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Original article

The effect of ciplukan (*Physalis angulate* Linn.) leaf extract on the testicles of rats (*Rattus norvegicus*) exposed to heat

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ABSTRACT

This study aims to determine the effect of administration of ciplukan (*Physalis angulate* Linn.) leaf extract on the variables of testicular weight, seminiferous tubules diameter, and spermatozoa plasma membrane integrity of rats (*Rattus norvegicus*) exposed to heat as model animals. Rats were divided randomly into five groups. In the NC group, rats were not exposed to heat and were only given 1% Na-CMC. In the PC, T1, T2, and T3 rats were exposed to heat followed by administration of 1% Na-CMC, 100, 200, and 400 mg/kg bw of ciplukan leaf extract (CLE) in 1% Na-CMC. Heat exposure at 40°C was conducted for 60 minutes every day for 21 days. On day-22, all rats were sacrificed for testicular evaluation. The results showed that all variables in the PC group were lower ($p < 0.05$) than in the NC group. All variables in the T3 group were higher ($p < 0.05$) than in the PC group. Testicular weight and spermatozoa plasma membrane integrity in the T3 group were not significantly different ($p > 0.05$) from the NC group. However, the diameter of the seminiferous tubules in the T3 group were lower ($p < 0.05$) than in the NC group. This study revealed that the administration of ciplukan leaf extract as an antioxidant increased testicular weight, seminiferous tubules diameter, and spermatozoa with intact plasma membrane in rats exposed to heat.

Keywords: antioxidant, seminiferous tubules diameter, spermatogenic cell count, spermatozoa plasma membrane integrity, testicular weight

INTRODUCTION

Global warming had a negative impact on livestock production performance so that it could disrupt the economic sustainability of the livestock industry. Livestock farming faced major challenges due to climate change. Animal welfare and male fertility were parameters that were easily affected by heat stress (Capela *et al*., 2022). Male fertility was determined by the quality of spermatozoa produced during the spermatogenesis process in the testicles.

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Spermatogenesis was a very thermosensitive process. Physiologically, the testicles had a mechanism to maintain the continuity of spermatogenesis against heat stress. In men and most mammalian species, testicular temperature was maintained at 2-6°C below body temperature so that physiologically normal spermatogenesis occured. There were two physiological thermoregulation systems of the testicles. The first was the countercurrent heat exchange between arterial blood and venous blood through the pampiniform plexus, the second was the loss of external heat to the outside of the body through passive convection and radiation by the scrotum (Barros Adwell *et al*., 2018; Gao *et al*., 2022). In bulls, the surface of the scrotum and its inner region had some temperature variations of approximately ± 1°C (Pham and Schultz, 2021). However, environmental temperatures that were higher than normal and persisted for a long time could disrupt male fertility. Several publications reported that increasing environmental temperature could reduce the semen quality of Mediterranean buffalo (Sun *et al*., 2022), Belgian Blue bulls (Gloria *et al*., 2021), Holstein Friesian bulls (Llamas-Luceño *et al*., 2020), ram (Moula *et al*., 2024), and buck (Mohamed *et al*., 2023).

Exposure to heat could produce excessive reactive oxygen species (ROS), thereby disrupting the oxidant-antioxidant balance, and causing oxidative stress (Utomo *et al*., 2019). Exposure to ROS damaged cell membranes and nucleic acids, resulting in apoptosis. Decreased testicular function due to oxidative stress resulted in a decrease in the number of Leydig cells, which had an important role in releasing testosterone (Ngoula *et al*., 2020). Too much ROS formation resulted in damage to the endogenous antioxidant defense, so exogenous antioxidants were needed so that the oxidation process returned to normal and homeostasis was achieved. Antioxidants were stable compounds that could neutralize free radicals by donating electrons (Jena *et al*., 2023). Flavonoids were polyphenol antioxidants that could inhibit lipid peroxidation and processes related to ROS (Speisky *et al*., 2022). One plant that contained flavonoids was ciplukan. Ciplukan leaves contained active flavonoids, terpenoid alkaloids, glycosides, and tannins (Alam *et al*., 2022; Ramakrishna *et al*., 2022).

Research on heat exposure in animal models had been carried out on rats (*Rattus norvegicus*) (Octaviani *et al*., 2021; Panggalih *et al*., 2021), and mice (*Mus musculus*) (Prastyaningtyas *et al*., 2021). The use of herbal plants to overcome the negative impscts of heat exposure on the reproductive systems of experimental animals had been carried out using ethanolic extract of watermelon (*Citrullus lanatus*) rind (Panggalih *et al*., 2021), Moringa (*Moringa oleifera* Lam.) leaf extract (Octaviani *et al*., 2021), red dragon fruit (*Hylocereus polyrhizus*) peel extract (Prastyaningtyas *et al*., 2021). The herbal extract of ciplukan (*Physalis angulate* Linn.) leaf had never been studied to treat heat exposure in the reproductive system of experimental animals. In connection with global warming, efforts are needed to maintain male fertility as a source of germplasm distribution. Therefore, this study aims to determine the effect of administering ciplukan leaf extract (CLE) on testicles, based on the variables of testicular weight, seminiferous tubules diameter, and spermatozoa plasma membrane integrity of heat-exposed rats (*Rattus norvegicus*) as model animals.

MATERIALS AND METHODS

This study used 25 white rats (*Rattus norvegicus*) aged 2.5 months with an average body weight of 200 grams. The research procedures were approved by the Experimental Animal Research Ethics Commission with number 1.KE.079.04.2021.

Extraction of *Physalis angulate* leaves

Ciplukan leaves were obtained from the Tempel area, Sleman regency, Jogjakarta, Indonesia. Ciplukan plants samples had been validated by the Purwodadi Botanical Gardens, Malang, Indonesia. Ciplukan leaves were

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washed, cut into small pieces, and then dried until they became simplicia. Two and a half kg of ciplukan leaf simplicia was put into a container for the maceration process with 96% ethanol solvent with a leaf-to-ethanol ratio of 1:10, and stored indoors for 24 hours at a temperature of 28°C. The macerate was filtered to remove the dregs and then concentrated using a rotary evaporator at a temperature of 40-50°C with a speed of 50 rpm until a thick extract was obtained (Iwansyah *et al*., 2020). Ciplukan (*Physalis angulata*) leaves contained alkaloids, glycosides, flavonoids, tannins, and phenolics which had strong antioxidant activity (Ramakrishna Pillai *et al*., 2022). Ciplukan leaf extract was dissolved according to the doses in 1% Na-CMC.

Treatment of rats

Rats were divided randomly, adapted for seven days in their respective cages, then treated for 21 days. In the negative control (NC) group, rats were not exposed to heat and were only given 1% Na-CMC (without CLE). Rats in the positive control (PC), T1, T2, and T3 groups were exposed to heat, and continued with administration of 1 % Na-CMC, 100, 200, and 400 mg/kg bw of CLE respectively. Heat exposure was carried out in an incubator chamber at 40°C (sourced from a light bulb with a thermostat) for 60 minutes once a day. On day-22, all rats were sacrificed for testicles collection.

Measurement of testicular weight and seminiferous tubule diameter

Rat testicles were cleaned with a physiological NaCl solution, drained with filter paper, and then weighed with a digital scale (Scale Lab High Precision Sensor 0.001 - 20 Grams). After weighing, the epididymis was separated from the testicles to collect epididymal spermatozoa and make histological slides of testicular tissue. Histological slides were stained with Hematoxylin Eosin. The diameter of the seminiferous tubules was measured under a light microscope (OlySPMIs CX-22 equipped with an Optilab microscope and Image Raster software) with a magnification of 400x. Seminiferous tubules diameter was measured at the shortest and longest distances from ten seminiferous tubules with the roundest shape, and averaged (Mardatillah *et al*., 2022) (Figure 1).

Examination of spermatozoa membrane integrity

Cauda epididymis was peeled off the covering tunica vaginalis, washed with normal saline, and sliced using a scalpel on a petri dish containing normal saline. Sliced cauda epididymis was left on the petri dish for 10 minutes at room temperature and then discarded (modified from Mujitaba *et al*., 2023). Spermatozoa suspension was collected to asses plasma membrane integrity using the hypoosmotic swelling test (HOS Test) with a microscope (OlySPMIs CX-22 with the help of an Optilab microscope camera) at 450x magnification. Spermatozoa with intact plasma membranes were characterized by bent or coiled tails, while spermatozoa with damaged plasma membranes were characterized by straight tails. The number of spermatozoa that experienced tail bending and coiling was recorded and the percentage out of the total spermatozoa observed was calculated (Alifia *et al*., 2023).

Data analysis

Data were tested for normality and homogeneity, followed by the One-way Anova and Duncan's Multiple Range Test ($p < 0.05$) using Statistical Product and Service Solution version 25 software.

RESULTS

Exposure to 40°C heat for 60 minutes a day for 21 days without the administration of CLE (PC group) caused a decrease ($p < 0.05$) in testicular weight, seminiferous tubules diameter, and percentage of spermatozoa with intact plasma membrane compared to group NC. Administration of CLE (groups T1, T2, and T3) increased ($p < 0.05$) testicular weight,

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seminiferous tubules diameter, and percentage of spermatozoa with intact plasma membrane compared to PC group rats. Testicular weight and spermatozoa with intact plasma membrane in group T3 were not significantly different ($p > 0.05$) compared to the normal group of rats (group NC). However, the diameter of the seminiferous tubules in the T3 group were still smaller ($p < 0.05$) than in the NC group (Table 1). Spermatozoa with intact plasma membranes show a bent or coiled tail, while spermatozoa with damaged plasma membranes have straight tails (Figure 2).

Table 1 Means ± SD of testicular weight (grams), seminiferous tubules (ST) diameter (µm), and spermatozoa plasma membrane integrity (SPMI, %) in rats (*Rattus norvegicus*) exposed to heat without or with the administration of leaf extract ciplukan (*Physalis angulate* Linn.) leaf extract

	testicular weight (gram)	ST diameter (µm)	SPMI (%)
NC	2.32 ± 0.09 ^{c}	330.58 ± 11.31 ^{e}	54.24 ± 10.22 ^{d}
PC	1.83 ± 0.11 ^{a}	226.42 ± 4.26 ^{a}	15.58 ± 3.52 ^{a}
T1	1.99 ± 0.09 ^{b}	244.57 ± 5.50 ^{b}	26.63 ± 4.65 ^{a}
T2	2.06 ± 0.09 ^{b}	278.86 ± 18.06 ^{c}	37.54 ± 6.98 ^{c}
T3	2.26 ± 0.20 ^{c}	313.18 ± 3.17 ^{d}	46.67 ± 4.78 ^{d}

Different superscripts in a column indicated significant difference ($p < 0.05$); NC: rats were not exposed to heat and were only given 1% Na-CMC; PC, T1, T2, and T3: rats were exposed to heat and continued to be given 1% Na-CMC, 100, 200, and 400 mg/kg bw of ciplukan leaf extract in 1% Na-CMC; treatment was carried out every day for 21 days; replicate= 5.

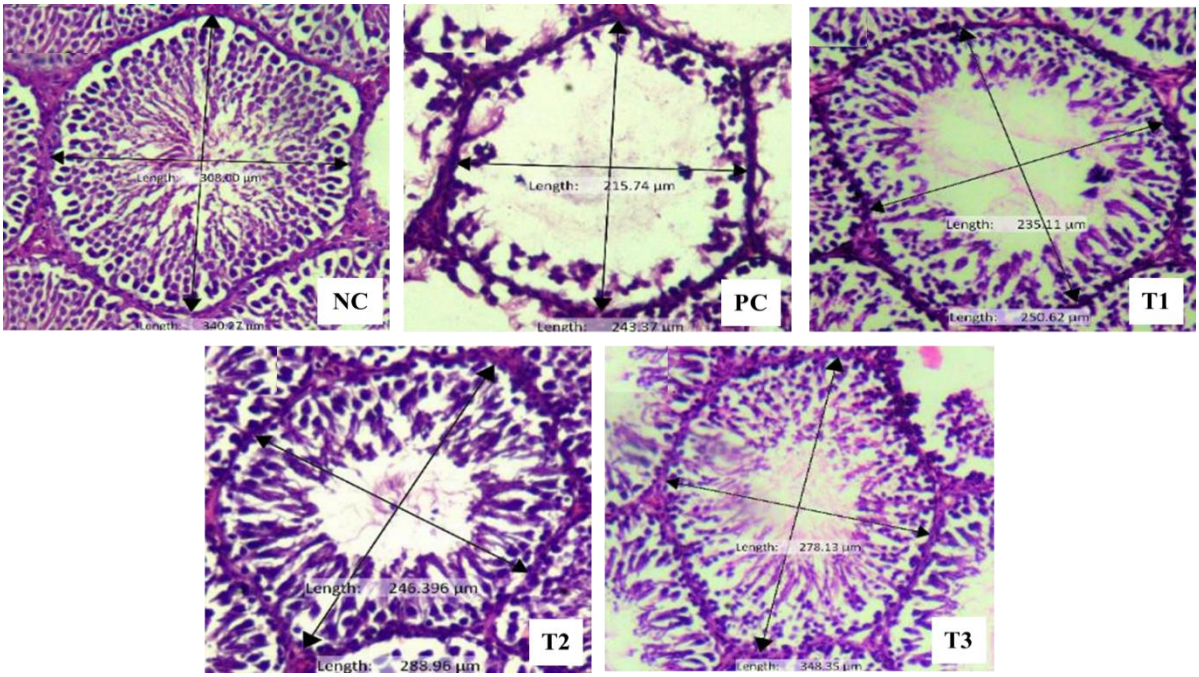

Figure 1 Diameter of the seminiferous tubules of rats (*Rattus norvegicus*) exposed to heat without or with administration of ciplukan leaf extract; NC: rats were not exposed to heat and were only given 1% Na-CMC; PC, T1, T2, and T3: rats were exposed to heat and continued to be given 1% Na-CMC, 100, 200, and 400 mg/kg bw of ciplukan leaf extract in 1% Na-CMC; treatment was carried out every day for 21 days; light microscope (OlySPMIs CX-22) at 400x magnification, Hematoxylin-Eosin staining.

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DISCUSSION

Exposure to a temperature of 40 °C for 60 minutes every day for 21 days without CLE administration caused a decrease in testicular weight, seminiferous tubules diameter, and percentage of spermatozoa with intact plasma membrane. Previous research confirmed that exposure to 40°C for 45-60 minutes per day for 36-52 days reduced the number of Leydig, Sertoli, and spermatogenic cells (Panggalih *et al*., 2021), seminiferous tubules diameter, and testicular weight (Prastyaningtyas *et al*., 2021), motility and viability of spermatozoa (Octaviani *et al*., 2021). These results were in accordance with the publication of Utomo *et al*. (2019) that heat exposure caused morphological changes, tubular atrophy, impaired spermatogenesis, and a decrease in the number and size of spermatogenic cells.

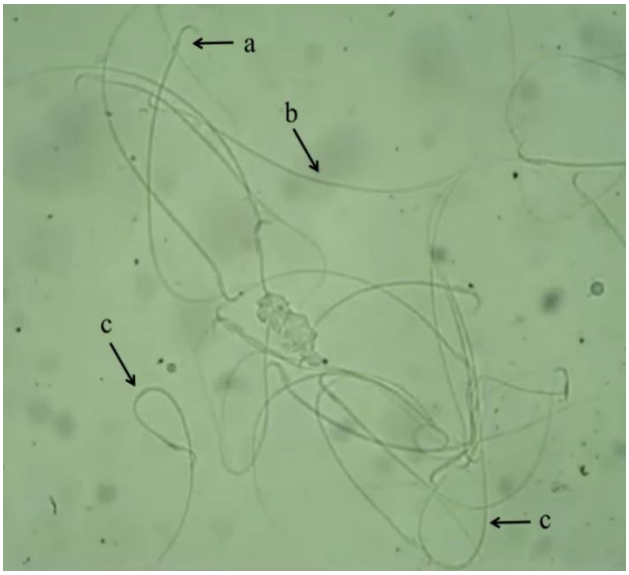

Figure 2 Integrity of the spermatozoa plasma membrane of rat (*Rattus norvegicus*) spermatozoa; examined with the HOS test under a microscope (OlySPMIs CX-22) at 450 x magnification; a: spermatozoa head; b: straight-tailed spermatozoa, reflecting plasma membrane damage; c: spermatozoa with coiled tail, reflecting an intact plasma membrane.

Heat exposure impaired male fertility via the pre-testicular and testicular pathways. In the pre-testicular pathway, heat exposure caused activation of the sympathetic-adrenal-medullary system and hypothalamic-pituitary-adrenal axis, followed by stimulation of adrenocorticotropic hormone secretion which then stimulated cortisol synthesis (Kaiser and Jaillardon, 2023). Increased levels of cortisol in the circulatory system caused a decrease in testosterone levels (Brownlee *et al*., 2005). As was known, testosterone played a systemic role in the emergence of male libido (Roychoudhury *et al*., 2021), and locally in the testicles, it played a role in the process of spermatogenesis (Pugliese *et al*., 2018).

In the testicular pathway, heat exposure disrupted testicular thermoregulatory mechanisms (Durairajanayagam *et al*., 2015). In humans and mammals, several structural and functional devices maintained the testicles at 2-6°C below body temperature for the normal physiological function of spermatozoa production. Testicular thermoregulator in bulls was the testicular vascular cone located above the testicles. The testicular vascular cone consisted of the testicular artery encircling the testis, surrounded by a complex network of small veins, the pampiniform plexus. The function of the testicular vascular cone was to transfer heat from the testicular artery to the testicular vein, cooled it, and then entered the testis again (Kastelic *et al*., 2018). However, long-term heat exposure disrupted testicular thermoregulation, resulting in higher blood perfusion. Testicular hyperthermia impacted semen quality after five days of continuous exposure (Barros Adwell *et al*., 2018). An increase in testicular temperature caused an increase in ROS, a decrease in cellular antioxidants, disrupted blood circulation in the blood-testis barrier, disrupted the structure and function of the blood-testis barrier, inhibited gene expression in Sertoli cells, and increased the production of cytokines and chemokines (Monageng *et al*., 2023). High ROS levels and

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reduced cellular antioxidants resulted in decreased spermatozoa quality. Low gene expression in Sertoli cells and high levels of cytokines and chemokines impacted Sertoli cell malfunction. Disruption of Sertoli cell function along with decreased blood circulation at the blood-testis barrier resulted in impaired growth of germ cells (Hussain *et al*., 2023). Structural damage and impaired function of the blood-testis barrier were followed by the entry of immune cells and toxins from the circulatory system into the seminiferous tubules, causing testicular inflammation, inhibiting the growth of germ cells and ultimately reducing the quality of spermatozoa (Moula *et al*., 2024).

The increase and accumulation of ROS caused lipid peroxidation in cell membranes and cell organelle membranes. Damage to the nuclear membrane would be followed by DNA damage. Damage to the mitochondrial membrane caused loss of membrane potential and leakage of electrons during oxidative phosphorylation, thereby reducing energy production capacity. Damage to the mitochondrial membrane also caused the release of cytochrome C which triggered apoptosis (Andres Juan *et al*., 2021). In testicular tissue, excessive exposure to ROS could damage Sertoli cells, Leydig cells, and spermatogenic cells (Monageng *et al*., 2023) resulting in a decrease in the weight and diameter of the seminiferous tubules. Disorders of the testicles by heat exposure by season were also similar in bucks. Testosterone level, semen volume, spermatozoa concentration, motility, viability, and intact acrosomes in summer were lower than in winter. Histopathologically, degeneration of the seminiferous tubules, excessive fragmentation, desquamation of epithelial cells in the tubule lumen, and vacuoles in the spermatogenic cell layer were found. Heat stress caused shrinkage of the seminiferous tubules walls and reduced the number of spermatocytes and spermatozoa. High temperatures during summer harmed semen quality (Mohamed *et al*., 2023).

In rams, it was also reported that heat stress resulted in a decrease in the quality and volume of fresh and cryopreserved semen. Heat stress greatly disrupted pachytene spermatocytes and spermatids which had high mitotic activity (Moula *et al*., 2024). In Belgian Blue bulls, high temperatures due to global warming reduced semen volume, spermatozoa concentration, percentage of live spermatozoa in fresh semen, motility, membrane integrity, and spermatozoa with normal morphology (Gloria *et al*., 2021). In FH bulls, it was also reported that an increase in environmental temperature caused a decrease in the quality of fresh and frozen semen, which could result in economic losses (Llamas-Luceño *et al*., 2020).

Administration of CLE increased testicular weight, seminiferous tubule diameter, and percentage of spermatozoa with intact plasma membrane compared to rats exposed to heat without CLE administration. Physalis leaf extract contained the highest total phenolics (144.4 mg gallic acid equivalent/g), total flavonoids (33.33 mg quercetin equivalent/g), and antioxidant activity (96.97 µg/mL) (Iwansyah *et al*., 2019). Another study (Kusumaningtyas *et al*., 2015) showed that the antioxidant activity of the ethanol extract of ciplukan leaf was 4.31 mg/g BHA (3-tert-butyl-4-hydroxyanisole). Ciplukan leaf ethanol extract had the highest total flavonoid value, compared to root extract and stem extract. Antioxidant activity based on DPPH (1,1-diphenyl-2-picrylhydrazyl) measurements shows that flavonoids were the main antioxidant components in CLE (Alam *et al*., 2022). Flavonoids could inhibit oxidation reactions through a radical scavenging mechanism which reduced the number of free radicals by giving one electron to free radical compounds that had unpaired electrons (Hassanpour and Doroudi, 2023). In the testicles, exogenous antioxidants played a role in improving the structure and function of the blood-testis barrier, Leydig and Sertoli cells, spermatogonia cells, spermatocytes, spermatids and spermatozoa (Aitken and Roman, 2008). The administration of exogenous antioxidants could also neutralize ROS, increase endogenous antioxidant capacity,

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and improve the plasma membrane of cells in the sympathetic-adrenal-medullary system and hypothalamic-pituitary-adrenal axis as a support system for spermatogenesis (Prevatto *et al*., 2017).

Administration of 400 mg/kg bw of CLE restored testicular weight and spermatozoa plasma membrane integrity so that it was the same as in normal rats. However, this dose was not sufficient to restore the diameter of the seminiferous tubules in the seminiferous tubules, so they were still smaller than in normal rats. Further research is needed to reveal this issue. However, what was certain was that increasing environmental temperature had been proven to reduce semen quality in experimental animals (Octaviani *et al*., 2021; Panggalih *et al*., 2021; Prastyaningtyas *et al*., 2021), as well as in livestock (Llamas-Luceño *et al*., 2020; Gloria *et al*., 2021; Sun *et al*., 2022; Mohamed *et al*., 2023; Moula *et al*., 2024). Therefore, efforts need to be made to maintain male fertility by controlling the temperature of the cage air and environment and providing antioxidants as additional feed (Pham and Schultz, 2021).

CONCLUSION

Administration of ciplukan leaf extract as an antioxidant improved testicular weight, seminiferous tubules diameter and spermatozoa plasma membrane integrity in rats exposed to heat.

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ANFR, SLC, RR, and TWS: conceived the idea. ANFR and SL: acquisition, analysis and interpretation of data. RR, TWS, EPH, and AM: analysis and interpretation of data, manuscript drafting. BU, NH, TH, HP, and KR: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

No potential conflict of interest was reported by the author(s).

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