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The Effect of Shrimp Shell (*Litopenaeus vannamei*) Extract on Testicular Parameters of Streptozotocin-induced Diabetic Rats

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder that has become a major health problem worldwide. Reproductive dysfunction is one of the main complications of DM, particularly in men. However, as is known, shrimp shell extract contains nutrients, such as astaxanthin, that affect reproductive traits. The present study aimed to evaluate the effect of shrimp shell extract on the volume, weight, and histological features of the testes of a DM rat model. Fifteen adult male rats were randomly divided into three groups. Group A (n = 5) was a healthy control group, group B (n = 5) was a DM control group, and group C (n = 5) was a DM group treated with shrimp shell extract. Rats in groups B and C were treated with streptozotocin to induce DM. Rats in group C were given shrimp shell extract at 25 mg/kg body weight for 30 consecutive days after DM induction. Testicles were collected and submitted to dimension, weight, and histological examinations. The testicle volume and weight of rats in group C were significantly higher and heavier, respectively, than rats in group B and did not differ from rats in group A. The seminiferous tubule diameter of rats in group C was significantly larger than rats in group B and did not differ from rats in group A. Rats in group B had a lower testicle volume and lighter testicle weight as well as a shorter seminiferous tubule diameter than rats in groups A and C. In conclusion, shrimp shell extract could improve male fertility parameters in a DM rat model. However, the mechanism of action needs to be studied further.

Keywords: Astaxanthin, Diabetes mellitus, Fertility, Seminiferous tubule, Testis

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder that has become a major health problem worldwide. A previous study reported that in 2015, there were 415 million people with DM worldwide; this number is predicted to rise to over 642 million by 2040 (Ogurtsova et al., 2017). In 2012, there were 1.5 million deaths due to DM (Ogurtsova et al., 2017). This disease could affect the quality of a patient's life due to its many complications. One of the complications is reproductive dysfunction (Shi et al., 2017). It has been recognized that abnormal blood glucose levels can impair reproductive function in men with DM (Maresch et al., 2018). DM can affect reproductive function, including ejaculation, penile erection, fertility, sperm maturation, and spermatogenesis (Ding et al., 2015; Nna et al., 2017). Insulin-based therapy was introduced in the 1920s and is still the primary treatment for patients with type 1 DM and some patients with advanced stages of type 2 DM (Tavares et al., 2018). In addition, there are some antidiabetic drugs for type 2 DM, such as sulfonylureas, meglitinide, biguanides, and thiazolidinediones (Skliros et al., 2016). Studies regarding the effectiveness of existing antidiabetic drugs on the male reproductive system have been carried out in animal models of DM (Adaramoye and Lawal, 2014; Alves et al., 2014; Ayuob et al., 2015; Zaidi et al., 2017; He et al., 2021). Although the use of antidiabetic drugs is relatively safe and they have been widely prescribed in patients with DM, some side effects, such as hypoglycemia, hyperlactatemia, or metabolic acidosis, may still result from long-term use of currently available antidiabetic drugs (Anagnostis et al., 2018; Wang and Hoyte, 2019).

Many studies have concerned the role of natural products on DM and male reproductive functions (Tran et al., 2020; Swelum et al., 2021; Thikekar et al., 2021; Fu et al., 2022). Some researchers have used whole plants or part of plant extracts, such as *Chlorophytum borivilianum* (root), *Amaranthus spinosus* (stem), *Danae racemosa* (leaves), and *Nigella sativa* (seeds), while others have used just specific compounds such as phenols, flavonoids, and flavanones isolated from plants (Nna et al., 2017). However, most of those natural products are not included in the food ingredients consumed daily by people. In this study, *Litopenaeus vannamei* shell extract was used in an animal model of DM to evaluate its effect on male reproductive organs. *Litopenaeus vannamei* is one of the most widely cultivated shrimp species besides *Penaeus monodon* and *Penaeus chinensis* (FAO, 2014). Shrimp is commercialized as seafood and is usually sold whole or sometimes only the meat of shrimp. Thus, shrimp shells are abundant in solid waste and underutilized in the food industry. Shrimp shells still contain some nutrients, such as minerals, proteins, chitin, and

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chitosan (Cavalcanti et al., 2016). Chitin has a variety of biological and biomedical uses, including tissue healing. Chitosan is also known for its potential therapeutic effects, including anti-inflammatory, antioxidant, antidiarrheal, and anti-Alzheimer's disease effects (Satitsri and Muanprasat, 2020). Chitosan can enhance the size of the antral follicle, the number of endometrial arterioles, and the endometrial thickness of female rats exposed to lead acetate (Purwitasari et al., 2019). Although mammals do not have endogenous chitin (Ohno et al., 2013), a previous study demonstrated that chitosan, a derivative of a natural carbohydrate biopolymer derived from chitin deacetylation, could improve sperm count and the motility of progressive sperm of lead-acetate-induced rats (Marianti et al., 2020). This suggests that chitin and/or chitosan may be involved in the male reproductive system. In addition, shrimp shell extract contains astaxanthin, which is known to have good effects on the male reproductive system in some species, such as rainbow trout and discus (Ahmadi et al., 2006; Haque et al., 2023). Astaxanthin could have a protective effect on sperm mitochondrial function and also ameliorate testicular heat stress and reproductive poison damage (Liu et al., 2016). However, research concerning the effect of astaxanthin supplementation via shrimp shell extract on the male reproductive organs in the DM condition is still limited. The present study aimed to evaluate the effect of shrimp shell extract on the volume, weight, and histological features of the testicular organ of a DM rat model.

MATERIALS AND METHODS

Ethical approval

The experimental protocols carried out in this study had been approved by Universitas Kristen Duta Wacana (UKDW) Medical Research Ethics Committee with Ethical Clearance Certificate Number: 1265/C.16/FK/2021.

Shrimp shell extraction

Fresh shrimp (*L. vannamei*) was obtained from a fish market located on the south beach of Java, Indonesia, during the rainy season in July 2021. Shrimp shell extraction was carried out at the Biotechnology for Health Laboratory (Indonesia). Shrimp shells were separated from the flesh manually and washed with running water. Shrimp shells were dried using an oven at 40°C for approximately a day. Then, shrimp shells were ground into powder. Dried shrimp shell powder was subjected to a 3-day extraction using a maceration method as described by Najoan et al. (2021). Ethanol (70%) was used as the solvent at a 1:10 (v/v) ratio with water. The maceration was repeated two times for 3 days, respectively. Subsequently, evaporation was carried out using a rotary evaporator at 5 rpm and 40°C, and then continued in an oven at 40°C until the consistency was like a paste (Najoan et al., 2021).

Experimental animals

A total of 15 male Wistar rats (*Rattus norvegicus*) aged 8-12 weeks with an average body weight of 190 g from the Faculty of Biology, Universitas Gadjah Mada, Indonesia, were used in this study. Before the experiment began, acclimatization was carried out for 7 days. The rats were given access to water and food *ad libitum* during this period. The rats were maintained in five plastic boxes, each with three rats at room temperature in a tropical environment with a 12-h photoperiod. After acclimatization, the rats were divided randomly into three groups. Group A (n = 5) was a healthy control group, group B (n = 5) was a DM control group, and group C (n = 5) was a DM group treated with shrimp shell extract. Group A did not receive any treatment during the experiment. In groups B and C, DM was induced by intraperitoneally injecting 50 mg/kg body weight (BW) of streptozotocin (STZ) (Cayman Chemical, USA) diluted in citrate buffer. The STZ dosage for DM induction was previously described by Suman et al. (2016). Three days after DM induction, a blood sample was collected via the caudal vena cava to measure blood glucose levels using glucose meters (OneTouch, USA). The diabetic condition was proven by high blood glucose levels (≥ 150 mg/dL) (Furman, 2021). After inducing DM, rats in group C were given shrimp shell extract at the dosage of 25 mg/kg BW, and rats in group B were given 1 ml of sterile water as a placebo. The shrimp shell extract dosage was chosen according to a previous study by Wisaksono et al. (2021). Sterile water for the rats in group B and shrimp shell extract for the rats in group C was administered orally via gavage for 30 consecutive days.

Sample collection and histology slide preparation

After 30 days of oral treatment using shrimp shell extract, all rats were euthanized for sample collection. Before euthanasia, rats were anesthetized using tiletamine and zolazepam (Zoletil, Virbac, India) at 20 mg/kg BW (Limprasutr et al., 2021). After the rat was fully anesthetized, indicated by the absence of a pedal reflex (Sivula and Suckow, 2018), it was euthanized by cutting the respiratory tract and carotid vessel in the cervix. The testicles were removed from their scrotum and fixed using a 10% formalin solution. The testicles were measured for dimension and weight before being processed for histological staining. Testicle tissue was trimmed, processed with paraffin, and cut at 5 µm thickness. The tissue slides were placed on the slide warmer for 30 minutes. Subsequently, tissue slides were deparaffinized using xylene and rehydrated using a graded series of alcohol. Haematoxylin and eosin (HE) staining was performed before the

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dehydration and clearing processes. Then, the slide was mounted with a cover slip. Histological examination was carried out using a light microscope (Olympus, Tokyo, Japan) with 40× magnification.

Data collection

The dimensions of the testicle, including length (l), width (w), and thickness (t) were measured using a vernier calliper. These dimensions were used to calculate the volume of the testicle by using the formula of volume (v) for ellipsoid ($v = [\pi/6] \times l \times w \times t$) (Van der Plas et al., 2013). The testicle weight was measured with a digital scale (Camry Scale, USA). The seminiferous tubule diameter was determined from histology slides. Three photomicrographs were taken for each histology slide using a digital camera connected to a light microscope. The objective lens was 4× magnification, and the total magnification was 40×. The diameter of 10 seminiferous tubules in each photomicrograph was measured using the Image Raster 3.0 software (Optilab, AZ 85012, USA). Finally, the mean seminiferous tubule for each sample was calculated.

Data analysis

Statistical analyses were conducted using SAS version 9.4 (SAS Inst. Cary, NC, USA.). The data are presented as mean ± standard error. Descriptive statistics were analyzed by using the MEANS Procedure. The testicle volume, testicle weight, and seminiferous tubule diameter of each group were analyzed by multiple analyses of variance using the generalized linear model procedure. Least-squares means were obtained from each group of the variables and were compared by using Tukey-Kramer adjustment for multiple comparisons. Correlation analysis between seminiferous tubule diameter and testicle volume, and testicle weight was carried out using Pearson correlation analysis of SAS. For all the statistical analyses, $p < 0.05$ was considered statistically significant.

RESULTS

There were 14 rats included at the end of this study. One rat from group C was excluded due to inferiority within the group. The average testicle volume and testicle weight of the rats differed significantly between the three groups ($p < 0.05$, Table 1). The testicle volume of rats in group B was significantly lower compared with the testicle volume of rats in groups A and C ($p < 0.05$). The rats in group C had the highest testicle volume, but it was not significantly different compared with the testicle volume of the rats in group A ($p > 0.05$). The testicle weight of rats in group B was significantly lower compared with the testicle weight of the rats in group A and group C ($p < 0.05$; Table 1). The rats in group C had the highest testicle weight, but it was not significantly different compared with the testicle weight of the rats in group A ($p > 0.05$).

The average seminiferous tubule diameter of the rats differed significantly between the three groups ($p < 0.05$) (Table 1). The seminiferous tubule diameter of the rats in group B was significantly shorter than that of the rats in groups A and C ($p < 0.05$). The rats in group C had the largest seminiferous tubule diameter, but it was not significantly different compared with the seminiferous tubule diameter of the rats in group A ($p > 0.05$). Pearson correlation analysis showed a strong and significant relationship between the seminiferous tubule diameter and testicle volume, and testicle weight ($p < 0.05$, Table 2).Histological observation showed that the rats in groups A and C had rounder seminiferous tubules compared with the rats in group B. Some grooved surfaces of seminiferous tubules could be observed in the rats in group B (Figure 1, black arrows).

Table 1. Testicular parameters in a diabetic rat model treated with shrimp shell (*Litopenaeus vannamei*) extract in Indonesia

Testicular parameters	Group A	Group B	Group C
Number of samples	5	5	4
Testicle volume (cm^3)	1.07 ± 0.07^a	0.54 ± 0.07^b	1.21 ± 0.08^a
Testicle weight (g)	1.17 ± 0.08^a	0.62 ± 0.08^b	1.26 ± 0.09^a
Seminiferous tubule diameter (µm)	329 ± 9^a	240 ± 9^b	339 ± 10^a

The values are presented as the mean ± standard error. Group A: healthy control group; group B: Diabetes mellitus control group; group C: Diabetes mellitus group treated with shrimp shell extract. Different superscripts indicate significant differences in rows ($p < 0.05$).

Table 2. Pearson correlation analysis of seminiferous tubule diameter, testicle volume, and testicle weight in a diabetic rat model treated with shrimp shell (*Litopenaeus vannamei*) extract in Indonesia

Variables \ Seminiferous tubule diameter	Correlation coefficient (r)	p value
Testicle volume	0.940	<0.001
Testicle weight	0.937	<0.001

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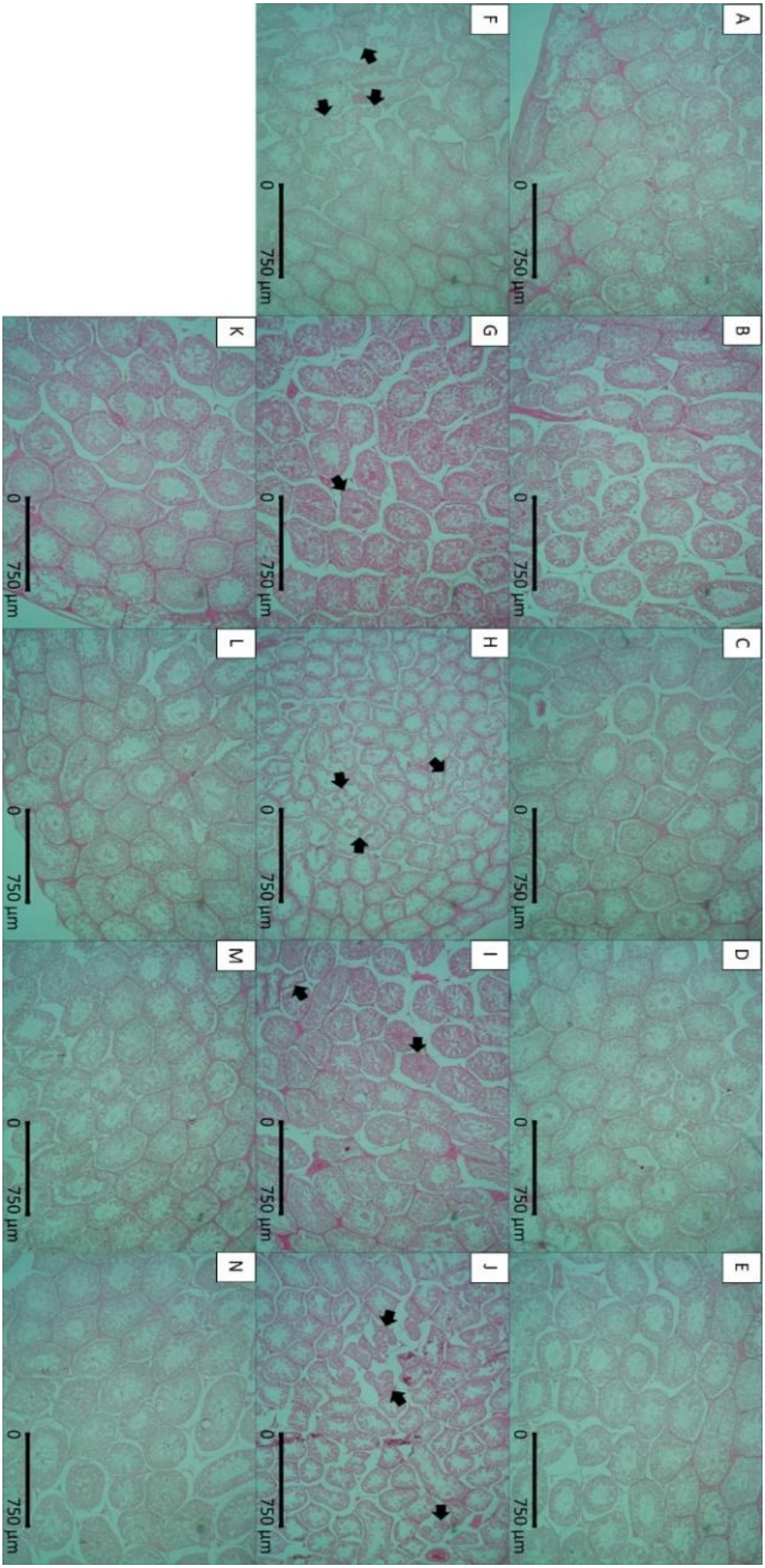

Figure 1. Histological changes in seminiferous tubules of diabetic rats. **A-E:** The healthy control rats (Group A); **F-J**: The diabetes mellitus (DM) rats (Group B); **K-N:** The diabetic rats treated with shrimp shell extract (Group C).

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DISCUSSION

The protocols for STZ-induced insulin deficiency and hyperglycemia in mice and rats have been well established (Furman, 2021). Suman et al. (2016) used the same STZ dosage to induce type 2 DM in combination with a high-fat diet. STZ injection increases glucose, insulin, free fatty acid, and triglyceride concentrations. A single intraperitoneal injection of a low STZ dose (30 mg/kg BW) in adult male Wistar rats affects pancreatic β-cells as well as the reproductive system via its diabetogenic effect (Omolaoye et al., 2018). Researchers have also reported adverse effects of STZ-induced DM on the male reproductive system in experimental animals (Omolaoye et al., 2018; Kotian et al., 2019; Sampannang et al., 2020). Maresch et al. (2019) demonstrated two major pathways of hyperglycemia-induced organ damage in the testis and epididymis, namely the diacylglycerol-protein kinase C pathway and the polyol pathway. The present study demonstrated that compared with the rats in group A, the rats in group B showed a significant decrease ($p < 0.05$) in the testicle volume, testicle weight, and seminiferous tubule diameter after 30 days of STZ injection from 1.07 cm^3, 1.17 g, and 329 µm, respectively, to 0.54 cm^3, 0.62 g, and 240 µm, respectively.

Shrimp shells are a waste product in the food industry. However, some nutrients contained in shrimp shells are still useful, such as chitosan (de Queiroz et al., 2017). *N,O*-Carboxymethyl chitosan, a derivative of chitosan, can be used to increase fiber contents; the resilience of food storage; and the stability of nutrients, including lowering the levels of dry substances, lowering the ash content, increasing the protein content, maintaining the fat content, and increasing the level of nitrogen-free extract (Kusuma et al., 2015). Nadapdap et al. (2014) demonstrated that supplementation with chitosan derived from shrimp shells could improve sperm count, normal sperm morphology, sperm motility, and sperm viability of Wistar rats treated with lead. The possible mechanism for this is that chitosan binds to the lead and forms bonds that make it hydrophilic and thus excretable via urine, thus reducing the male reproductive side effects of lead. However, the direct mechanism of action of chitosan on the male reproductive system is still unclear. Abd El-Hakim et al. (2020) demonstrated that a combination of chitosan-stabilized selenium nanoparticles and metformin could increase sperm motility, sperm viability, and sperm concentration and reduce sperm abnormality in an STZ-induced DM rat model. This suggests that chitosan may act as a delivery agent for the other substance. In addition, astaxanthin can be found in the shrimp shell when extracted using a maceration method with 70% ethanol as the solvent (Wisaksono et al., 2021). Astaxanthin is a xanthophyll carotenoid found in various microorganisms, marine animals, and crustaceans, including shrimp shells (Higuera-Ciapara et al., 2006; Ambati et al., 2014; Wisaksono et al., 2021). Astaxanthin has many biological activities and health benefits, such as antioxidant, anti-lipid peroxidation, anti-inflammatory, and anticancer activities; cardiovascular disease prevention; and immunomodulation (Visioli and Artaria, 2017; Faraone et al., 2020; Fouad et al., 2021). Martínez-Álvarez et al. (2020) stated that the use of astaxanthin and astaxanthin-containing lipid extracts as a food ingredient might have a double function: a technological function because they can provide foods with attractive reddish color and a bioactive function (for example, antioxidant activity) when consumed. Moreover, astaxanthin is safe to consume daily at a dosage ranging from 2 to 24 mg (Brendler and Williamson, 2019).

The use of astaxanthin in DM has been studied by many researchers (Feng et al., 2020; Landon et al., 2020; Ahriyasna et al., 2021; Wisaksono et al., 2021). However, the reproductive aspect in such studies has not been evaluated. The present study revealed that shrimp shell extract could protect STZ-induced rats from testicular damage. This was denoted by the improvement in testicle volume, testicle weight, and seminiferous tubule diameter in STZ-induced rats that were supplemented with shrimp shell extract for 30 days. However, the effect of shrimp shell extract on sperm parameters and reproductive hormones still needs to be clarified. Bašković et al. (2021) reported that intraperitoneal injection of astaxanthin has a favorable effect on histological morphometric testicular parameters (mean seminiferous tubule diameter, mean seminiferous lumen diameter, epithelial height, tubular area, luminal area, and Johnsen score) in testicular torsion/detorsion-induced rats. This effect is mediated by the antioxidant activity of astaxanthin (Demir et al., 2022). Astaxanthin supplementation of 50-100 mg/kg feed for 6 weeks to improve reproductive performance has been reported in many studies in various species such as *Nodipecten nodosus* (Linnaeus, 1758), *Procambarus clarkia*, and layer breeder roosters (Suhnel et al., 2014; Zhenhua et al., 2020; Gao et al., 2021). Wisaksono et al. (2021) reported that supplementation with shrimp shell extract could reduce blood glucose levels in STZ-induced rats. This strengthens the notion that the mechanism by which shrimp shell extract protects STZ-induced rats from reproductive organ damage not only comes from its astaxanthin content, which has bioactive activity but might also be caused by lowering hyperglycemia.

It has been reported that long-term hyperglycemia can increase levels of reactive oxygen species and advanced glycation end products, inhibits endothelial nitric oxide synthase metabolism, and decrease endothelial synthesis and the release of nitric oxide, which leads to erectile dysfunction in patients with DM (He et al., 2021). In addition, hyperglycemia interferes with gonadotropin-releasing hormone secretion, thus reducing gonadotropin and prolactin secretion, which in turn leads to a significant decrease in testosterone secretion from Leydig cells and ultimately to spermatogenesis disorders (He et al., 2021).

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CONCLUSION

In conclusion, supplementation of shrimp shell extract in STZ-induced rats could improve testicle volume, testicle weight, and seminiferous tubule diameter, which are fertility parameters in males. Shrimp shells are a waste product of the food industry that might be useful in preventing reproductive problems in patients with DM in the future. However, the mechanism of action in reproductive health, especially in pathological conditions, needs to be studied further.

DECLARATIONS

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Authors' contribution

Aniek Prasetyaningsih and Vinsa Cantya Prakasita designed the research project and obtained the funding. Aniek Prasetyaningsih, Abner Amadeuz Wicaksono, and Yosua Kristian Adi conducted the experiments and collected the samples. Vinsa Cantya Prakasita analyzed the data and prepared the manuscript. All authors read and contributed to evaluating the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

Plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by the authors.

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