are comparable to the negative control and

approach to the histology of normal seminiferous tubules.

The effect of improving spermatogenesis score is due to the bioactive compounds in breadfruit leaf extract, namely flavonoids, which act as antioxidants. Antioxidants are defense substances that neutralize free radicals. Research by Effendy et al. proved that breadfruit leaves have very strong antioxidant activity with levels of 29.60 ± 0.14 μg/mL[20]. Flavonoids are essential in antioxidant properties because the hydroxyl groups bond to the aromatic carbon ring and can capture free radicals and prevent cellular damage[21].

Breadfruit leaf extract at 200 mg/kg BW and 400 mg/kg BW doses increased the seminiferous tubules' histological features by improving spermatogenesis. That might be associated with its antioxidant properties. However, administering breadfruit leaf extract at a dose of 800 mg/kg BW decreases spermatogenesis. This may result from the interaction between the secondary technical implementation of the study's procedure and the uncontrolled environmental conditions of the experiment

CONCLUSION

Based on this study, it can be concluded that there is a significant difference in histological features of seminiferous tubules of Wistar rats with streptozotocin-induced diabetes that receive breadfruit leaf extract at a dose of 200 mg/kg BW and 400 mg/kg BW orally after 30 days of treatment. The highest mean Johnsen score on seminiferous tubules was found in the diabetic group treatment 2 with a 400 mg/kg BW dose of extract.

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