Original Article

The effect of red dragon fruit *(Hylocereus polyrhizus)* peel extract on Leydig cells number, seminiferous tubules diameter, and testicular weight of mice *(Mus musculus)* exposed to heat

Pengaruh ekstrak kulit buah naga merah *(Hylocereus polyrhizus)* terhadap jumlah sel Leydig, diameter tubulus seminiferus, dan berat testis pada mencit *(Mus musculus) yang* dipapar panas

Kukuh Prastyaningtyas[1], Rochmah Kurnijasanti[2], Suherni Susilowati[3*], Tri Wahyu Suprayogi[3], Hana Eliyani[4], Rahmi Sugihartuti[2]

[1] Student, [2] Division of Basic Veterinary Medicine,
[3] Division of Veterinary Reproduction, [4] Division of Veterinary Anatomy,
Faculty of Veterinary Medicine, Universitas Airlangga
[*] Corresponding author, e-mail: suhernifkhunair@gmail.com

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ABSTRACT

This study aims to determine the effect of red dragon (*Hylocereus polyrhizus*) fruit peel extract (RDFPE) on the parameters of Leydig cells number, seminiferous tubules diameter, and testicular weight of mice (*Mus musculus*) exposed to heat (40°C). Twenty adult male mice were divided randomly into five groups. The control group (C) mice only received a placebo. Meanwhile, the treatment groups mice were exposed to heat for 45 minutes daily for 36 days and oral administration of placebo, RDFPE of 250, 500, and 1000mg/kg BW for T0, T1, T2, and T3, respectively. The result showed that heat exposure on mice (T0 group) caused a lower of all of the parameters ($p < 0.05$) than normal mice (control group, C). RDFPE administration at a dose of 250 mg/kg BW (T1 group) and 500 mg/kg BW (T2 group) resulted in a higher value of those parameters ($p < 0.05$) compared to the T0 group. All those parameters of the T2 group (dose of 500 mg/kg BW) were not significantly different ($p > 0.05$) than the control group (normal mice). However, the higher dose of RDFPE (1000 mg/kg BW, T3 group) resulted in the lower values of those parameters ($p < 0.05$) than those of the T2 group. It could be concluded that 500mg/kg BW dose of RDFPE could return Leydig cells number, seminiferous tubules diameter, and testicular weight of mice (*Mus musculus*) exposed to heat.

Keywords: *Hylocereus polyrhizus*, Leydig cells, *Mus musculus*, seminiferous tubules, testicular weight

INTRODUCTION

Heat stress caused by high ambient temperature can result in increased body temperatures and decrease males' fertility. In males, testis is suspended in a scrotum outside the body to keep the temperature 2-8°C lower than the body temperature to maintain the normal spermatogenesis (Takahashi *et al*., 2011). Heat stress increased the oxidant level and decreased the antioxidant enzyme (Morrell, 2020). The testicular tissue is susceptible to oxidative stress (Asadi *et al*., 2017). The increased testicular temperature will induce oxidative stress by mitochondria-derived Reactive Oxygen Species (ROS), and lipid peroxidation occurs in cell

membranes (Shiraishi, 2002). ROS damaged the Leydig cell membrane, thereby, inhibits the process of steroidogenesis, decreases the testosterone hormone level, followed by the disturbance on spermatogenesis process (Walker *et al*., 2009).

Antioxidants are needed to protect the body from attacks by free radicals, which in certain amounts can inhibit or slow the damage caused by the oxidation process (Sayuti and Yenrina, 2015). The antioxidant in red dragon fruit (*Hylocereus polyrhizus*) peel is more than the fruit flesh, which is 1 mg/ml red dragon fruit peel can inhibit 83.48 ± 5.03% of free radicals, while the dragon fruit flesh is only able to inhibit 27.45 ± 1.02% of free radicals. Vitamins A, C, E, polyphenols, flavonoids, and lycopene are capable of being electron donors to prevent oxidative damage by excess free radicals of ROS (Durairajanayagam *et al*., 2014). Therefore, this study was aimed to determine the effect of red dragon fruit peel extract (RDFPE) on Leydig cells number, seminiferous tubules diameter, and testicular weight of mice (*Mus musculus*) exposed to heat.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Animal Care and Use Committee, Universitas Airlangga, No. 247/HRECC.FDRM/IX/2018.

Red dragon fruits peel extract preparation

The peel of red dragon fruit was washed, and cut into small pieces. The pieces were dried at room temperature, and then pounded. One kg of pounded fruits peel was put in an Erlenmeyer flask and soaked in 96% ethanol for maceration. Maceration was conducted for 3 days and stirred daily. Macerate was dried in a rotary evaporator at 50 rpm, 40°C, and resuspended in 1% sodium Carboxymethyl cellulose (Na-CMC) for oral use on mice.

Treatment

This study used 20, three months old male mice weighed 20 grams obtained from Pusat Veteriner Farma, Surabaya. All mice were acclimatized for a week before treatment. Mice were divided randomly into five equal groups. The control group (C) was administered with placebo (1% Na-CMC) only. Meanwhile, the treatment groups mice were exposed to heat (40°C) for 45 minutes daily and administered with placebo, RDFPE dose of 250, 500, and 1000mg/kg BW respectively for T0, T1, T2, and T3, for 36 days. Heat exposure was conducted in a 50 x 30 x 40 cm chamber with heat derived from two, 5 Watts light bulbs and a thermostat to keep the temperature steady at 40°C (Rohmah *et al*., 2018). The RDFPE was administered 0.5 ml per mouse orally through at a stomach tube. On day-37, rats were sacrificed. Testicles were dissected, weighed, and fixed for histological specimens with hematoxylin-eosin staining.

Measurement of testicular weight

The testicles of mice were separated from fatty and connective tissue (Pujianti, 2016), and weighed individually on an electronic scale.

Measurement of seminiferous tubules diameter

The diameter (µm) of five round or nearly round cross sections of seminiferous tubules was measured at 100 x magnification on a light microscope equipped with the Image Raster Software Version 3.7. The diameter was defined as the distance from the two points opposite the center point. In one seminiferous tubule, four diameters were measured with an angular distance of 90° each other and then averaged (Alamsyah *et al*., 2018; Fitri *et al*., 2019).

Leydig cells count

The number of Leydig cells was counted on five interspaces among seminiferous tubules and then averaged (Adikara *et al*., 2018). Observations were made on a 400 x magnification light microscope (Nikon E200) equipped with Optilab Viewer Software Version 2.2.

Data analysis

Data were analyzed statistically using one-way Anova, continued with Duncan's Multiple Range Test at a 95% level of confidence in SPSS 23.

RESULTS

Heat exposure on T0 group mice caused a lower Leydig cell number, seminiferous tubules diameter, and testicular weight ($p < 0.05$) than normal mice (control group, C). The RDFPE administration at a dose of 250 mg/kg BW (T1 group) and 500 mg/kg BW (T2 group) resulted in a higher Leydig cells number, seminiferous tubules diameter, and testicular weight ($p < 0.05$) compared to the T0 group. The number of Leydig cells, seminiferous tubules diameter, and testicular weight of the T2 group (dose of 500 mg/kg BW) were not significantly different ($p > 0.05$) than the control group (normal mice). However, the higher dose of RDFPE (1000 mg/kg BW, T3 group) actually resulted in a lower number of Leydig cells, seminiferous tubules diameter, and testicular weight ($p < 0.05$) than those of the T2 group.

Table 1 Testicular weight (mg), seminiferous tubules diameter (µm), and number of Leydig cells in mice (*Mus musculus*) exposed to heat and treated with red dragon fruit peel extract (RDFPE)

	means ± standard deviations		
	Leydig cells	seminiferous tubules diameter	testicular weight
C	10.75 ± 1.50 [a]	157.11 ± 6.64 [a]	95 ± 12 [a]
T0	4.75 ± 0.66 [d]	110.30 ± 8.62 [c]	65 ± 12 [bc]
T1	8.00 ± 0.37 [b]	123.63 ± 7.05[b]	75 ± 12 [b]
T2	10.35 ± 1.45 [a]	150.78 ± 3.51[a]	95 ± 12 [a]
T3	6.45 ± 0.34 [c]	107.07 ± 0.08 [c]	50 ± 0,8 [c]

C= the mice received oral 0.5 mL 1% Na-CMC daily for 36 days; T0= mice exposed to heat for 45 minutes and administered 0.5 mL 1% Na-CMC daily for 36 days; T1, T2, T3= mice exposed to heat for 45 minutes daily and administered RDFPE orally 250, 500, and 1000mg/kg BW daily for 36 days; heat exposure was conducted in a heating chamber (40 ^{o}C, with two, 500 watts light bulbs and a thermostat)

DISCUSSION

Heat exposure on mice (T0 group) caused lower Leydig cell number, seminiferous tubules diameter, and testicular weight ($p < 0.05$) than normal mice (control group, C). Testis exposed to temperature of up to 40°C will result in hyperthermia and a reduced blood flow to the testicles, thereby caused cellular hypoxia (Paul *et al*., 2009). Heat stress will induce oxidative stress in cells that experience hypoxia (Saha *et al*., 2014). Heat is a form of physical stress that will trigger the formation of reactive oxygen species (ROS) or free radicals (Ermiza, 2012). ROS causes oxidative stress due to the overproduction of free radicals, and thereby the oxidant level was higher than antioxidants (Umar *et al*., 2015). The oxidation of cell membranes also harms DNA and RNA (Thompson *et al*., 2014; Yusrizal, 2017) and triggers apoptosis (Shiraishi *et al*., 2010). Oxidative stress will cause damage to Leydig cell membranes (Umar *et al*., 2015) and caused a decreasing number of Leydig cells (Yusrizal, 2017). The Leydig cell is a producer of testosterone. Therefore, a decreased number of Leydig cells will be followed by a decrease in testosterone level. Testosterone deficiency can disturb the process of spermatogenesis in seminiferous tubules (Kaiin *et al*., 2013), cause degeneration of seminiferous tubular epithelial cells (Zhang *et al*., 2012), followed by a decrease in the diameter of the seminiferous tubules (Kumar and Nagar, 2014). The massive decrease of the number of cells in the seminiferous and interstitial tubules and the decrease of seminiferous tubules will be followed by decreased testicular weight (Paul *et al*., 2008).

The RDFPE administration at a dose of 250 mg/kg BW (T1 group) and 500 mg/kg BW (T2 group) resulted in a higher Leydig cells number, seminiferous tubules diameter, and testicular weight ($p < 0.05$) compared to the T0 group. The red dragon fruit peel (*Hylocereus polyrhizus*)

has antioxidant content in the form of vitamins A, C, E, polyphenols, flavonoids, and carotenes that functioned in cell regeneration (Barakat *et al*., 2014). These substances can directly react with superoxide anions, hydroxyl radicals, singlet oxygen, and lipid peroxide. As a reducing agent, the antioxidant will donate one electron to form a semi dehydroascorbate which is not reactive and subsequently undergoes a disproportionation reaction to form an unstable dehydroascorbate (Werdhasari, 2014). Vitamin E can reduce superoxide radicals because vitamin E can be an antioxidant chain breaker in the membrane that can prevent cell damage by lipid peroxidation and inhibit the formation of free radicals. Lipid peroxidation in cell membranes is divided into three stages, namely initiation, propagation, and termination. Vitamin E breaks the chain in the propagation phase, contributing its phenolic hydrogen atom to peroxyl radicals and converting it to hydroperoxide. Radical tocopherol is quite stable and out of the cycle of lipid peroxidation reacts with other peroxyl radicals to form non-radical products (Hajibabaei, 2016). Vitamin E in binding or reacting with oxygen radicals is faster than PUFA (poly unsaturated fatty acid), so that vitamin E is effective as an antioxidant membrane in inhibiting oxygen radicals with PUFA in cell membranes (Vinnata *et al*., 2018). Vitamin E is a source of antioxidants that prevent lipid peroxidation from unsaturated fatty acids in cell membranes and helps oxidize vitamin A and maintain fertility (Simanjuntak, 2012).

The number of Leydig cells, seminiferous tubules diameter, and testicular weight of the T2 group (dose of 500 mg/kg BW) were not significantly different ($p > 0.05$) than the control group (normal mice). As mentioned earlier, RDFPE contains polyphenol and flavonoids. The polyphenol group activity is antioxidant and chelating agents, whereas flavonoids act as catalysts on the lipid bilayers and membrane function (Roychoudhury *et al*., 2017). On molecular level, polyphenol acts on the signaling pathway of adenosine monophosphate-activated protein kinase, cyclic adenosine monophosphate, calcium ion (Rahman *et al*., 2018), ferrous and ferric iron, and low-density lipoproteins (Selvam *et al*., 2018). The RDFPE dose of 500 mg/kg BW proved returned the number of Leydig cells, seminiferous tubules diameter, and testicular weight of heat exposed mice similar to normal mice (C).

The higher dose of RDFPE (1000 mg/kg BW, T3 group) actually resulted in the lower number of Leydig cells, seminiferous tubules diameter, and testicular weight ($p < 0.05$) than those of the T2 group. The excessive consumption of flavonoids can act as a pro-oxidant as a mutagen and a key inhibitor of enzymes involved in hormone metabolism (Prochazkova *et al*., 2011). Excessive flavonoid compounds will inhibit topoisomerase DNA II and p53 regulation or by causing mitochondrial toxicity, which initiates mitochondrial apoptosis (Saraswat *et al*., 2016). This caused a decrease in the number of Leydig cells, seminiferous tubules diameter, and testicular weight compared to the P2 group, this phenomenon was due to the antioxidant paradox. Higher exposure to antioxidants shifted the reduction-oxidation balance to the reduction of stress. The antioxidant paradox significantly decreases male fertility (Majzoub and Agarwal, 2018).

Physiologically, testicles require low quantities of ROS for the healthy functioning of spermatozoa (Wagner *et al*., 2017). The changes in both ROS and antioxidant levels caused a redox imbalance. Excessive ROS or excessive antioxidants disrupted the balance state needed for healthy spermatozoa (Salehi *et al*., 2018). The higher dose of RDFPE (T3 group) causes a lacking level of ROS for normal physiology of the testicle. Therefore, a higher exposure to antioxidants (reductants) also resulted in an antioxidant paradox that significantly affects male fertility (Majzoub *et al*., 2018).

The changes pattern of Leydig cells number, seminiferous tubules diameter, and testicular weight among the treatments were similar. Oxidative stress caused by heat stress inhibits testosterone production (Wang *et al*., 2017) by damaging Leydig cells, derangement of other reproductive hormonal profiles (Darbandi *et al*., 2018). Leydig cells produce testosterone due to LH stimulation produced by the anterior pituitary (Ramaswamy and Weinbauer, 2014). Testosterone regulates signaling pathways in Sertoli cells to maintain

spermatogenesis (Smith and Walker, 2014). The decline in the number of Leydig cells due to heat stress exposure resulted in a reduction in testosterone production, causing decreased spermatogenesis. Intratubular testosterone concentration greatly influences the early stages of germ cell development. Emphasis on testosterone biosynthesis can inhibit spermatogenesis. Testosterone in the seminiferous intratubular significantly affects the blood-testis barrier; moreover, testosterone is required for Sertoli-spermatid adhesion, and mature spermatozoa release into the lumen of the seminiferous tubules (Walker, 2009). Testosterone controls developing germ cells, especially spermatids, and acts as an adhesive between spermatids and Sertoli cells. A disruption in testosterone will cause spermatids to be released from the epithelium (Lui and Lee, 2009).

CONCLUSION

Based on this research, it could be concluded that 500mg/kg BW dose of Hylocereus *polyrhizus peel* could maintain Leydig cells number, seminiferous tubules diameter, and testicular weight of mice (*Mus musculus*) exposed to heat.

REFERENCES

Adikara YAR, Utama S, Yudaniayanti IS, Adikara RTS, Samik A, Hestianah EP. 2018. Effect of laser acupuncture shoot on ova point of male Mojosari Duck (Anas Plathyrhynchos) on the number of sertoli and Leydig cells. Ovozoa 7: 109-13.

Alamsyah RH, Hestianah EP, Juniastuti T, Wurlina W. 2018. Potensi ekstrak etanol seledri (apium graveolens) terhadap diameter tubulus seminiferus dan jumlah sel spermatogenik mencit (Mus musculus). Ovozoa 7: 37-41.

Asadi N, Bahmani M, Kheradmand A, Rafieian-Kopaei M. 2017. The Impact of Oxidative Stress on Testicular Function and the Role of Antioxidants in Improving it: A Review. J Clin Diagn Res. 11: IE01-IE05.

Barakat IAH, Al-Himaidi AR, Rady AM. 2014. Antioxidant effect of green tea leaves extract on in vitro production of sheep embryos. Pakistan J. Zool. 46: 167-75.

Darbandi M, Darbandi S, Agarwal A, Sengupta P, Durairajanayagam D, Henkel R, Sadeghi MR 2018. Reactive oxygen species and male reproductive hormones. Reprod Biol Endocrinol 16: 87.

Durairajanayagam D, Agarwal A, Ong C. 2015. Causes, effects and molecular mechanisms of testicular heat stress. Reprod Biomed Online 30: 14-27.

Ermiza. 2012. Pengaruh paparan suhu terhadap kualitas spermatozoa mencit jantan (*Mus musculus*) strain Jepang. Sainstis 1: 20-7.

Fitri PE, Wurlina W, Chusniati S, Suwanti LT, Plumeriastuti H, Mufasirin M. 2019. Pengaruh pemberian madu terhadap staging spermatogenesis dan sel Leydig pada mencit (Mus musculus) yang diinfeksi Toxoplasma gondii. Ovozoa 8: 10-5.

Hajibabaei, K. 2016. Antioxidant Properties of Vitamin E. Ann Res Antioxid. 1: e22.

Kaiin E, Djuwita I, Yusuf TL, Setiadi MA. 2013. Konsentrasi, kemurnian, dan viabilitas sel leydig dari hasil purifikasi dengan gradien Nycodenz dan kultur *in vitro*. J Kedokteran Hewan 7: 75-80.

Kumar A, Nagar M. 2014. Histomorphometric study of testis in deltamethrin treated albino rats. Toxicol Rep. 1: 401-10.

Lui WY, Lee WM. 2009. Molecular mechanisms by which hormones and cytokines regulate cell junction dynamics in the testis. J Mol Endocrinol. 43: 43-51.

Majzoub A, Agarwal A, Esteves SC. 2017. Antioxidants for elevated sperm DNA fragmentation: a mini-review. Transl Androl Urol. 6: S649-S653.

Majzoub A, Agarwal A. 2018. Systematic review of antioxidant types and doses in male infertility: Benefits on semen parameters, advanced sperm function, assisted reproduction and live-birth mice, Arab J Urol. 16: 113-24.

Morrell JM. 2020. Heat stress and bull fertility. Theriogenology 153: 62-7.

Paul C, Murray AA, Spears N, Saunders PT. 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. Reproduction. 136: 73-84.

Procházková D, Boušová I, Wilhelmová N. 2011. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82: 513-23.

Pujianti P. 2016. Pengaruh pemberian pasta tomat (Solanum lycopersicum) terhadap jumlah sel spermatogonium pada testis mencit (Mus musculus) yang dipapar boraks. Ovozoa 5: 87-92.

Rahman SU, Huang Y, Zhu L, Feng S, Khan IM, Wu J, Li Y, Wang X. 2018. Therapeutic role of green tea polyphenols in improving fertility: A review. Nutrients 10: 834

Ramaswamy S, Weinbauer GF. 2015. Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone. Spermatogenesis 4: e996025.

Rohmah L, Triana IN, Sunarso A, Susilowati S, Hidajati N, Kurnijasanti R. 2018. Pengaruh pemberian ekstrak kulit semangka (*Citrullus lanatus*) terhadap motilitas dan viabilitas spermatozoa tikus (*Rattus norvegicus*) dengan paparan suhu panas. Ovozoa 7: 131-6.

Roychoudhury S, Agarwal A, Virk G, Cho CL. 2017. Potential role of green tea catechins in the management of oxidative stress-associated infertility. Reprod Biomed Online 34: 487-98.

Saha S, Dungdung SR, Majumder GC. 2014. Determination of The Antioxidant Potential of Goat Sperm Cells. Oxid Antioxid Med Sci. 3: 195-200.

Salehi B, Martorell M, Arbiser JL, Sureda A, Martins N, Maurya PK, Sharifi-Rad M, Kumar P, Sharifi-Rad J. 2018. Antioxidants: Positive or negative actors? Biomolecules 8: 1-11.

Saraswat S, Jindal SK, Karcher SD. 2016. Antioxidant and spermatozoa: A complex story-A review. Indian J Anim Sci. 86: 495-501.

Sayuti K, Yenrina R. 2015. Antioksidan Alami dan Sintetik. Padang. Andalas University Press. 7-29.

Selvam MKP, Agarwal A. 2018. A systematic review on sperm DNA fragmentation in male factor infertility: Laboratory assessment. Arab J Urol. 16: 65–76.

Shiraishi K, Takihara H, Matsuyama H. 2010. Elevated scrotal temperature, but not varicocele grade, reflects testicular oxidative stress-mediated apoptosis. World J Urol. 28: 359-64.

Shiraishi, T., M. Abe and T. Miyagawa, 2002. Cheese foods containing conjugated polyunsaturated fatty acid glycerides. Japanese Patent: JP 2002176913.

Simanjuntak, K. 2012. Peran antioksidan flavonoid dalam meningkatkan kesehatan. Bina Widya 23: 135-40.

Smith LB, Walker WH. 2014. The regulation of spermatogenesis by androgens. Semin Cell Dev Biol. 30: 2-13.

Takahashi M. 2011. Heat stress on reproductive function and fertility in mammals. Reprod Med Biol. 11: 37-47.

Thompson A, Agarwal A, Du Plessis SS. 2013. Physiological role of reactive oxygen species in sperm function: a review. In: Parekattil SJ, Agarwal A (eds). Antioxidants in male infertility: a guide for clinicians and researchers. New York, USA: Springer Science and Business Media. 69–89.

Umar S, Queljoe E, Tendean L. 2015. Pengaruh pemberian ekstrak kulit buah manggis *(Garcinia mangostana L.)* terhadap kualitas spermatozoa wistar jantan *(*Rattus *norvegicus)* yang diberi paparan suhu panas. J e-Biomedik 3: 670-5.

Vinnata NN, Sali S, Nita S. 2018. Pengaruh pemberian daun kemangi (*Ocinum Americanum L*) terhadap berat testes, diameter dan tebal epitel tubulus seminiferus, morfologi dan jumlah spermatozoa tikus putih jantan (Rattus Norvegicus). J Kesehatan 9: 366-75.

Wagner H, Cheng JW, Ko EY. 2017. Role of reactive oxygen species in male infertility: An updated review of literature. Arab J Urol. 16: 35-43.

Walker WH 2009. Molecular mechanisms of testosterone action in spermatogenesis. Steroids 74: 602-7.

Wang Y, Chen F, Ye L, Zirkin B, Chen H. 2017. Steroidogenesis in Leydig cells: effects of aging and environmental factors. Reproduction 154: R111-R122.

Werdhasari A. 2014. Peran antioksidan bagi Kesehatan. J Biotek Medisiana Indonesia 3: 59-68.

Yusrizal A. 2017. Pengaruh karbon aktif terhadap penurunan jumlah sel Leydig tikus putih (*Rattus norvegicus*). Skripsi. Universitas Muhammadiyah Yogyakarta.

Zhang M, Jiang M, Bi Y, Zhu H, Zhou Z, Sha J. 2012. Autophagy and apoptosis act as partners to induce germ cell death after heat stress in mice. PLoS One 7: e41412.