Original article

**Red dragon (Hylocereus polyrhizus) fruit peel extract increased the motility and viability of spermatozoa of hypercholesterolemic rats (Rattus norvegicus)**

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**ABSTRACT**

This study aims to determine the effect of red dragon (Hylocereus polyrhizus) fruit peel extract (RDFPE) on spermatozoa motility and viability of hypercholesterolemic rats (Rattus norvegicus) as a model. Twenty male rats were randomly divided into negative control (NC), positive control (PC), treatment 1 (T1), treatment 2 (T2), and treatment 3 (T3) groups. All rats were given 2 mL of high cholesterol feed orally every day for 28 days. On day-15, all rats were measured for their blood cholesterol levels, followed by treatment for 14 days. Rats in the NC, PC, T1, T2, and T3 groups were treated with 1% Na-CMC, Simvastatin 10 mg/kg BW, and RDFPE of 500, 750, and 1000 mg/kg BW, respectively. On day-29, all rats were sacrificed to evaluate spermatozoa viability and motility. The results showed that spermatozoa viability and motility in the hypercholesterolemic rats (NC) group were the lowest (p <0.05) among the groups. Treatment of hypercholesterolemic rats with Simvastatin 10 mg/kg BW (PC) group showed higher (p <0.05) spermatozoa viability and motility compared to the NC group. RDFPE dose of 1000 mg/kg BW (T3 group) resulted in higher (p <0.05) spermatozoa viability and motility compared to other RDFPE doses (T1 and T2) and the control (NC) groups, and it was similar (p >0.05) compared with the Simvastatin treated (PC) group. It could be concluded that the administration of 1000 mg/kg BW ethanolic extract of red dragon fruit (Hylocereus polyrhizus) peel increased the viability and motility of spermatozoa of hypercholesterolemic rats (Rattus norvegicus) which were the same as the group of rats given Simvastatin 10 mg/kg BW.

**Keywords:** cholesterol, motility, red dragon fruit, spermatozoa, viability

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INTRODUCTION

Infertility in men was caused by low spermatozoa quality in more than 90% of cases. Parameters of spermatozoa quality are spermatozoa motility, morphology, concentration, and spermatozoa viability. Hypercholesterolemia had an impact on decreasing spermatozoa quality (Nuramisa et al., 2020). A high-fat diet negatively affected spermatozoa viability and motility, followed by low spermatozoa quality and low fertility (Mortazavi et al., 2014) due to mitochondrial respiration of rat spermatozoa (Ferramosca et al., 2016).

Hypercholesterolemia increased reactive oxygen species (ROS), reduced tissue antioxidant levels. Free radicals in high amounts could induce lipid peroxidation due to a complex reaction between unsaturated fatty acids that make up cell membranes and free radicals (Singh et al., 2017). The spermatozoa plasma membrane was composed of polyunsaturated fatty acids (PUFA) and sensitive to lipid peroxidation (Van Tran et al., 2017).

Lipid peroxidation caused damage to protein stability and mutagenic damage to DNA (Juan et al., 2021). Lipid peroxidation could cause damage to spermatozoa by changing the stability, structure, and function of cell membranes so that it interfered with spermatozoa metabolism which causes death. Damage to the spermatozoa membrane affects spermatozoa motility and viability (Alahmar, 2019). Lipid peroxidation could be inhibited by antioxidant bio-active compounds (Nguyen et al., 2017), among others by providing antioxidants such as red dragon fruit. The main antioxidant activity of red dragon fruits was polyphenols (Paško et al., 2021).

Antioxidant compounds in red dragon fruit included phenols, flavonoids, phytoalbumin, and betalains such as betacyanin and betaxanthin (Harahap et al., 2020). Dragon fruit peel extract has better antioxidant activity than the fruit extract because of its higher phenolic content (Febrianti et al., 2020). The effect of red dragon (Hylocereus polyrhizus) fruit peel extract (RDFPE) on hypercholesterolemic male fertility subjects has never been studied. Therefore, this study aims to determine the effect of RDFPE on spermatozoa motility and viability of hypercholesterolemic rats (Rattus norvegicus) as a model.

MATERIALS AND METHODS

The study was conducted from February to April 2022 at the Experimental Animal Unit, Faculty of Veterinary Medicine, Universitas Airlangga. Red dragon fruit peel extract was made at the Laboratory of Pharmacology of the Department of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga. Cholesterol levels were measured at the Indonesian Animal Health Clinical Laboratory, Malang. Evaluation of spermatozoa motility and viability was carried out at the Laboratory of Artificial Insemination, Faculty of Veterinary Medicine, Universitas Airlangga. The proposal for this study was approved by the Animal Care and Use Committee (IACUC) Faculty of Veterinary Medicine, Universitas Airlangga No. 2. KEH.017.03.2022.

Table 1 The nutritional content of broiler finisher BR II pellet, duck egg yolk, and lard

<table>
<thead>
<tr>
<th>nutritional content</th>
<th>BR II ¹</th>
<th>duck egg yolk ²</th>
<th>lard ³</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein</td>
<td>19-20%</td>
<td>12.19</td>
<td>-</td>
</tr>
<tr>
<td>fat</td>
<td>5 %</td>
<td>12.23</td>
<td>160</td>
</tr>
<tr>
<td>total cholesterol (mg/100g)</td>
<td>-</td>
<td>878.7 ± 7.6</td>
<td>32</td>
</tr>
</tbody>
</table>

¹,²,³ References, ¹ Comfeed, 2021; ² Sudarman et al., 2018; ³ Ardilla et al., 2018; -: there is no data on the reference.

High cholesterol feed and RDFPEx preparations

High cholesterol feed was made by diluting 3 g of boiled duck egg yolk in 1 mL of lard (Kusmita and Puspitaningrum, 2020). The nutritional content of duck egg yolk and lard is presented in Table 1. Extract of red dragon (Hylocereus polyrhizus) fruit peel was obtained
by maceration method. Red dragon fruit peel was cut into small pieces, and dried in an oven at 50°C. As much as 50 grams dried red dragon fruit peel was crushed using a grinder to a coarse powder, then extracted using 96% ethanol for 3 days and stirred periodically. Dragon fruit peel powder that has been soaked was filtered, then filtrate was evaporated in a rotary evaporator to obtain viscous extract (Prastyaningtyas et al., 2021).

**Experimental animals**

This study used 20 male rats (*Rattus norvegicus*) of the Wistar strain aged 12 weeks weighing about 200 grams. The rats were adapted to the cage with standard feed BR II Japfa Comfeed (nutrient content as shown in Table 1) and drinking water ad libitum for seven days. Rats were randomly divided evenly into five groups for negative control (NC), positive control (PC), treatment 1 (T1), treatment 2 (T2), and treatment 3 (T3) groups, respectively. All rats in each group were given 2 mL of high-cholesterol feed every day using an oral sonde before being given regular feed, for 28 days (Alaydrus et al., 2020).

**Cholesterol analysis**

On day-15 of treatment, all rats were fasted and blood samples were taken to measure cholesterol levels using the Multi-Monitoring System Autocheck®. This device was prepared before use. The total cholesterol code strip was inserted. Subsequently code number and test mode would be displayed shown on the screen. The code number and test mode on the screen should be the same as those on the strip label. The code strip was removed from the device, and replaced with a cholesterol test strip. Blood sample was taken as much as 0.5 mL from the lateral vein of the rat tail with a tuberculin syringe (Lee, 2015). The blood sample was dripped onto the test strip and the cholesterol level will automatically be displayed on the screen.

**Treatment of rats**

From day-15 (after measuring blood cholesterol) until day-28, rats in the NC, PC, T1, T2, and T3 groups were given 0.3 mL of 1% of Na-CMC, Simvastatin 10 mg/kg BW, 500, 750, and 1000 mg/kg BW of RDFPE successively through a gastric tube (Sahin et al., 2021). On day-29, all rats were injected intraperitoneally with 100 mg/kg BW of ketamine HCl (Widyawati and Ayomi, 2015) and then sacrificed for dissection to collect cauda epididymis. Two mL of 0.9% NaCl was placed in a 3.5 cm petri dish (Nunc™, Thermo Scientific). Cauda epididymis was cut into small pieces and put in the petri dish to disperse the spermatozoa in sodium chloride. The spermatozoa suspension was stirred homogeneously and evaluated for spermatozoa motility and viability (Octaviani et al., 2021).

**Spermatozoa motility examination**

Spermatozoa suspension was dripped on an object glass and observed under a light microscope (Nikon Eclipse E100) with a magnification of 400 times. Spermatozoa motility was assessed based on the percentage of spermatozoa with progressive movement out of 100 spermatozoa observed (Octaviani et al., 2021).

**Spermatozoa viability examination**

Spermatozoa suspension was dripped onto an object glass, added with a same volume of eosin-nigrosin, mixed, smeared and then dried over a flame for 15 seconds. Observations were performed under a light microscope (Nikon Eclipse E100) with a magnification of 400 times. Live spermatozoa cells were translucent (unstained), while dead spermatozoa were purplish-red. Spermatozoa viability (percentage of live spermatozoa) was obtained by examining 100 spermatozoa (Octaviani et al., 2021).

**Data analysis**

Data were analyzed using the Analysis of Variance followed by Duncan’s Multiple Range Test with a significance level of 5%. Statistical analysis was performed using Statistical Product and Service Solution (SPSS) version 23 software for Windows (IBM Corp, Chicago, USA).
RESULTS

Cholesterol levels of all rats were more than 97 mg/dL (Table 2). Spermatozoa viability (Figure 1) and spermatozoa motility of the hypercholesterolemic rats (NC group) were the lowest (p < 0.05) among the groups. Treatment of hypercholesterolemic rats with Simvastatin 10 mg/kg BW (PC groups) showed higher (p < 0.05) spermatozoa viability and motility compared to the NC group.

Spermatozoa viability and motility of hypercholesterolemic rats given RDFPE of 500 mg/kg BW (group T1) did not show any different results (p > 0.05) to those of the 750 mg/kg BW dose (group T2). Meanwhile, the dose of 1000 mg/kg BW (group T3) resulted in higher (p < 0.05) spermatozoa viability and motility compared to other doses of RDFPE (groups T1 and T2) and the control group (NC). The spermatozoa viability and motility of the T3 group were similar (p > 0.05) to those of the Simvastatin-treated (PC) group (Table 3).

Table 2 Cholesterol levels of rats (Rattus norvegicus) fed high cholesterol feed for 14 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>97.17 ± 1.15</td>
</tr>
<tr>
<td>PC</td>
<td>97.81 ± 0.90</td>
</tr>
<tr>
<td>T1</td>
<td>97.55 ± 1.06</td>
</tr>
<tr>
<td>T2</td>
<td>97.29 ± 0.55</td>
</tr>
<tr>
<td>T3</td>
<td>97.67 ± 1.26</td>
</tr>
</tbody>
</table>

NC: negative control group, rats were treated with 0.3 mL of 1% Na-CMC; PC: positive control, rats were treated with 10 mg/kg BW of Simvastatin; T1, T2, T3: rats were treated 500, 750, 1000 mg/kg BW of red Dragon fruit peel extract, respectively; rats from all groups were fed high cholesterol feed for 28 days; treatment was given for 14 days, starting 14 days after the start of high cholesterol feeding.

DISCUSSION

Hypercholesterolemia in animal models mimicked dyslipidemia in humans. Wistar rats induced by a high cholesterol feed can become a model of hypercholesterolemia (Cunha et al., 2021). Cholesterol levels of hypercholesterolemic rats in this study were more than 97 mg/dL.

Table 3 Spermatozoa viability and motility of rats (Rattus norvegicus) after being fed high cholesterol feed and red Dragon fruit peel extract

<table>
<thead>
<tr>
<th>Group</th>
<th>Viability</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>20.19 ± 3.21 c</td>
<td>15.57 ± 3.88 c</td>
</tr>
</tbody>
</table>

Different superscripts in the same column show significant differences (p < 0.05); NC: negative control group, rats were treated with 0.3 mL of 1% Na-CMC; PC: positive control, rats were treated with 10 mg/kg BW of Simvastatin; T1, T2, T3: rats were treated 500, 750, 1000 mg/kg BW of red Dragon fruit peel extract, respectively; rats from all groups were fed high cholesterol feed for 28 days; treatment was given for 14 days, starting 14 days after the start of high cholesterol feeding.
Eclipse E100) with 400x magnification; a: live rat spermatozoa (clear/unstained spermatozoa head); b: dead rat spermatozoa (reddish head).

This was higher than the average total cholesterol level in normal rats, which was between 45.57 mg/dL (Nugroho et al., 2021) and 50.42 mg/dL (Harini and Astirin, 2009).

This study revealed that hypercholesterolemic rats showed low spermatozoa viability and motility. Live spermatozoa with an intact plasma membrane are microscopically characterized by a translucent color because they were impermeable to dyes, and subsequently the cytoplasm was not stained. Whereas in dead spermatozoa, eosin-nigrosin penetrated the spermatozoa membrane, stained the spermatozoa cytoplasm purplish-pink (Silviani et al., 2022). High cholesterol increased the production of ROS in the circulation system (Bin-Jumah, 2018). There are endogenous antioxidants in the biological system of rats; however, higher ROS levels were followed by an imbalanced oxidant-antioxidants which induced oxidative stress and increased lipid peroxidation (Amiya, 2016). ROS reacted with nitric oxide to produce peroxynitrite. Peroxynitrite mediated protein oxidation, nitration, lipid peroxidation, mitochondrial dysfunction, and cell death (Radi et al., 2018).

The spermatozoa plasma membrane contained poly unsaturated fatty acids (PUFA), which were susceptible to ROS. PUFA oxidation disrupted membrane integrity (Van Tran et al., 2017). PUFA oxidation consists of three main stages, namely initiation, propagation, and termination. In the initiation stage, fatty acid radicals were formed, which were highly reactive and unstable fatty acid derivatives due to the loss of a hydrogen atom or the addition of a carbon double bond. The carbon double bonds were capable of weakening the carbon-hydrogen bonds. In the propagation stage, hydrogen atoms are removed by rearranging the bonds to stabilize the peroxy radical. Through a chain reaction, peroxy radicals attacked other fatty acids, producing hydroperoxides and new fatty acid radicals to produce more hydroperoxides (Ayala et al., 2014). Furthermore, ROS enters the cell, damaging structural proteins, functional proteins, and DNA (Deoxyribonucleic Acid) fragmentation (Juan et al., 2021). Therefore, the plasma membrane would cause leakage of intracellular organelles, affecting spermatozoa motility and viability (Vetter et al., 1998).

The decrease in the percentage of spermatozoa motility in hypercholesterolemic rats was associated with abnormal cell function, especially Sertoli and Leydig cells. Disruption of testosterone synthesis by Leydig cells (Feng et al., 2005) had implications for the process of spermatogenesis and spermatozoa maturation in the epididymis which resulted in morphological abnormalities and decreased spermatozoa motility (Bashandy, 2007). A previous study reported that red dragon (Hylocereus polyrhizus) fruit peel extract restored the number of Leydig cell of mice (Mus musculus) that were exposed to heat (Prastyaningtyas et al., 2021). In addition, spermatozoa motility occurs due to the vibration of the spermatozoa tail. The contraction of the fibrils in the principal piece and end piece of spermatozoa tail were energized by the mitochondria in the midpiece of the spermatozoa tail. Aspartate transerase enzyme is a mediator of the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) in energy production for spermatozoa motility (Amaral, 2022). Damage to the plasma membrane in the midpiece of the spermatozoa tail was followed by loss of function of the aspartate transferase enzyme, which resulted in non-motile spermatozoa because there is no energy source (Fraser et al., 2001).

Viability and motility of spermatozoa in hypercholesterolemic rats treated with Simvastatin 10 mg/kg BW were higher than rats without any treatment. This study did not measure cholesterol levels after Simvastatin treatment. However, Harini and Astirin (2009) reported that the cholesterol level of the Wistar rats after Simvastatin treatment was 60.44 ± 2.56 mg/dL. This cholesterol level was lower than the hypercholesterolemic level of the rats before treatment (97.81 ± 0.90 mg/dL) in this study. Simvastatin is one of the statins commonly used for the treatment of hypercholesterolemia in humans. Simvastatin works by blocking substances needed for cholesterol synthesis by...
competitively inhibiting 3-hydroxy-3-methylglutaryl-CoA, a reductase enzyme involved in cholesterol biosynthesis (Talreja et al., 2022). Simvastatin treatment in this study assumed lower cholesterol levels followed by decreased ROS production, restoring spermatogenesis to produce spermatozoa with higher viability and motility.

Treatment of hypercholesterolemic rats with RDFPE increased spermatozoa viability and motility. The RDFPE of 1000 mg/kg BW was proved to be the optimum dose to restore spermatozoa viability and motility in hypercholesterolemia rats, similar to spermatozoa viability and motility after treatment with Simvastatin at a dose of 10 mg/kg BW. These results were similar to those of ethanolic extract of Hylocereus polyrhizus peel orally, which showed an increase in spermatozoa count, spermatozoa viability (Aziz and Noor, 2010), and spermatogenesis cells in rats induced by a high-fat diet (Cantika et al., 2019).

Oxidative stress could be inhibited by antioxidants. Cells use enzymatic antioxidants, namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to protect cells from damage by ROS (Sharifi-Rad et al., 2020). However, excessive ROS production was followed by unbalanced oxidant-antioxidants (Amiya, 2016). Dragon fruit peel contains betacyanins, flavonoids and phenols, vitamins C, E, and A, terpenoids, thiamine, niacin, pyridoxine, cobalamin, carotene, and phytoalbumin, which had antioxidant benefits (Hendra et al., 2016). Flavonoids are antioxidant compounds that inhibit lipid peroxidation at the initiation stage of lipid oxidation. Flavonoids can prevent the generation of ROS by capturing ROS or indirectly by increasing enzymes (Ullah et al., 2020). Flavonoids could provide hydrogen atoms to lipid radicals to become more stable compounds. The addition of antioxidants controlled lipid peroxidation by blocking oxidation at the initiation and propagation stages. Derivatives of antioxidant radical are more stable than lipid radicals and cannot react with other lipid molecules; thereby, new lipid radicals were not formed. Flavonoid compounds provide hydrogen donors and bind lipid radicals to become stable lipid radicals, and finally stop the chain reaction of lipid peroxidation (Zheng et al., 2022). Physiologically, ROS was vital for spermatozoa maturation, viability, motility, capacitation, acrosome reaction, and fertilization (Dutta et al., 2019).

CONCLUSION

Administration of red dragon fruit (Hylocereus polyrhizus) peel ethanolic extract increased the viability and motility of spermatozoa of hypercholesterolemic rats (Rattus norvegicus), which was the same as the effect of Simvastatin. Further studies are needed to analyze several oxidative stress biomarkers and antioxidants along with cholesterol that was considered to explain the mechanism of protection by red Dragon fruit peel extract.

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AUTHORS' CONTRIBUTIONS

Niken Meyliana Sari (NMS), Gandul Atik Yuliani (GAY), Nurhusien Yimer (NY), Tatik Hernawati (TH), Eduardus Bimo Aksono Herupradoto (ENAH), Nanik Hidayatikat (NH). NMS conceived and designed the study, collected data, analyzed data and drafted the manuscript under supervision of GAY. TH, NY, ENAH, and NH read and reviewed the manuscript for intellectual content. All authors approved the final draft.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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