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Original article

Porang (*Amorphophallus oncophyllus*) tuber extract improved the histopathological features of diabetic rat (*Rattus norvegicus*) testicles

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ABSTRACT

This study aims to determine the effect of porang (*Amorphophallus oncophyllus*) tuber extract on the histopathological features of the testicle of alloxan induced rats (*Rattus norvegicus*). Thirty male rats were randomly divided into six groups. Rats in group C- were injected with distilled water, while rats in groups C+, T0, T1, T2, and T3 were injected with 120 mg/kg bw alloxan. Blood glucose was measured three days after alloxan injection. Rats in groups C- and C+ were then administered with 1% Na-CMC, whereas rats in groups T0, T1, T2, and T3 were then administered with 45mg/kg bw metformin, and 100, 200, and 400 mg/kg bw of porang tuber extract, respectively. All solution were given orally once every day for 14 days. Spermatogenic activity was assessed using the Johnsen scoring system and analyzed by Kruskal-Wallis, followed by the Mann-Whitney test. The diameter and epithelial thickness of the seminiferous tubule were measured using image raster software and analyzed using Anova followed by Duncan's test. The results showed that spermatogenesis score, diameter, and epithelial thickness of seminiferous tubule of group C+ were smaller ($p < 0.05$) than group C-. Spermatogenesis scores of groups T0, T1, T2, and T3 were higher ($p < 0.05$) than group C+. Seminiferous tubule diameter and epithelial thickness in groups T0, T1, and T2 were greater ($p < 0.05$) than group C+. It could be concluded that porang tuber extract at a dose of 200 mg/kg bw improved the spermatogenesis score, diameter seminiferous tubule, and thickness of the epithelium of diabetic rats.

Keywords: alloxan, diameter, epithelium thickness, Johnsen score, seminiferous tubules

INTRODUCTION

Diabetes mellitus was an endocrine disorder characterized by increased blood glucose levels. In Indonesia, human diabetes mellitus in 2045 was predicted at 28.6 million and was ranked 5th in the world (Dysted *et al.*, 2021). In pets, diabetes mellitus included all types of diabetes found in humans (Niaz *et al.*, 2018). The incidence of diabetes in dogs correlated with the

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incidence of diabetes in their owners, due to similar health behaviors such as physical activity levels and diet (Delicano *et al*., 2020). In cats, the prevalence of type 2 diabetes was more common than type 1 diabetes. Environmental risk factors that triggered type 2 diabetes in cats were old age, obesity due to a high-carbohydrate diet, male gender, sterilization, prolonged drug use, and lack of physical activity. In dogs, the prevalence of type 1 and 2 diabetes was almost the same. Latent autoimmune factors caused type 1 diabetes in dogs. Environmental factors such as feeding high-fat foods caused pancreatitis which then developed into diabetes (Rand *et al*., 2004).

A hyperglycemic state caused a decrease in antioxidant activity, thereby cell protection against free radicals was also weakened and worsens the damage caused by ROS (Papachristoforou *et al*., 2020). Oxidative stress induced DNA damage (Kilarkaje and Al-Bader, 2015), and mitochondrial damage, causing the release of cytochrome C and activate caspases which could cause apoptosis of cells in the testicular tissue (Takeshima *et al*., 2020). In addition, hyperglycemia caused damage to the blood vessel endothelium followed by microangiopathy, and interfered with the delivery of nutrients to the tissues (Kolluru *et al*., 2012).

The treatment of diabetes included oral hypoglycemic drugs, insulin injections, diet, or a combination of the three (Feingold, 2022). Only a few antihyperglycemic drugs were allowed to be given to pets (dogs and cats) such as insulin preparations and some oral hypoglycemic drugs (Shiel and Mooney *et al*., 2022). Metformin was a drug to control blood glucose levels in diabetes patients (Feingold, 2022). However, using metformin could cause side effects such as vomiting, lethargy, loss of appetite, and weight loss (Barrella *et al*., 2017). In dogs, insulin use could cause side effects of intracranial pressure followed by convulsions, hypoglycemia, hypoglycemic seizures, insulin resistance, and skin allergic reactions to the injections (Park *et al*., 2023). Meanwhile, long-term treatment of canine diabetes with herbal extracts was reported to cause no side effects and tolerability in animals (Suemanotham *et al*., 2023).

Herbal therapy had the potential to treat diabetes and could be studied in laboratory animals as a model (Niaz *et al*., 2018). Porang tubers (*Amorphophallus oncophyllus*) contained glucomannan, which was a water-soluble fiber that forms a gel in the stomach, thereby slowing down the process of releasing food substances from the stomach to the intestines (Anggela *et al*., 2020). The gel would fill the stomach and send a signal to the brain that the stomach is full. In diabetes patients, this would help relieving the burden on Langerhans beta cells (Rix *et al*., 2019). Glucomannan was fermented by the microflora of the large intestine and triggered the production of glucagon-like peptide (GLP-1) (Xu *et al*., 2023). GLP-1 was an incretin hormone that could stimulate insulin release by pancreatic beta cells and also improved insulin sensitivity (Müller *et al*., 2019). In addition, GLP-1 protected tissues against oxidative damage via the activation of the Nrf2 signaling pathway (Oh and Jun, 2017), and inhibited the chain reaction of ROS formation by completing the lack of electrons (Li *et al*., 2022). Therefore, this study aims to determine the effect of porang (*Amorphophallus oncophyllus*) tuber extract on the histopathological feature of the testicles of diabetic rats.

MATERIALS AND METHODS

This study's proposal had been ethically approved by the experimental animal research ethics committee of the Faculty of Veterinary Medicine Universitas Airlangga with reference number 1.Ke.041.04.2021. Extraction of Porang tubers was carried out in the Laboratory of Pharmacology, histological preparations were made in the Laboratory of Veterinary Pathology, and treatment of rats was carried out in the Laboratory of Experimental Animal, Faculty of Veterinary Medicine, Universitas Airlangga.

Porang tuber extraction

Porang tubers were peeled, sliced, washed, dried, ground, and sieved using a 60-mesh sieve.

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One kilogram of porang tuber powder was macerated using five liters of 96% ethanol for 3 x 24 hours and stirred once a day. The macerate was evaporated using a rotary evaporator at a speed of 50 rpm at 45°C until a thick extract was obtained (Agustina *et al*., 2021). The extract was redissolved in 1% Na-CMC to adjust the dose and volume.

Animal treatments

Thirty male Wistar rats aged 2-3 months, weighing 150-200 grams were acclimatized for seven days, then their blood glucose levels were measured (glucometer, Nesco). Blood samples were taken from the coccygeal vein. The rat's tail was cleaned with wet cotton wool and then cleaned again with 70% alcohol. The base of the tail was pricked with a small needle, then the blood that came out was then touched on the glucometer strip. Blood glucose levels was displayed on the screen after 10 seconds, expressed in mg/dL. Next, the rats were randomly divided into six groups. Rats in group C-were injected with 0.5 mL of distilled water, while rats in groups C+, T0, T1, T2, and T3 were injected with a single dose of 120mg/kg bw alloxan (Sigma Aldrich) intraperitoneally (Fajarwati *et al*., 2023). Blood glucose levels were measured again three days after alloxan injection. Rats were said to have diabetes if their blood glucose was more than 150 mg/dL (Furman, 2021). Rats in groups C- and C+ were then administered with 1% Na-CMC, whereas rats in groups T0, T1, T2, and T3 were then administered with 1% Na-CMC, 45mg/kg bw metformin (Akram, 2021), and 100, 200, and 400 mg/ kg bw/day of porang tuber extract, respectively. All solution were given orally in a volume of 1 mL once every day for 14 days. On day-15 (after treatment), the rats were sacrificed, and their testicles were taken for making histological preparations with Haematoxylin Eosin staining.

Spermatogenic activity scores

Assessment of spermatogenic activity was carried out using the Johnsen scoring system (Teixeira *et al*., 2019) (Table 1). Testicular histopathological scoring was carried out under a light microscope (Nikon Eclipse E100) with a magnification of 400 x in five fields of view, then averaged.

Table 1 Johnsen scoring system for evaluating spermatogenic activity (Teixeira *et al*., 2019)

score	histological criteria
10	Tubule epithelium is normal with complete spermatogenesis, tubule lumen is open containing more than ten spermatozoa.
9	Tubule epithelium is partially damaged with incomplete spermatogenesis, tubule lumen is closed but still contains many late spermatids, more than ten spermatozoa.
8	Tubule epithelium is damaged, tubule lumen is closed; there are few late spermatids and less than five spermatozoa.
7	Tubule epithelium is damaged, tubule lumen is closed; there are many early spermatids but no late spermatids or spermatozoa.
6	Tubule epithelium is damaged, tubule lumen is closed; there are a few (less than ten) early spermatids but no late spermatids or spermatozoa.
5	There are many (more or equal to five) spermatocytes, no spermatids or spermatozoa.
4	There are a few (less than five) spermatocytes, no spermatids or spermatozoa.
3	There are only spermatogonia.
2	There are only Sertoli cells
1	There are no cells in the tubules

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Diameter and epithelial thickness of seminiferous tubules

Measurement of the diameter and epithelial thickness of the seminiferous tubules was carried out under a light microscope (Nikon Eclipse E100) equipped with image raster software with 100x magnification for five replicates. The diameter of the roundest seminiferous tubules was measured from a point on the basement membrane to the opposite basement membrane on the same seminiferous tubule. The thickness of the seminiferous tubule epithelium was the distance from the point of the basement membrane to the tip of the epithelium in the lumen of the seminiferous tubule (Ravi *et al*., 2022).

Data analysis

Spermatogenesis score data were analyzed using the Kruskal Wallis test, followed by the Mann-Whitney test ($p < 0.05$). The diameter and epithelial thickness of the seminiferous tubule were analyzed using Anova followed by the Duncan's test ($p < 0.05$). Statistical analysis was conducted using Statistical Product and Service Solution for Windows version 23 software.

RESULTS

Blood glucose levels before injection in rats among treatment groups were not significantly different ($p > 0.05$). Three days after alloxan injection, the blood glucose levels of rats in groups C+, T0, T1, T2, and T3 group rats increased ($p < 0.05$) compared to before alloxan injection. There was no significant difference ($p > 0.05$) in the blood glucose levels of group C- rats three days later (after distilled water injection), and also between treatment groups after alloxan injection (Table 2).

Table 2 Rat blood glucose levels before and three days after alloxan injection

	before alloxan injection	after alloxan injection
C-	83.50 ± 14.50 ^{a}	(97.20 ± 8.29 ^{a})
C+	92.25 ± 10.76 ^{a}	205.75 ± 86.93 ^{b}
T0	109.00 ± 8.57 ^{a}	368.67 ± 97.88 ^{b}
T1	84.33 ± 3.34 ^{a}	358.00 ± 149.46 ^{b}
T2	104.00 ± 25.13 ^{a}	217.67 ± 51.03 ^{b}
T3	96.50 ± 6.01 ^{a}	192.35 ± 92.76 ^{b}

Different superscripts in one row indicate significant difference ($p < 0.05$); C-: normal rats as control; C+: alloxan induced diabetic rats untreated; T0: alloxan induced diabetic rats treated with metformin (45mg/kg bw); T1, T2, and T3: alloxan induced diabetic rats treated with porang (*Amorphophallus oncophyllus*) tuber extract at 100, 200 and 400 mg/kg bw, respectively; diabetes was induced by a single injection of 120mg/kg bw alloxan; treatment was given orally once daily for 14 days; replicate= 5.

Table 3 Means ± SD of spermatogenesis score, diameter, and epithelial thickness of seminiferous tubules of diabetic rats treated with porang (*Amorphophallus oncophyllus*) tuber extract

	spermatogenesis score	diameter (µm)	epithelial thickness (µm)
C-	9.80 ± 0.70 ^{d}	471.51 ± 10.06 ^{d}	93.56 ± 3.18 ^{d}
C+	5.18 ± 0.48 ^{a}	364.72 ± 19.50 ^{a}	71.63 ± 3.09 ^{a}
T0	8.20 ± 0.27 ^{c}	425.81 ± 13.32 ^{b}	78.20 ± 4.86 ^{b}
T1	8.46 ± 0.35 ^{cd}	426.57 ± 16.10 ^{b}	83.59 ± 1.20 ^{c}
T2	8.84 ± 0.18 ^{c}	449.49 ± 13.95 ^{c}	87.70 ± 4.19 ^{c}
T3	7.08 ± 0.58 ^{b}	377.46 ± 19.25 ^{a}	74.79 ± 3.15 ^{ab}

Different superscripts in one column indicate significant differences ($p < 0.05$); C-: normal rats as control; C+: alloxan induced diabetic rats untreated; T0: alloxan induced diabetic rats treated with metformin (45mg/kg bw); T1, T2, and T3: alloxan induced diabetic rats treated with porang

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(*Amorphophallus oncophyllus*) tuber extract at 100, 200 and 400 mg/kg bw, respectively; diabetes was induced by a single injection of 120mg/kg bw alloxan; treatment was given orally once daily for 14 days; replicate= 5.

In group C+, the spermatogenesis score, diameter, and epithelium thickness of the testicular seminiferous tubule were smaller ($p < 0.05$) compared to group C- (normal rats). Administration of metformin (group T0) or porang tuber extract (groups T1, T2, and T3) was followed by an increase ($p < 0.05$) in spermatogenesis scores compared to C+ group. The diameter and epithelial thickness of the seminiferous tubule in groups T0, T1, and T2 were higher ($p < 0.05$) than in group C+. However, the diameter and epithelial thickness of the seminiferous tubule in group T3 were smaller ($p < 0.05$) compared to those in groups C-, T1, and T2, and were not significantly different ($p > 0.05$) from group C+ (Table 3).

Histopathological features of the testicle of group C- rats showed normal tubular epithelium, complete spermatogenesis, open tubules lumen, and more than ten spermatozoa cells. In group C+ rats, there was damage to the epithelial structure of the seminiferous tubules, the number of spermatozoa and spermatid was less than five, and the number of spermatocytes was more than five. In group T0 rats, the seminiferous tubule epithelium was damaged, and the tubule lumen was closed, but there were more than ten spermatozoa. In groups T1 and T2 rats, the number of spermatozoa was less than ten, whereas in group T3, there was no spermatozoa and there were less than ten spermatids (Figure 1).

In group C+, the diameter and epithelial thickness of the testicular seminiferous tubule appeared smaller and there were empty areas between the seminiferous tubules. The diameter and epithelial thickness of the seminiferous tubule in groups T0, T1, and T2 were greater than those in group C+. There were empty areas between the seminiferous tubules in group T1. However, the diameter and thickness of the seminiferous tubule epithelium in group T3 appeared smaller and there were empty areas between seminiferous tubules compared to groups T0, T1, and T2 (Figure 2).

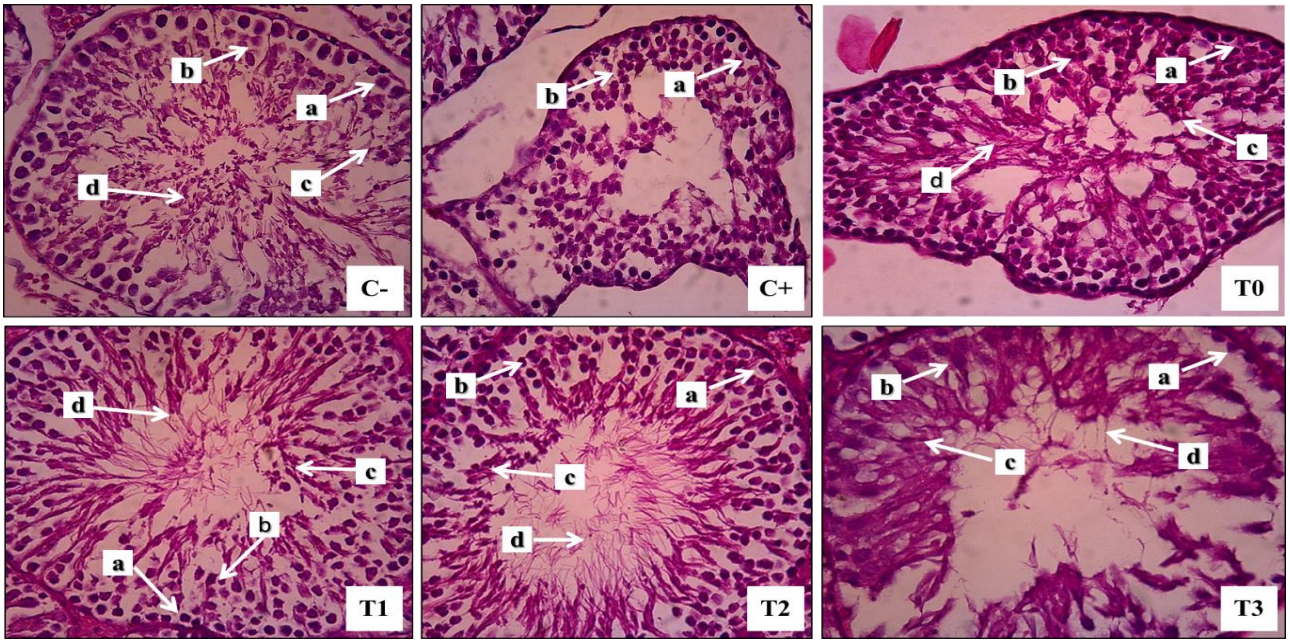

Figure 1 Representative histopathological image of spermatogenic activity in the seminiferous tubules of diabetic rats (*Rattus norvegicus*) treated with porang (*Amorphophallus oncophyllus*) tuber

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extract; C-: normal rats as control; C+: alloxan induced diabetic rats untreated; T0: alloxan induced diabetic rats treated with metformin (45mg/kg bw); T1, T2, and T3: alloxan induced diabetic rats treated with porang tuber extract at 100, 200 and 400 mg/kg bw, respectively; diabetes was induced by a single injection of 120mg/kg bw alloxan; treatment was given orally once daily for 14 days; Hematoxylin and Eosin staining; 400x magnification; a = spermatogonia; b = spermatocytes; c = spermatid; d = spermatozoa.

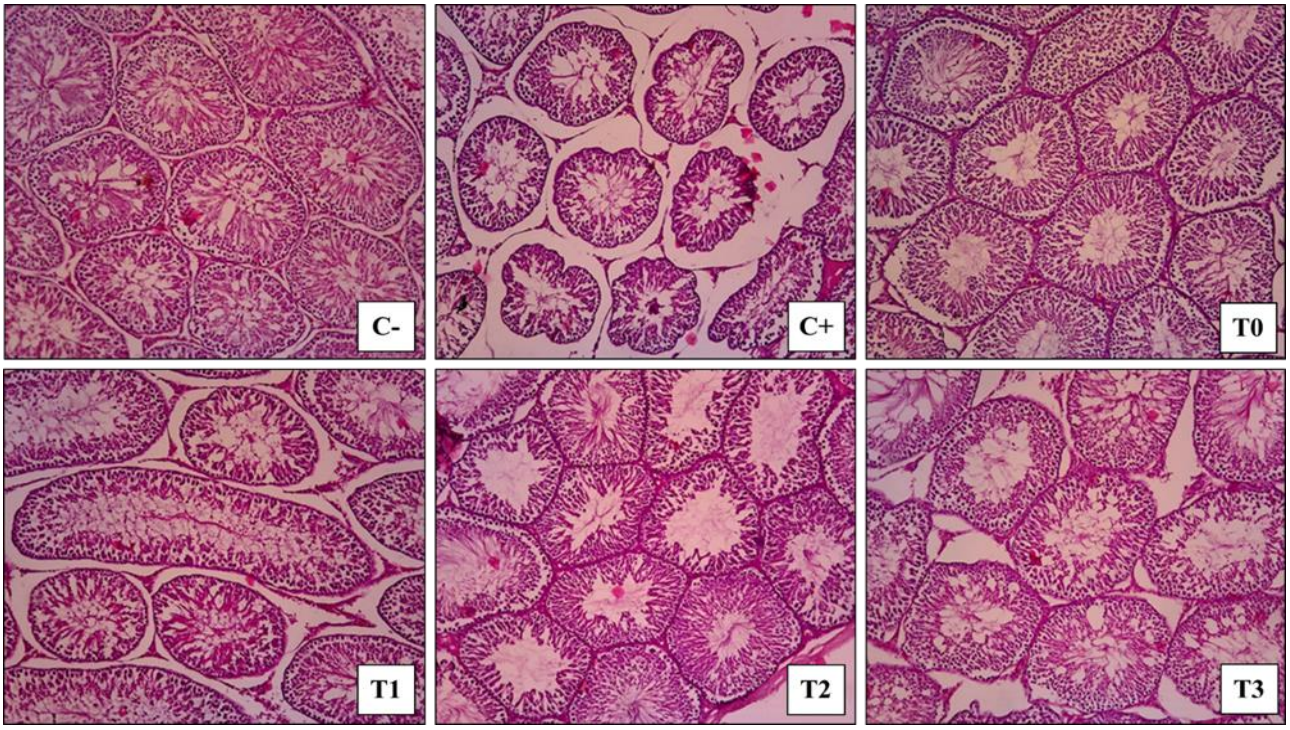

Figure 2 Representative histopathological images of diameter and epithelial thickness of seminiferous tubules of diabetic rats (*Rattus norvegicus*) treated with porang (*Amorphophallus oncophyllus*) tuber extract; C-: normal rats as control; C+: alloxan induced diabetic rats untreated; T0: alloxan induced diabetic rats treated with metformin (45mg/kg bw); T1, T2, and T3: alloxan induced diabetic rats treated with porang tuber extract at 100, 200 and 400 mg/kg bw, respectively; diabetes was induced by a single injection of 120mg/kg bw alloxan; treatment was given orally once daily for 14 days; Hematoxylin and Eosin staining; 100x magnification.

DISCUSSION

The blood glucose levels of rats before alloxan injection in this study (83.50-109.00 mg/dL) were in the normal range, the same as reported by Rehman *et al.* (2023) (50-135 mg/dL). In rats injected with alloxan, blood glucose levels increased to 192.35-368.67 mg/dL, while in negative control, it was relatively normal (97.20 ± 8.29 mg/dL). Rats became diabetic with glucose levels of 254.71 ± 7.43 mg/dL seven days after injection, whereas in normal rats it was 88.60 ± 2.99 mg/dL (Ibrahim *et al*., 2023). Alloxan induced diabetic hyperglycemia and could reach blood glucose levels of more than 200 mg/dL in rats one hour after administration (Ighodaro *et al*., 2017). Their blood glucose levels could reach 4.26 times higher than normal rats (Ojiako *et al*., 2015).

Spermatogenesis scores, diameter, and epithelial thickness of the seminiferous tubules in the testicles of alloxan induced diabetic rats without treatment were smaller than those of normal rats. Hyperglycemia induced dysfunction of glucose metabolism in rat testicles leading to

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increased oxidative stress. Normally, in the testicles there were antioxidant enzymes (catalase, CAT, glutathione peroxidase, GPX) which played a role in cleaning excess ROS and preventing lipid peroxidation. This mechanism would reduce the risk of DNA damage and cell death. However, continuous oxidative stress due to hyperglycemia caused CAT and GPX activity to be unable to maintain ROS balance due to glucose autoxidation and non-enzymatic glycation. The hyperglycemic state affected Sertoli cell metabolism (Brodjonegoro *et al*., 2021). In diabetic patients, long-term hyperglycemia caused insulin resistance syndrome. Insulin deficiency and resistance could damage the hypothalamus, pituitary gland, gonads, and perigonads. This could decrease the secretion of sex hormones including GnRH, FSH, LH, and testosterone. Impaired reproductive hormone function could cause testicular atrophy, stromal cell atrophy, damage to seminiferous tubules, Leydig cells, Sertoli cells, spermatogenic cell damage, sperm DNA, and sperm apoptosis (He *et al*., 2021). Changes in sperm characteristics were often observed in male diabetic animals (Ding *et al*., 2015). Sperm number, motility, and morphology were considered markers of sperm characteristics (Krausz and Farnetani, 2023). Spermatogenesis and testosterone levels were lower in humans with diabetes than in healthy individuals (Zhong *et al*., 2021). Diabetes disrupted sperm characteristics through mechanisms involving ROS production and the formation of lipid peroxidation (González *et al*., 2023). Excessive ROS production disrupted mitochondrial membrane potential and reduced energy availability, so that it could inhibit sperm motility (Nowicka-Bauer and Nixon, 2020).

Administration of metformin at a dose of 45 mg/kg bw to diabetic rats was followed by an increase in the spermatogenesis scores, diameter, and thickness of seminiferous tubules. The spermatogenesis scores in these rats were the same as in normal rats, however, the diameter and thickness of the seminiferous tubules were still lower than in normal rats. Metformin worked directly or indirectly on the liver to reduce glucose production. Metformin acted on the intestine to increase glucose utilization, increase GLP-1, and alter the microbiome, activating AMPC-dependent duodenal pathways to decrease hepatic glucose production (Almuttairi, 2023). Metformin inhibited the mitochondrial respiratory chain in the liver, causing AMPK activation, increasing insulin sensitivity, and reducing cAMP, thereby reducing the expression of gluconeogenic enzymes (Rena *et al*., 2017).

Administration of 100, 200, and 400 mg/kg bw/day of porang tuber extract to diabetic rats was followed by an increase in spermatogenesis scores compared to diabetic rats. The ethanol extract of porang leaf contained alkaloids, flavonoids, saponins, tannins, and steroids. The ethanol extract of porang leaf had antioxidant activity in the strong category with an IC50 value of 93.04 µg/mL and vitamin C had antioxidant activity in the very strong category with an IC50 value of 1.01 µg/mL (Pulungan *et al*., 2022). Several studies using extracts that had antioxidant properties had been reported to improve the reproductive system in diabetic males. Johnson *et al*. (2019) showed that the use of *Antrodia cinnamomea* extract in diabetic rats resulted in a reduction hyperglycemia and insulin resistance, followed by a reduction in lipid peroxidation levels, a reduction in oxidative stress in the endoplasmic reticulum and mitochondria, an improvement in sperm parameters, and an increase testicular structural integrity. In addition, LH, FSH, and testosterone levels increased (Johnson *et al*., 2019). Administration of *Momordica charantia* extract resulted in a decrease in blood glucose and HbA1c as well as an increase in serum insulin, TST, and gonadotropin levels in diabetic rats. Momordica charantia extract induced the recovery of testicular antioxidant enzymes, improved testicular histopathological structure, and reduced spermatogenic and Sertoli cell apoptosis. Decreased testicular cell apoptosis was characterized by an increase in Bcl-2 and a decrease in Bax and caspase-3 (Soliman *et al*., 2020).

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Reproductive hormones such as testosterone, FSH and LH were biomarkers of male reproductive function. LH influenced the release of testosterone by Leydig cells and stimulated FSH to bind to Sertoli cells to stimulate the spermatogenesis process (Oduwole *et al*., 2021). Serum testosterone and gonadotropin levels were lower in diabetic rats than in healthy rats (Soliman *et al*., 2020). Low serum testosterone levels associated with testicular dysfunction had been reported in diabetic rats due to glycemia and oxidative stress in Leydig cells caused by diabetes (Dimakopoulou *et al*., 2019). Low insulin levels inhibited gonadotropin secretion, thereby reducing testosterone release in testicular tissue (Ahn *et al*., 2013). The results of this study were in line with the report that the administration of *Momordica charantia* as an antioxidant could ward off free radicals, and increase testosterone, FSH, and LH levels in diabetic rats (Soliman *et al*., 2020). Testosterone levels were found to be negatively correlated with HbA1c values (Hu *et al*., 2023).

CONCLUSION

Administration of 100 and 200 mg/kg bw of porang tuber extract to diabetic rats improved the histopathological features of the seminiferous tubules, where a dose of 200 mg/kg bw gave better results. Meanwhile, administration of 400 mg/kg bw of porang tuber extract did not provide any improvement.

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AUTHOR'S CONTRIBUTIONS

Dwi Puspa Sukma Viranda (DPSV), Rochmah Kurnijasanti (RK), Pudji Srianto (PS), Hani Plumeriastuti (HP), Kadek Rachmawati (KR), Iwan Sahrial Hamid (ISH), Lita Rakhma Yustinasari (LRY).

DPSV and RK: conceived the idea, designed the mainframe of this manuscript, , and manuscript drafting under the supervision DPSV and PS: acquisition, analysis and interpretation of data. KR, ISH, LRY: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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