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EFFECT OF HESPERETIN TREATMENT ON BLOOD GLUCOSE LEVEL, SPERMATOZOA QUALITY, AND SPERMATOZOA QUANTITY IN ALLOXAN-INDUCED DIABETIC MICE

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ABSTRACT

Male infertility has occurred rapidly in the last few decades, primarily in developing countries. An antioxidant, hesperetin is a flavonoid that is found in abundance in orange peels. The aims of this research were to determine the effect of hesperetin on blood sugar levels, spermatozoa quality, and spermatozoa quantity. The research structure included induction of diabetes mellitus and treatment for 8 weeks, followed by determination of blood sugar levels, spermatozoa quality, and spermatozoa quantity. Hesperetin has the ability to restore blood sugar levels, spermatozoa quantity, seminiferous tubules diameter, and testicular weight, volume, and germinal epithelial layer thickness with significant difference from the normal control group. Hesperetin did not fully restore spermatozoa motility, viability, and morphology with significant difference from the normal control group, nor from the positive control group. However, overall, hesperetin decreased blood glucose levels, increased spermatozoa quantity, and improved the spermatozoa quality in alloxan-induced diabetes mellitus mice. Dose-dependent activity was observed with the optimum dose at 200 mg/kg body weight.

Key words: alloxan, blood glucose, diabetic, hesperetin, spermatozoa

ABSTRAK

Peningkatan kejadian infertilitas secara pesat terhadap kaum pria yang mayoritas terjadi pada negara berkembang dalam beberapa dekade terakhir. Hesperetin merupakan flavonoid yang terdapat dalam jumlah melimpah dari bahan alam kulit jeruk. Penelitian ini bertujuan mengetahui pengaruh pemberian hesperetin terhadap kadar gula darah, kualitas spermatozoa, dan kuantitas spermatozoa. Alur penelitian mencakup induksi diabetes melitus dan perlakuan selama 8 minggu, yang selanjutnya dilakukan pemeriksaan kadar gula darah, kualitas spermatozoa dan kuantitas spermatozoa. Hesperetin dapat memulihkan kadar gula darah, kuantitas spermatozoa, massa testikular, volume testikular, diameter tubulus seminiferous, dan ketebalan lapisan epitel germinal testicular yang tidak berbeda signifikan dengan kelompok kontrol normal. Kelompok pemberian hesperetin tidak dapat memulihkan motilitas spermatozoa, viabilitas spermatozoa, dan morfologi spermatozoa yang berbeda signifikan dengan kelompok kontrol normal, namun tidak berbeda signifikan dengan kelompok kontrol positif. Hesperetin menurunkan kadar gula darah, meningkatkan kuantitas spermatozoa, dan memperbaiki kualitas spermatozoa, pada tikus diabetes melitus yang diinduksi dengan aloksan dengan aktivitas yang bergantung terhadap dosis dan dosis optimum 200 mg/kg berat badan.

Kata kunci: aloksan, gula darah, diabetes, hesperetin, spermatozoa

INTRODUCTION

Diabetes mellitus is a chronic disease and one of the most important public health problems to be resolved globally. Diabetes mellitus has a very high prevalence in Indonesia (Ligita *et al*., 2019). Diabetes mellitus increases morbidity and mortality along with complications in various organs. Malfunction of male reproductive organs is one of the most widely reported complications in people with diabetes mellitus and can cause infertility in men and women (Deyhoul *et al.*, 2017). Previous research on diabetes mellitus finds that decreases in the hormone testosterone and disruption of spermatogenesis are closely related to uncontrolled increases in blood glucose levels (Salimnejad *et al.*, 2017; Soliman *et al*., 2019).

Hyperglycemia can also interfere with hypothalamus-pituitary-gonad axis function and cause local damage to the proliferation of Leydig and Sertoli cells. Leydig and Sertoli cells are intratesticular functional cells that have a role in testosterone biosynthesis and spermatogenesis (Petersen *et al.*, 2015). Flavonoid is a compound which is found to reduce blood glucose level by antioxidant activity, recovering spermatozoa abnormalies caused by diabetes mellitus (Sarian *et al.*, 2017; Jalili *et al.*, 2018). Hesperetin is a class of flavonoid that can reduce blood sugar levels and can be extracted abundantly from citrus fruit peels (Meneguzzo *et al.*, 2020). However, the effect of hesperetin on spermatozoa, and spermatozoa disorders caused by alloxan-induced diabetes mellitus in testes, is not yet known. Based on the findings above, the researchers are interested to examine the effect of hesperetin treatment on blood glucose level, spermatozoa quality, and spermatozoa quantity in alloxan-induced diabetes mellitus mice (*Mus musculus* L.).

MATERIALS AND METHODS

This study used 48 male mice obtained from the Department of Pharmacy at Universitas Sumatera Utara, Medan, Indonesia. Average age and weight for the mice were 60 days ±5 days and 30±5 g,

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respectively. Mice were placed in a plastic cage covered with husks; husks were changed twice a week. The cage was covered with wire and the mice were given food and drink *ad libitum* every day in accordance with the Ethical Clearance from the Department of Biology at Universitas Sumatera Utara.

The experiment used a completely randomized design with 8 treatments composed of 3 controls (normal, negative, and positive) and 5 treatments of hesperetin (Sigma Aldrich) in various concentrations. The number of treatments and replications in both tests fulfilled the Federer formula, $(t-1) \times (n-1) \geq 15$. Each treatment was replicated 6 times. The distribution of each treatment is C(0): normal control (without alloxan (Sigma Aldrich) induction and without treatment); C(–): negative control (with alloxan induction and without treatment); C(+): positive control (with alloxan induction and 150 mg/kg metformin treatment); T(1): treatment one (with alloxan induction and 50 mg/kg hesperetin treatment ; T(2): treatment two (with alloxan induction and 100 mg/kg hesperetin treatment); T(3): treatment three (with alloxan induction and 150 mg/kg hesperetin treatment); T(4): treatment four (with alloxan induction and 200 mg/kg hesperetin treatment); and T(5): treatment five (with alloxan induction and 250 mg/kg hesperetin treatment).

Alloxan solution in water was administered to the animals by intramuscular induction with a dose of 150 mg/kg body weight. Blood glucose level was checked on the 7th day after induction using a glucometer. The tail of each mouse was cleaned with cotton dipped in warm water. Blood from the tail tip dripped onto a strip test and was read with a glucometer. Mice with blood glucose levels >200 mg/dL were diabetic and ready to be tested. The test-ready mice were given an extract according to the treatment dose. Hesperetin was dissolved with water and given to the mice orally at 0.3 mL for 8 weeks. During 8 weeks trial, the blood glucose level of mice was measured once a week after the mice fasted for 24 hours.

The Examination of Sperm Quality

Mice were killed by anaesthetic with ether and neck dislocation before being operated on to isolate the testes and epididymis. The epididymis was taken from the testes, and spermatozoa samples were taken immediately after operating by slicing and pressing the cauda epididymis slowly. Spermatozoa motility, viability, morphology, and quantity were then observed. Testicular weight and volume, diameter of seminiferous tubules, and thickness of testicular germinal epithelial layer were also determined. These methods were modified from Nak-Ung *et al*. (2018); and Al-Saeed *et al*. (2019).

Observation of spermatozoa motility was performed using a 100 fold magnification microscope. Spermatozoa motility was calculated by the formula $(A + B) = (A + B + C + D) \times 100$. The motility of spermatozoa was grouped into the categories: fast progressive (A), slow progressive (B), non-progressive (C), and immotile spermatozoa (D) as they were counted. Observations of spermatozoa viability was conducted by placing one drop of spermatozoa sample in a petri dish. One drop of 0.9% NaCl solution was added and mixed using a glass rod. The suspension was observed.

Morphology of spermatozoa was examined as follows: spermatozoa suspensions were placed on a glass object, to which 1 drop of 70% methanol was added. This was fixed to dry with Canadian balsam and covered with a glass cover. The number of normal spermatozoa and abnormal spermatozoa was observed under a microscope with a 100-fold magnification microscope. The results obtained were calculated with the formula $a = (a + b) \times 100$. Spermatozoa morphology was grouped into the categories normal morphology (a) and abnormal morphology (b). Normal mouse spermatozoa consist of a head (caput) that forms a hook like head, a short middle section, and a very long cauda.

For measuring the spermatozoa quantity, suspended spermatozoa were homogenized with 0.9% NaCl. Then 0.1 mL of the sample was added to the Neubauer improved hemocytometer and topped with a glass cover. Under a light microscope with a 100-fold magnification, a hemocytometer was placed and the number of spermatozoa was counted in rooms 1, 2, 3, 4, and 5. The spermatozoa quantity was calculated by $N = 2 \times 10^5$. N is the number of spermatozoa calculated in boxes 1, 2, 3, 4, and 5 on the Neubauer improved hemocytometer.

The Examination of Testicle

The testicular weight was measured by weighing the testes. The testicular volume was calculated by measuring the length and width of the testes, using a calliper and millimetre paper. The testicular volume was calculated according to the formula $(\pi \times W2 \times L) = 6$. The testicular volume in mm^3 was determined with π, testicular width (W), and testicular length (L).

Preparations of testicular histology were carried out using the paraffin method with hematoxylin eosin double stain. The paraffin method required fixation, washing, dehydration, purification, paraffin infiltration, implantation, incision, sticking, deparaffinization, staining, closing, and labelling.

The diameter of the seminiferous tubules and the thickness of the testicular germinal epithelial layer were determined using a 100x microscope and computer program Axiocam 8.0. Measurements were conducted on seminiferous tubules which were round or close to each other. Figure 1 shows the overview of the seminiferous tubules diameter and testicular germinal epithelial layer thickness. D is the diameter of the seminiferous tubules; E is the thickness of the testicular germinal epithelial layer with Hematoxylin Eosin and 100x magnification (Altoe *et al*., 2014).

Data Analysis

Data were arranged with the software Statistical Product and Service Solutions. The observed data were analyzed statistically using the parametric analysis of variance test. If the results were significant, analysis continued via Duncan test with degree of significance $\alpha = 0.05$.

RESULTS AND DISCUSSION

The blood glucose level in the negative control group, positive control group, and the treatment group after alloxan induction was greater compared to the normal group without alloxan induction. This might be observed because alloxan compound is toxic and can damage pancreatic ß cells which produce insulin (Lenzen, 2008). Figure 2 shows the blood glucose level with various treatments from the first week to the eighth week.

After alloxan induction, blood glucose levels increased in the negative control group, positive control group, and treatment groups. Hesperetin and metformin treatments in alloxan-induced mice resulted in lower blood glucose levels than in the negative alloxan-induced control group. In the final week, it was found that the hesperetin and metformin treatment groups had significantly reduced blood glucose levels ($P<0.05$). The antidiabetic effect of hesperetin at 200 mg/kg body weight was not significantly different from the antidiabetic effect of metformin with dose 150 mg/kg body weight. In the eighth week, antidiabetic effects of hesperetin at all doses were not significantly different from the normal control group. Flavonoid acted as an active antidiabetic compound triggered insulin production and reduced blood glucose levels (Al-Ishaq *et al*., 2019; Caro-Ordieres *et al*., 2020).

All hesperetin treatment groups and metformin treatment groups declined in blood glucose level from the first week (W1). The greatest decrease in glucose level occurred in the 250 mg/kg hesperetin treatment group. The higher the dose, the greater the antidiabetic effect, indicating that flavonoid antidiabetic activity is dose dependent (Ahmed *et al*., 2012). As antioxidants, flavonoids play a role in lowering blood glucose levels (Banjarnahor and Artanti, 2014). Flavonoids can also prevent damage to insulin-producing β-cells by suppressing β cell apoptosis without changing the proliferation of pancreatic β cells (Gregory and Kelly, 2011). Oral antidiabetic drugs can trigger diabetic complications, including reproductive system disorders (Maiorino *et al*., 2014; Bahar *et al*., 2020). Therefore, antidiabetic drugs also have the potential to treat reproductive system disorders for people with diabetes (Meneses *et al*., 2015).

The spermatozoa quality and quantity decreased after alloxan induction. High blood glucose level disrupts blood flow due to vessel damage in several parts of the body, affecting spermatogenesis (Mangoli *et al*., 2013). Table 1 shows spermatozoa motility, viability, normal morphology, and quantity in the eight week, with various treatments. Table 2 shows the testicular weight, volume, and germinal epithelium thickness, along with the diameter of seminiferous tubules in the eight week, with various treatments.

Compared to the negative control group, the hesperetin treatment groups significantly affected the percentage of spermatozoa motility, viability, normal morphology, and quantity, as well as testicular weight,

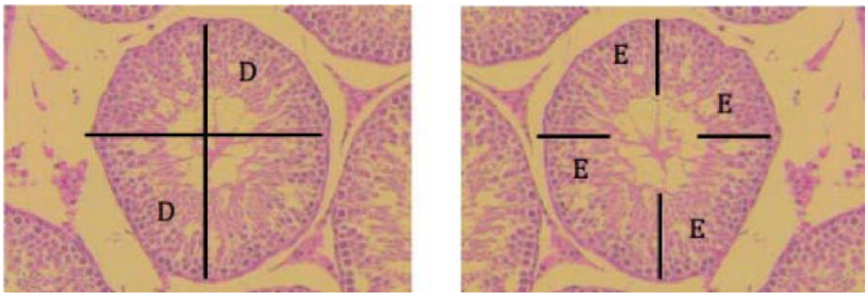

Figure 1. Overview of seminiferous tubules diameter and testicular germinal epithelium thickness

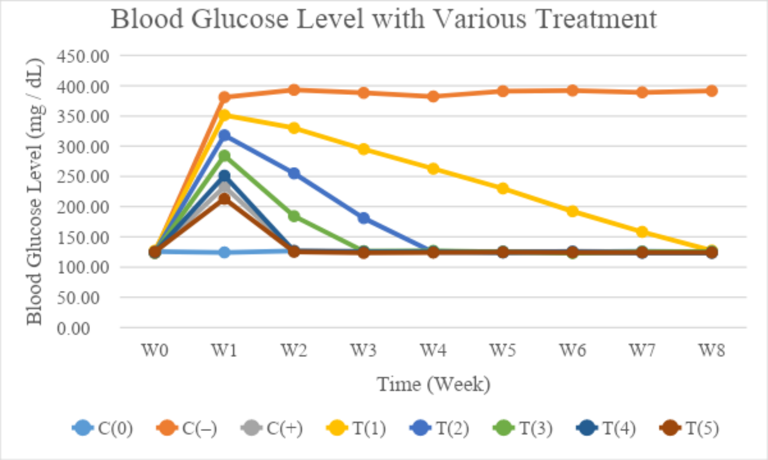

Figure 2. Blood glucose levels with various treatments from the first week to the eighth week. C(0)= Normal control (without alloxan and treatment); C(–)= Negative control (with alloxan and without treatment); C(+)= Positive control (with alloxan and with metformin 150 mg/kg body weight); T(1)= Treatment one (with alloxan and with hesperetin 50 mg/kg body weight); T(2)= Treatment two (with alloxan and hesperetin 100 mg/kg body weight); T(3)= Treatment three (with alloxan and hesperetin 150 mg/kg body weight); T(4)= Treatment four (with alloxan and with hesperetin 200 mg/kg body weight); and T(5)= Treatment five (with alloxan and hesperetin 250 mg/kg body weight)

Table 1. The spermatozoa motility, viability, normal morphology, and quantity in the eight week, with various treatments

Treatment	Motility (%)	Viability (%)	Normal morphology (%)	Quantity (n)
C(0)	81.32±13.03^{a}	98.31±3.56^{a}	97.55±1.82^{a}	253.15±51.35^{a}
C(–)	50.25±8.05^{b}	65.14±2.36^{b}	70.11±1.31^{b}	133.22±27.02^{b}
C(+)	64.05±10.26^{c}	89.33±3.24^{c}	88.93±1.66^{c}	249.12±50.53^{a}
T(1)	53.05±8.50^{b}	77.52±2.81^{d}	75.31±1.41^{d}	167.31±33.94^{c}
T(2)	57.77±9.26^{d}	82.24±2.98^{e}	79.96±1.49^{e}	201.19±40.81^{d}
T(3)	60.31±9.66^{d}	86.29±3.13^{c}	84.34±1.57^{f}	225.18±45.68^{e}
T(4)	63.82±10.23^{c}	88.82±3.22^{c}	89.02±1.66^{c}	266.21±54.00^{a}
T(5)	68.02±10.90^{e}	90.01±3.26^{c}	92.35±1.72^{c}	312.14±63.32^{f}

$^{a, b, c, d, e, f}$ Different superscripts within the same column indicate significant differences (P<0.05). C(0)= Normal control (without alloxan and treatment); C(–)= Negative control (with alloxan and without treatment); C(+)= Positive control (with alloxan and with metformin 150 mg/kg body weight); T(1)= Treatment one (with alloxan and with hesperetin 50 mg/kg body weight); T(2)= Treatment two (with alloxan and hesperetin 100 mg/kg body weight); T(3)= Treatment three (with alloxan and hesperetin 150 mg/kg body weight); T(4)= Treatment four (with alloxan and with hesperetin 200 mg/kg body weight); and T(5)= Treatment five (with alloxan and hesperetin 250 mg/kg body weight)

Table 2. The testicular weight, volume, and germinal epithelial layer thickness, in addition to the diameter of seminiferous tubules in the eigth week, with various treatments

Treatment	Testicular weight (mg)	Testicular volume (mm³)	Diameter (μm)	Thickness (μm)
C(0)	135.15±5.37^{a}	145.05±27.15^{a}	556.17±91.24^{a}	150.34±28.22^{a}
C(–)	99.23±3.94^{b}	82.31±15.41^{b}	331.29±54.35^{b}	64.51±12.11^{b}
C(+)	134.84±5.36^{a}	146.34±27.39^{a}	475.16±77.95^{c}	123.51±23.18^{c}
T(1)	107.14±4.26^{c}	105.34±19.72^{c}	400.16±65.65^{d}	96.55±18.12^{d}
T(2)	118.35±4.70^{d}	124.16±23.24^{d}	443.14±72.70^{e}	109.24±20.51^{e}
T(3)	134.14±5.33^{a}	144.92±27.13^{a}	479.02±78.58^{c}	122.94±23.08^{c}
T(4)	135.24±5.37^{a}	146.55±27.43^{a}	516.12±84.67^{f}	135.28±25.39^{f}
T(5)	135.08±5.37^{a}	145.34±27.20^{a}	560.15±91.89^{a}	148.22±27.82^{a}

volume, germinal epithelial layer thickness, and diameter of seminiferous tubules after alloxan induction and hesperetin treatment (P<0.05). Hesperetin treatment fully recovered the spermatozoa quantity, along with seminiferous tubules diameter and testicular weight, volume, and germinal epithelial layer thickness compared to the normal control. Hesperetin treatment could not fully recover but closely resembled the spermatozoa motility, viability, and normal morphology of the normal control. However, the effect of 200 mg/kg hesperetin treatment was not significantly different from 150 mg/kg metformin treatment. Hesperetin treatment improved spermatozoa quantity and quality compared to the negative control. This corroborates previous studies which reveal that plant extracts with high flavonoid content can significantly improve spermatozoa quality and quantity (Benko *et al*., 2019).

Flavonoids can affect the process of spermatozoa transport to increase motility (Jofré *et al*., 2019). Adenosine triphosphatase enzyme maintains internal homeostasis for sodium and potassium ions. Ion concentrations maintain the ability of the membrane to transport dissolved materials across the cell membrane. A disrupted sperm membrane interferes with the transport of spermatozoa essential nutrients (Saez *et al*., 2011; Delpire and Gagnon, 2018). Alloxan induction causes damage to the cells of the pancreas, interferes with insulin production, causes hyperglycemia, and increases reactive oxygen species. Reactive oxygen species damage the mitochondrial membrane, potentially causing a loss of function which can induce sperm cell apoptosis (Loeken, 2012; Noh *et al*., 2020).

Flavonoids thought to increase testosterone production play an important role in spermatozoa viability. They maintain the epididymis, which is a temporary storage area for mature spermatozoa, allowing them to survive until they are excreted through the penis (Martin and Touaibia, 2020). Good epididymis function increases the percentage of spermatozoa viability. When dye is applied, viable sperm will appear colourless, however, non-viable sperm will appear red due to the entry of dye across the damaged sperm head membrane (Popal and Nagy, 2013).

Increased spermatozoa abnormalities may also occur due to various disturbances, particularly during spermatogenesis at spermiogenesis (Abdelhamid *et al*., 2019). The shape of the spermatozoa changes based on decreased testosterone levels, on which the epididymis also depends. Thus decreased testosterone levels cause abnormal spermatozoa morphology (Orieke *et al*., 2019). Flavonoid can repair spermatozoa morphology by decreasing free radical activity. This increases fertility and decreases the percentage of abnormal spermatozoa. It produces a positive feedback loop that also leads to increased testosterone levels (Olayinka *et al*., 2019). The normal shape of the spermatozoa will directly affect the spermatozoa motility and integrity (Jakubik-Uljasz *et al*., 2020). Spermatozoa morphology also plays a role in fertilization, wherein if the abnormal number of spermatozoa is too high, fertility will decrease (Alahmar, 2019).

Flavonoid increases spermatozoa quantity because of hypoglycemic activity; flavonoid reduces the blood glucose level in diabetic mice (Shi *et al*., 2019). Phytochemicals such as flavonoids increase the number of spermatozoa in mice due to antioxidant activity in which they neutralize free radicals (Biagi *et al*., 2019). Antioxidants are hydrogen and electron donors for free radicals and thus they neutralize free radical activity (Aryal *et al*., 2019).

Increased testicular weight is probably due to the increased activity of spermatogenic cells that make up the structure and the weight of the testicles. Spermatogenic cells also increase spermatogonia and increase testicular weight (Parhizkar *et al*., 2014). Flavonoid increases testicular weight by increasing secretion of gonadotropin releasing hormone that can affect the anterior pituitary, which in turn secretes follicle stimulating hormone and luteinizing hormone (Das and Kumar, 2018). Luteinizing hormone maintains the growth of Leydig cells, increases follicle stimulating hormone, maintains spermatogenesis, and increases testicular weight (Ramaswamy and Weinbauer, 2014).

Citrus flavonoid has antioxidant properties that restore testicular volume after exposure to certain substances (Gandhi *et al*., 2020). Testicular volume is related to semen quality, which is influenced by male sexual libido. The existence of repeated stimulation increases the gonadotropin hormone, which induces the hormone testosterone for optimum spermatogenesis (Dutta *et al*., 2019; Swee and Quinton, 2019). Semen fluid is secreted from the accessory gland stimulated by testosterone. Semen production and the volume are related to testicular volume (Noda, 2019).

Diameter and thickness of seminiferous tubules are determined by the interaction between follicle stimulating hormone and luteinizing hormone (Oduwole *et al*., 2018; Kalwar *et al*., 2019). If follicle stimulating hormone is not produced, luteinizing hormone fails to maintain homeostasis, causing the diameter of the seminiferous tubules to shrink. Normal levels of follicle stimulating hormone maintain the diameter and the thickness of the seminiferous tubules (Gupta and Verma, 2018). A diabetic condition can inhibit supply of nutrients to the testicles and disrupt spermatogenesis (Ding *et al*., 2015). Flavonoids provide antioxidant effects which restore the diameter and thickness of seminiferous tubules because antioxidant compounds neutralize free radicals (Gayatri *et al*., 2017).

CONCLUSION

Hesperetin decreases blood glucose levels, increases spermatozoa quantity, and improves spermatozoa quality in alloxan-induced diabetes mellitus mice. Activity was dose-dependent with the optimum dose observed at 200 mg/kg body weight. It is recommended that the next study be conducted with a longer treatment period to see the extent of damage to the reproductive organs due to complications of alloxan-induced diabetes.

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