Original article

Moringa oleifera leaf extract restored the diameter and epithelium thickness of the seminiferous tubules of rat (*Rattus norvegicus*) injected with gentamicin

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ABSTRACT

This research aims to determine the effect of *Moringa oleifera* leaf extract (MLE) on the diameter and epithelium thickness of the seminiferous tubules of rats induced with gentamicin. This study used 25 male rats (*Rattus norvegicus)* aged 8-12 weeks with 150-200 grams body weight. Negative control group (C-) was injected with aquadest and given 0.5% Na-CMC orally, positive control group (C+) was induced with gentamicin injection of 5 mg/kg BW/day and given 0.5% Na-CMC orally, while groups T1, T2, and T3 were induced with gentamicin at the same dose and treated orally with MLE of 200, 316, 500 mg/kg BW/day. Analysis of variance showed significant differences and further analysis using Duncan Multiple Range Test showed difference in each group. The results showed that treatment with MLE of 500 mg/kg BW/day improved the diameter and epithelium thickness of the seminiferous tubules of rats injected with gentamicin. It could be concluded that MLE restored the diameter and epithelium thickness of the seminiferous tubules of rats induced with gentamicin.

Keywords: gentamicin, Moringa oleifera leaves, rat (*Rattus norvegicus*), seminiferous tubules

INTRODUCTION

Gentamicin is an effective aminoglycoside antibiotic widely used to treat life-threatening Gram-negative bacteria infections (Edeogu *et al*., 2020), including mycobacterium infections, septicemia, complications of urinary tract infections, endocarditis, peritonitis, and others in humans and animals (Chaves and Tadi, 2021). So far, it is known that gentamicin had toxic effects, including nephrotoxic effects (Saleh *et al*., 2016) and neurotoxic (Rezaei *et al*., 2018) as well as toxic effects on male reproductive organs (Aly, 2019).

Male fertility is driven by spermatogenic stem cells, which renew themselves and give rise to differentiated spermatogonia. The spermatogonial transition is accompanied by a shift in gene expression, and changes in metabolism. The process consisted of the upregulation of genes involved in mitochondrial function, biogenesis, oxidative phosphorylation,

and glycolysis (Lord and Nixon, 2020). Spermatogenesis was a complex process of proliferation and maturation of male germ cells from diploid spermatogonia, through meiosis, into mature haploid spermatozoa. The process involved dynamic interactions between the developing germ cell and its supporting Sertoli cells. The gonadal tissues, with their high abundance of unsaturated fatty acids, high rates of cell division, and various testicular enzymes produce were particularly susceptible to overexpression of reactive oxygen species (ROS). The testis had developed a sophisticated array of antioxidant systems with enzymes and scavenging free radicals (Guerriero *et al*., 2014). Gentamicin was an aminoglycoside-class antibiotic that could increase ROS and decrease antioxidant reserves (Rahayu *et al*., 2019). Aminoglycosides passed through the gram-negative membrane in an oxygen-dependent active transport (Chaves and Tadi, 2021). ROS exposure can be neutralized using antioxidants (He *et al*., 2017).

Moringa oleifera had many health benefits (Gopalakrishnan *et al*., 2016). One of the most prominent contents of the *Moringa oleifera* was antioxidants, especially in the leaves. *Moringa oleifera* leaves contained antioxidants such as flavonoids, tannins, steroids and triterpenoids, alkaloids, and saponins based on phytochemical tests. *Moringa oleifera* leaves were rich in minerals, vitamins, and phytochemicals, and antioxidants (Falowo *et al*., 2018).

Study on the effect of *Moringa oleifera* leaf extract (MLE) on male animal fertility has not been reported. Therefore, this study aimed to determine the effectiveness of MLE on the diameter and epithelium thickness of the seminiferous tubules of the testes of white rats (*Rattus norvegicus*) induced by gentamicin.

MATERIALS AND METHODS

Ethics commission approval

This study used 25 heads of white male rats (*Rattus norvegicus*) Wistar strain aged ten weeks with an average body weight of 200 grams. This research procedure has been approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine Universitas Airlangga No. 1.KE.013.01.2020.

Moringa leaf extraction

Moringa oleifera leaves were dried in a dark room at room temperature (27°C) for one week. Then Moringa leaves were pulverized using a powder making machine (Primtech, PDK-2000 N, Indonesia). Moringa leaf powder (150 grams) was put in an erlenmeyer and added with 96% ethanol. Maceration was carried out for 3 x 24 hours and stirred everyday. Macerate was filtered and dried in a rotary evaporator (Rotavapor Buchi RE301 Malaysia) at 40°C 50 rpm until all the solvent and water content of the Moringa leaf were evaporated, leaving the Moringa leaf extract (MLE). MLE was resuspended in 0.5% sodium carboxymethyl cellulose (Na-CMC) for the treatment of the experimental animals (Das *et al*., 2012).

Determination of the dosage of ingredients

The dose of gentamicin was determined based on previous study (Khaki, 2009), which was 5 mg/kg BW/day for 14 days. Determination of the dose of MLE refered to the research of Ogunsola *et al*. (2017), with the minimum dose of 200 mg/kg BW/day and the maximum dose of 500 mg/kg BW/day. The dose between the minimum and maximum doses was determined by multiplying the dosing interval with the lowest dose, thereby a dose of 316 mg/kg BW/day was obtained.

Experimental animals

White rats (*Rattus norvegicus*) were placed in individual cages, where husks were added as a bedding to the cage. Feed pellets and drinking water were given in the morning and evening. During the study, the health of rats was observed concurrently with feeding. Rats were considered healthy if there was no sign of weight loss or changes in body weight of not more than 10% of the sample criteria, the color and condition of the fur were clean and not shedding, and the mouse movement was active, the color of the ears and extremities was red and not pale.

Treatment of rats

Rats were adapted in their respective cages for 7 days. After the adaptation, the rats in group C+, T1, T2, and T3 were injected (sc) with Gentamicin 5 mg/kg BW/day for 14 days, while rats in group C- were injected with 0.5 ml aquadest (sc). At day-15 the rats in group T1, T2, and T3 were treated with 0.5 ml MLE of respectively 200, 316, and 500 mg/kg BW/day, while the rats in group C- and C+ were treated with 0.5 ml of 0.5% Na-CMC. Treatment was given orally for 14 days.

Measurement of variables

After the completion of treatment, all rats were sacrificed by cervical dislocation and dissected for testicle collection. The testes were immersed in 10% formalin for 24 hours, and prepared for Hematoxylin Eosin (HE) stained histological slides (Machmudia *et al*., 2021). The research variables were examined using a light microscope with a magnification of 100x and photographed using Optilab. Diameter and epithelium thickness of the seminiferous tubules were measured using Image Raster Program.

Diameter of seminiferous tubules

The diameter of the seminiferous tubules was measured from the farthest distance from two opposite points that pass through the midpoint, then a diameter perpendicular to the first diameter that have to pass the midpoint of the tubule was measured, then two perpendicular diameters between the previous two diameters were measured. The four measurements obtained were then averaged (Wurlina *et al*., 2021).

Epithelium thickness of seminiferous tubules

The epithelium thickness of the seminiferous tubules was measured by drawing a line from the closest distance at the boundary between the layers of spermatogonia cells to the lumen surface of each seminiferous tubule. Epithelial thickness measurement was repeated four times at different places in each seminiferous tubule. The results of epithelial thickness measurements in five seminiferous tubules of each preparation were averaged (Wurlina *et al*., 2021).

Data analysis

The data on diameter and epithelium thickness were analyzed using one-way Analysis of Variance and Duncan's Multiple Range Test. The statistical analysis was conducted at a 95% level of confidence by using Statistical Product and Service Solutions (SPSS) version 20 International Business Machines (IBM) Corporation (Armonk, New York, USA).

RESULTS

Subcutaneous injection of 0.5 ml of gentamicin 5 mg/kg BW/day for 14 days without MLE administration (C+ group) caused a decrease ($p < 0.05$) in diameter and epithelium thickness of the seminiferous tubules compared to those of the normal rats (C- group). The diameter and the epithelium thickness of the seminiferous tubule in the T3 group (rats injected with 0.5 ml of gentamicin 5 mg/kg BW/day for 14 days and treated with 500 mg/kg BW/day MLE orally for the next 14 days) were higher ($p < 0.05$) than those of the C+ group, and not significantly different ($p > 0.05$) than those of C- group (Table 1).

Table 1 Diameter and epithelium thickness (μm, means ± SD) of the seminiferous tubules of white rats (*Rattus norvegicus*) injected with gentamicin and treated with Moringa leaf extract

	diameter (μm)	epithelium thickness (μm)
C-	381.80 ± 22.48 ^{a}	99.01 ± 4.63 ^{a}
C+	328.33 ± 15.06 ^{c}	67.77 ± 8.35 ^{c}
T1	338.34 ± 20.40 ^{bc}	75.69 ± 9.31 ^{c}
T2	352.58 ± 28.92 ^{ab}	85.67 ± 8.31 ^{b}
T3	361.48 ±18.76 ^{ab}	92.00 ± 4.62 ^{ab}

Different superscripts in the same column show significant differences ($p < 0.05$); C-: rats were injected with 0.5 ml of distilled water subcutaneously for 14 days, followed by administration of 0.5% Na-CMC orally 0.5 ml/day/head for the next 14 days; C+, T1, T2, and T3 groups: rats were injected with 0.5 ml gentamicin (5 mg/kg BW/day for 14 days), followed by the oral administration of 0.5% Na-CMC, 0.5 ml MLE (in 0.5% Na-CMC) (200,

316, and 500 mg/kg BW/day respectively for the next 14 days.

DISCUSSION

Gentamicin could increase the number of reactive oxygen species (ROS) in the testes (El-maddawy, 2014). ROS was one of the free radicals that trigger lipid peroxidation, including in the testicular tissue (Su *et al*., 2019). The functional development of the testes was played by spermatogenic cells, Sertoli as nursing cells, Leydig cells as testosterone producers, and other testicular stromal tissues. Like other cells, cells in testicular tissue were also rich in polyunsaturated fatty acids (PUFA) (Van Tran *et al*., 2017). High levels of ROS could damage cells in testicular tissue (Aly and Hassan, 2018).

ROS weree short-lived and highly reactive molecules. Low levels of ROS played a role in activating cell survival signaling pathways. However, ROS activated cell death signaling pathways from apoptosis and necroptosis at high levels. ROS activated mitochondria, death receptors, and endoplasmic reticulum apoptotic pathways (Redza-Dutordoir *et al*., 2016). ROS were free radicals derived from oxygen during normal cellular metabolism by nature.

ROS played a vital role in physiological processes and signaling pathways associated with male fertility. ROS acted as molecular mediators of signal transduction pathways at physiological concentrations involved in regulating the hypothalamic-pituitary-gonadal axis, spermatogenesis, and steroidogenesis. In contrast, oxidative stress occured when ROS concentrations are higher than the body's antioxidant. Therefore, maintaining a balanced redox state was essential for normal male reproductive function (Baskaran *et al*., 2021).

Oxidative stress caused by gentamicin injection could directly or indirectly affect testicular tissue. The direct effect of oxidative stress on functional cells and supporting cells of testicular tissue would cause tissue damage, resulting in a decrease in the diameter and thickness of the seminiferous tubules epithelium. Exposure to substances that were thought to increase ROS resulted in a decrease in the number of spermatogenic cells, and Leydig cells, and Sertoli cells (Pratama *et al*., 2021). The death of some Sertoli cells reduced the capacity of nursing functions for the process of spermatogenesis (Griswold, 2018). The death of some Leydig cells also decreased spermatogenesis. Leydig cells were cells that produce testosterone, which was essential for spermatogenesis. Testosterone was transported by androgen-binding proteins to Sertoli cells, binding to androgen receptors to regulate spermatogenesis (Ge *et al*., 2021). Treatment which was also thought to trigger an increase in ROS in rats, caused a decrease in the diameter of the seminiferous tubules and the epithelium thickness of the seminiferous tubules (Wurlina *et al*., 2021).

Apoptosis and necrosis caused by ROS exposure (Redza-Dutordoir *et al*., 2016) due to gentamicin injection would be followed by a decrease in epithelium thickness of the seminiferous tubules, which in turn caused shrinkage and a decrease in the diameter of the seminiferous tubules. Gentamicin induction could result in testicular atrophy, degeneration, and loss of spermatogenesis. This was because gentamicin induced oxidative stress associated with spermatogenic disorders (Aly and Hassan, 2018). Sperm viability, sperm motility, total sperm count, testicular weight, seminal vesicle weight, epididymal weight, percentage of total apoptotic cells, and seminiferous tubule diameter were significantly decreased with gentamicin induction (Khaki, 2015). Animals treated with gentamicin decreased testosterone and luteinizing hormone (LH) levels, sperm count, viability, motility and increase sperm abnormalities (Yahya *et al*., 2019).

Oxidative stress could also occur in cells on the hypothalamic-pituitary-testis axis, thereby inhibiting the secretion of the follicle-stimulating hormone (FSH) and LH and ultimately reducing the synthesis of the hormone testosterone (Al-Damegh, 2014). FSH produced from the pituitary played a role in supporting spermatogonia's structural and metabolic development into mature spermatids through receptors bound to the Sertoli cell membrane. FSH also played a vital role in determining the number of Sertoli cells to regulate the production capacity of spermatogenesis (Oduwole *et al*., 2018).

The seminiferous tubules could make up to 90 percent of the testes. The walls of the tubules were composed of stratified germinal epithelium that contained spermatogenic cells and Sertoli cells, nutritive cells that have a mature sperm head embedded in them. Sertoli cells helped facilitating the process of spermiogenesis and thus the production of viable sperm. Sertoli cells also secreted many vital molecules, including androgen binding protein (ABP), Inhibin B, and Activin. This secretion facilitated spermatogenesis directly or indirectly through a hormonal negative feedback system. Sertoli cells also responded to pituitary hormones such as FSH to initiate the process of spermatogenesis, complementing adjacent spermatogonia (Wong and Khan, 2021). If the seminiferous tubule tissue was damaged, it could affect the process of spermatogenesis, the inhibition of cell division to differentiate, affecting the number of spermatogenic cells and the quality of the spermatozoa produced.

Damage to the seminiferous tubules of the testes after gentamicin injection was the result of ROS causing oxidative stress. Moringa leaves contain antioxidants, such as flavonoids, polyphenols (Pizzino *et al*., 2017), and quercetin (Dong *et al*., 2019). Moringa leaves are also known to contain vitamin C (Dafaalla *et al*., 2017) and vitamin E (Fejér *et al*., 2019). Flavonoids acted as antioxidants in preventing male reproductive system (Ye *et al*., 2020). The flavonoids in Moringa leaves were antioxidants that prevented oxidative stress, increased testosterone secretion (Ogunsola *et al*., 2017) and reduced testicular disorders due to ROS exposure (Kathun and Varma, 2017). Administration of quercetin prevented the histopathological changes of testicular tissue (seminiferous tubule diameter, epithelial height) exposed to ROS from bisphenol A (Jahan *et al*., 2020). Vitamin C in MLE functioned as an antioxidant or as a free radical scavenger (Vergara-Jimenez *et al*., 2017). Vitamin C was able to improve the epithelium thickness of the seminiferous tubules. The administration of vitamin C could increase the previously reduced number of spermatogenic cells, increasing the epithelium thickness of the seminiferous tubules, which eventually affected the weight of the testes (Vijayprasad *et al*., 2014). Administration of vitamin E in the form of α-tocopherol maintained the number of spermatogenic and Leydig cells in white rats (*Rattus norvegicus*) exposed to ROS from 2,3,7,8 Tetrachlorodibenzo-p-dioxin (Machmudia *et al*., 2021).

Antioxidant flavonoids and polyphenols, quercetin, vitamin C, and vitamin E in *Moringa oleifera* leaves prevented oxidative stress, thereby restoring the health of cells involved in spermatogenesis after the exposure to ROS from gentamicin. The study at this stage was limited to knowing the potential of MLE to restore the diameter and epithelium thickness of the seminiferous tubules in rats receiving gentamicin injection.

CONCLUSION

The administration of MLE restored the diameter and epithelium thickness of the seminiferous tubules of white male rats (*Rattus norvegicus*) induced with gentamicin. The MLE dose of 500mg/kg BW/day was the most effective in restoring the diameter and epithelium thickness of the seminiferous tubules. Further research is needed to determine the effect of MLE on semen quality in male animals exposed to ROS.

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