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EFFECT OF BREADFRUIT LEAF EXTRACT ON SPERM QUALITY IN DIABETIC MALE WISTAR RATS

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ABSTRACT

Background: Diabetes mellitus can lead to various complications, one of the most devastating complications is infertility. Meanwhile, the antioxidant compounds of breadfruit leaves have been shown to help heal oxidative stress and therefore prevent infertility in diabetic patients. Therefore, this study aimed to determine the effect of breadfruit leaves in improving sperm quality parameters in male diabetic Wistar rats. **Methods:** In this study, twenty four male rats were used and were classified into four groups. Two of the four groups were alloxan-induced diabetics and were treated with a graded dose of breadfruit leaf extract. The two control groups received no therapy and the treatment was administered for 30 days. The rats were terminated after day 31 and sperm concentration, morphology, motility, and spermatogenesis were examined. **Result:** The results showed that breadfruit leaves increase sperm quality parameter levels compared to the control groups. The group that received a 400 mg/kg dose of breadfruit leaves showed the most significant improvement in terms of the level of sperm parameters compared to the other group that received a dose of 100 mg/kg breadfruit leaves. **Conclusions:** The protective effect of breadfruit leaves significantly reduced sperm damage caused by diabetes mellitus in Wistar Rats.
Keywords: *Breadfruit Leaves, Diabetes Mellitus, Sperm Concentration, Sperm Morphology, Sperm Motility, Sperm Morphology*

INTRODUCTION

Diabetes mellitus can lead to various complications, one of the most devastating complications is infertility. Clinically, infertility is defined as the inability to conceive after 12 months of regular unprotected sex.[1] Infertility is divided into primary and secondary based on women's inability to carry a pregnancy to full term. It is estimated that 8-12% of couples of reproductive ages worldwide experience infertility. Subsequently, it is believed that men alone are responsible for 20-30% of infertility cases, but they are usually responsible for 50%.[2]

The main cause of male infertility is poor sperm quality, which is the result of drug use, radiation, infections, anatomical deformities, and endocrine problems.[3] Also, systemic disease such as diabetes mellitus is believed to play an important role in male infertility.[4] Meanwhile, oxidative stress is considered to be the main cause that contributes to pathogenesis. Stressors stimulate Reactive Oxygen Species (ROS) production rather than antioxidant production, causing cells to enter a state of necrosis and apoptosis as well as generating oxidative stress.[4,5] Therefore, men with diabetes will experience a decrease in sperm count, sperm motility, sperm concentration, androgen levels, and impaired sperm morphology.[6] In addition to pathology, diabetes mellitus can cause damage to the majority of blood vessels, including dysfunction of penis blood flow, affecting nutritional distribution toward the tubule of seminiferous, which affects sperm quality.[7]

Interventions are needed to prevent infertility caused by diabetes mellitus, including the use of chemical drugs and herbal medicines. However, treatment with chemical drugs has some weaknesses, including side effects, long-term estimates, and high treatment costs.[8] Herbal medicine can be adopted as a supplementary treatment for the prevention of diabetic infertility. Breadfruit leaves are believed to contain high levels of antioxidants. Subsequently, breadfruit leaves contain antioxidants such as flavonoids, saponin, and tannin, which are commonly known as anti-diabetic, anti-inflammation, and antivirus.[9] Compounds in breadfruit leaves can capture free radicals and form more stable compounds to terminate the free radical chain. The antioxidant compounds in breadfruit leaves have been shown to help heal oxidative stress, thereby preventing diabetic infertility.[10,11]

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In addition, the antioxidant activity of breadfruit leaves affects the pancreatic β-cell, leading to the normalization of insulin secretion.[12] The normal concentration of insulin stimulates the production of hormones LH and FSH, leading to an increase in testosterone level, which is important for spermatogenesis.[12] Therefore, this study was carried out to determine the effect of breadfruit leaf extract on improving sperm quality in diabetic rats.

METHODS

This study is based on a real experiment, a post-test-only group design, which was conducted from May to June 2021. Furthermore, twenty four male Wistar rats, aged 2-3 months with mean weights of 150-250 grams, were randomly divided into four study groups. Each group consists of 6 rats, and this study was carried out over 30 days.

The group consists of three diabetic groups and one non-diabetic group. The diabetic groups received different doses of treatment. Out of the three diabetic groups, two are treatment groups, the first group received a dosage extract of 100 mg/kg and the second group received a dosage extract of 400 mg/kg. The last diabetic group was assigned as the diabetic-control group, which received no breadfruit leaves extract. The remaining non-diabetic group was assigned as a non-diabetic control group, which received no treatment and served as the negative control.

All rats were kept in the Animal Study Laboratory, Faculty of Math and Science, State University of Semarang. They were kept in a study-grade animal cage with constant room temperature between 25 ° C and 28 ° C and were provided with a sufficient amount of standard rodent diet and drinking water ad libitum.

Diabetes Induction and Monitoring

Diabetes induction was performed by alloxan injection, which was injected intraperitoneally using a sterile syringe and needle. Blood glucose was measured one week after injection using the PCOT technique from a lateral saphenous vein sample. The rats were considered diabetic with a blood glucose level of 200 mg/dl and were randomly assigned to the three diabetic groups as mentioned above.12 The blood glucose level measure was carried out once again before the rats termination to check whether the rats were still in the diabetic state or not.

Preparation of Breadfruit Leaves Extract

Mature breadfruit leaves were used as a material for the extract, and a total of 4kg of mature breadfruit leaves were cut into pieces and dried without direct sunlight, then mixed and sieved to powder. The powder was macerated in 96% ethanol for 48 hours. The results of the ethanol maceration are filtered to obtain a filtrate. The filtrate is then evaporated in a rotary vacuum evaporator, forming a crude extract from a paste that is used as experimental material.

Sperm Concentration and Motility Analysis

The rats were eliminated with a high dose of chloroform inhalation, followed by dislocation of the cervical vertebrae. Then, spermatozoa were obtained by removing and massaging the epididymis of the rat. The obtained sperm were diluted to 20 µL with NaCl 0.9%, and the sperm of all rats received the same treatment. The sample was then placed in object glass and covered with glass. The sample was examined at 400X five-field magnification using a light electron microscope.

Sperm Morphology Analysis

The spermatozoa were obtained by removing and massaging the rat epididymis, homogenization, and dripping into the edge of the object glass. Then, another object of glass with a 30° slope, the dripped sample was leveled. After waiting 5 minutes for the sample to dry, the process continues with an ether-alcohol fix for 5 minutes. Then, 10% Giemsa staining was used for the Sorensen buffer for 20-30 minutes. The sample was placed in object glass and covered with glass. The sample was examined under 1000x magnification using an electronic light microscope.

Testicular Histopathology Examination

A testis sample was obtained from the rats after elimination. The testes were placed in 10% buffer formalin, then dehydrated, and placed inside a paraffin block. Furthermore, paraffin blocks were cut into 5-6 µm using a microtome. Then, the sample was stained using hematoxylin. The samples were examined at 400x magnification using an electronic light microscope.

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Statistical analysis

Statistical analysis was performed using SPSS 26.0 for windows. The Saphiro-wilk test was used to check the normality of the data distribution. Based on the Saphiro-wilk test result, the analysis of normally distributed data was carried out using a one-way ANOVA test followed by the pots-hoc test. The significance was established at 0.05.

RESULTS

Rats (Rattus novergicus L.) Blood glucose level

Based on the blood glucose analysis results and statistics of rats in the table below. In both treatments 1 and 2, the diabetic group's blood glucose levels were much lower than the diabetic control group that received no treatment. The mean blood glucose levels in diabetic group treatments 1 and 2 are significantly different between the pre-test and post-test.

Rat Sperm Quality Parameters

Based on analytical statistics, the diabetic treatment groups had higher sperm concentrations than the control groups. Although sperm morphology, spermatogenesis, and motility were higher in the diabetic treatment groups than in the diabetic control group lower than in the non-diabetic control group. Most results of the sperm and testicular analysis indicate that a higher dosage of breadfruit leaf extract gave better results in both the sperm and the testicular analysis (Table 1).

Table 1. Blood Glucose Level

Group	Pre-Test (mg/dL)	Post-test (mg/dL)	P
C1	126±8.09	101,0±29.2	0.006*§
C2	271±17.15	261,6±19.2	0.044*§
P1	272±14.12	146±22.2	0.001*§
P2	299,6±22.19	175,6±21.9	0.002*§

§ Paired sample T-test; *Significant
C1 : Non-diabetic control group
C2 : Diabetic control group
P1 : Diabetic group treatment 1 (100 mg/kg dosage of breadfruit leaves extract)
P2 : Diabetic group treatment 2 (400 mg / kg dose of breadfruit leaf extract)

The post-hoc test showed that the treated diabetic group differed significantly from the diabetic control groups with a significant value of $p<0.05$. In the variable sperm concentration, the diabetic group treated with treatment 1 showed a significant difference from the non-diabetic control group, but the diabetic group with treatment 2 showed no significant difference. Furthermore, the post-hoc test was also performed by comparing mean data from the diabetic group with treatments 1 and 2, the results showed that there was no significant difference between these two groups. In the variable of sperm morphology, the diabetic group with treatments 1 and 2 showed no significant differences compared to the non-diabetic control group. Compared to the diabetic control group, all treatment groups showed significant differences. The post-hoc test was also carried out by comparing mean data from the diabetic group with treatments 1 and 2, the results indicated there was no significant difference between these two groups (Table 2 and 3).

Table 2. Sperm Quality Parameter Data

Sperm Quality Parameter	Group				
	C1	C2	P1	P2	P†
Sperm Concentration (x 10^6 /mL)	20±0.71	16.4±8.85	26.7±9.82	21.7±6.19	0.006*
Sperm Morphology (%)	85.50±7.53	74.83±5.49	82.17±5.88	86.17±3.43	0.011*
Testicular Histopathology (Johnson Score)	8.93±0.45	6.20±0.71	7.57±0.56	8.83±0.53	0.023*
Sperm Progressive Motility (%)	36.82±6.19	19.74±5.32	22.60±8.85	18.05±9.82	0.002*

†One-Way ANOVA; *significant
C1 : Non-diabetic control group
C2 : Diabetic control group
P1 : Diabetic group treatment 1 (100 mg/kg dosage of breadfruit leaves extract)
P2 : Diabetic group treatment 2 (400 mg / kg dose of breadfruit leaf extract)

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Table 3. Sperm Quality Parameter Post-Hoc Analysis

Sperm Quality Parameter	Group					
	C1-C2	C1-P1	C1-P2	C2-P1	C2-P2	P1-P2
Sperm Concentration	0.160‡	0.015*‡	0.497‡	0.001*‡	0.046*‡	0.058‡
Sperm Morphology	0,004*‡	0,329‡	0,843‡	0,040*‡	0,003*‡	0,244‡
Testicular Histopathology	<0.001*‡	<0.001*‡	0.668‡	0.001*‡	<0.001*‡	<0.001*‡
Sperm Progressive Motility	0.001*‡	0.005*‡	<0.001*‡	0.531‡	0.711‡	0.323‡

‡Post-Hoc LSD; *significant
C1 : Non-diabetic control group
C2 : Diabetic control group
P1 : Diabetic group treatment 1 (100 mg/kg dosage of breadfruit leaves extract)
P2 : Diabetic group treatment 2 (400 mg / kg dose of breadfruit leaf extract)

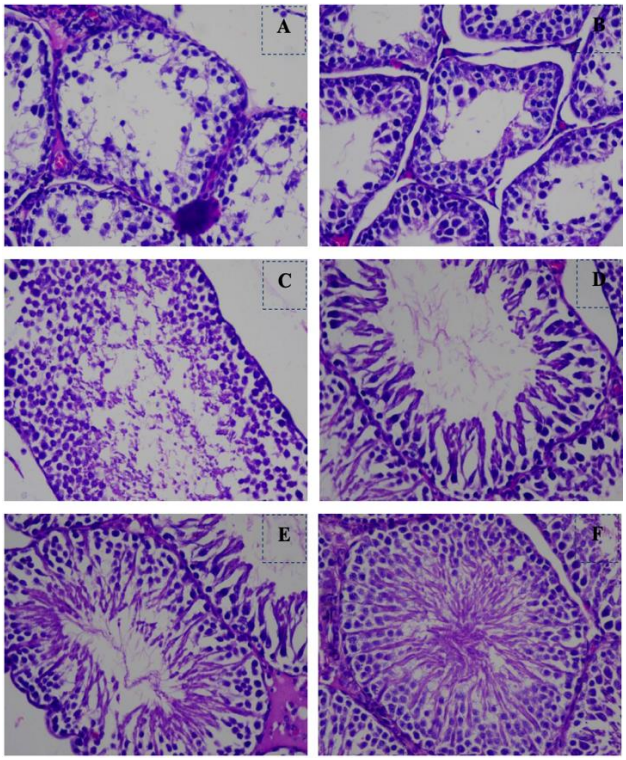

Figure 1. Histopathology of testicular rats. A. Johsen score 5; B. Johsen Score 6; C, Johnson Score 7; D. Johnson score 8; E. Johsen Score 9; F. Johnson Score 10

DISCUSSION

This study was conducted on 24 Wistar rats randomly grouped into two control groups, non-diabetic and diabetic, as well as two treatment groups, diabetic group, with a 100 mg/kg dosage and 400 mg/kg dosage treatment with breadfruit leaves extract. This study was carried out to show that there was a significant effect on sperm quality measured using sperm concentration, motility, morphology, and testicular histology in diabetic Wistar rats treated with breadfruit leaf extract treatment compared to the control groups.

Additionally, 24 Wistar rats were injected with alloxan to induce a diabetic condition in the rats. Induction of alloxan caused symptoms similar to diabetes mellitus in Wistar rats due to its destructive effect on pancreatic cells.[13,14] Alloxan, which is chemically known as 5,5-dihydroxyl pyrimidine-2,4,5-trione is a urea derivate, a carcinogen and cytotoxic glucose analogue, infiltrates pancreatic cells and forms superoxide radicals (O_2^-) through an oxidation-reduction (redox) cycle. O_2^- radicals undergo dismutation into hydrogen peroxide (H_2O_2). H_2O_2 turns into reactive hydroxyl radicals (OH^-), thereby accelerating the destruction of pancreatic cells by increasing the concentration of cytosolic calcium.[13,15]

Complications of male reproductive disorders are caused due to diabetes mellitus oxidative stress processes in tissues that play a role in the spermatogenesis process.[16] Oxidative stress is caused by increased ROS production, which is a type of reactive free radical.[16] Increased ROS production causes mitochondrial breakdown, thereby triggering the release of cytochrome C and triggering the activation of caspases that cause apoptosis.[17] Increased ROS causes a state of oxidative stress, hence, cells are injured through the mechanism of lipid peroxidation and oxidative damage to proteins and DNA.[18] Indication of failure of spermatogenesis is shown by the reduction of spermatogenic cells that cause testicular dysfunction.[17]

Moreover, diabetes affects testicular tissue in men and has led to insufficient insulin production, which in turn reduces the function of Leydig and Sertoli cells.[19] Diabetes has created a condition in which Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and testosterone levels were low in diabetic rats, while also decreased levels of FSH affect the spermatogenesis and endocrine function of the testis.[20-22]

The compound used in this study already tested with phytochemical analysis to ensure compound contained flavonoid extract inside. This method has

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been widely used in others study quantitatively measure flavonoid content inside solution.[23-25] Breadfruit leaves was extracted using 96% ethanol through maceration process with 1:1 proportion which revealed later has the highest extraction efficiency.[26]

Flavonoid compounds have protective antioxidant and anti-inflammatory effects in cells by stimulating survival-promoting signalling pathways and inhibiting the expression of pro-apoptotic proteins. They inhibit ROS and lipid peroxidase accumulation in cells by increasing the antioxidant capacity of pancreatic cells.[27] This process prevents autophagy, apoptosis, and necrosis of pancreatic cells. Additionally, a flavonoid also plays an important role in enhancing mitochondrial function and stimulating amplifying pathways in insulin secretion.[28] Glucotoxicity, lipid toxicity, and exposure to chronic oxidative stress in diabetes will increase the expression of pro-apoptotic genes (caspases) and result in the downregulation of anti-apoptotic genes (Bel-2) in cells.[29] Flavonoids inhibit the process of gene expression changes - the gene and therefore the progression of apoptosis in β cells can be prevented.[30,31] The antioxidant content in breadfruit leaves has the potential to increase the concentration of spermatozoa. Increased insulin levels stimulate the production of the LH and FSH hormones in Leydig and Sertoli cells, then trigger the production of the testosterone hormone. Increased testosterone hormone stimulates the spermatogenesis process in the testes, thereby producing more sperm cells.[12,20] Furthermore, flavonoids prevent degeneration of testicular structure and function due to the accumulation of free radicals by diabetes mellitus and increase sperm concentration.[5,32]

Besides flavonoid, breadfruit leave also contains champerol, artoindonesianin, and quercetin, commonly described as one of the powerful antioxidants that can prevent alloxan oxidation.12 Chemical component of breadfruit leaves has a strong potential, which can reduce blood glucose levels through flavonoid as an inhibitor of alpha-glucosidase, playing a role in inhibiting glucose uptake in the small intestine.[33] In this study, researchers has validated the total phenolic and flavonoids content in this extract through phytochemical analysis. The results showed that total phenolic and flavonoids content was expressed as mg Catechin and gallic acid equivalents/ g of extract. Study showed the potent antioxidant effect of breadfruit leaves as antioxidant comparable to green tea.[34]

The mean quality parameter of sperm (concentration, morphology, motility, and spermatogenesis) in the treatment group increased with the addition of the dose of breadfruit leaf extract. Based on the data obtained, the dose of breadfruit leaf extract that showed the most significant effect on sperm concentration was 400 mg/kg with the highest mean sperm concentration, morphology, motility, and spermatogenesis compared to other groups. Statistically, the two treatment groups of breadfruit leaf extract were not significantly different between treatments (concentration: p 0.058; morphology p 0.244; motility p 0.52), except for testicular histopathology, the mean value was statistically significant ($p<0.001$), and therefore the determination of the most effective dose would be reviewed through the average concentration of spermatozoa.

When all semen quality parameters are examined, it can be concluded that all testicular motility, morphology, and histopathology parameters showed that mean values were higher in diabetic treatment group 2 (breadfruit leaf extract 400 mg/kg), while the only parameter of sperm concentration showed a greater value in the diabetic treatment group 1 (breadfruit leaf extract 100 mg/kg). It can be concluded that breadfruit leaf extract showed increasing effectiveness with the addition of the dosage.

CONCLUSION

Based on this study, it can be concluded that there is a significant difference in the sperm quality of male Wistar rats with alloxan-induced diabetes mellitus that received breadfruit leaf extract (Artocarpus altilis) at a dose of 100 mg/kg and 400 mg/kg orally after 30 days of treatment in comparisons with both of control groups. The protective effect of flavonoid compounds in breadfruit leaf extract was directly proportional to the addition of the dose. The highest mean quality value of sperm was found in the diabetic group treatment 2 with a 400 mg/kg dose of breadfruit leaf extract.

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ETHICAL APPROVAL

In this study, the animal sample was treated accordingly with the experimental animal treatment protocol. Furthermore, this study ethical clearance was issued by the Medical Health and Research Ethics Commission (KEPK), Faculty of Medicine, Diponegoro University. The ethical number is 47/EC/H/FK-UNDIP/V/2021.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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