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THE EFFECT OF SEMBUNG LEAF EXTRACT (*BLUMEA BALSAMIFERA*) ON THE NUMBER AND DIAMETER OF RATS LEYDIG CELLS INDUCED BY HIGH-FAT DIET

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ABSTRACT

This study aims to determine the morphology of Leydig cells in hyperlipidemic wistar rats after having administered with sembung (*Blumea balsamifera*) extract orally. This study utilised a randomized post-test only control group design. The sample in this study were 16 adult male wistar rats (Rattus novergicus) aged 3-4 months with a body weight of 150-200 grams, which were equally and randomly divided into two groups, namely control group (hyperlipidaemia and sterile aquadest) and treatment group (hyperlipidaemia and sembung leaf extract). Hyperlipidaemia was induced with lard administration for 50 days. Data were analysed statistically using the Independent t-test. The results showed that the number of Leydig cells in the treatment group was higher than the control group with 68.13 ± 1.89 and 55.63 ± 1.92 cells respectively ($P < 0.05$). In addition, the mean Leydig cell core diameter of the control group, 5.00 ± 0.34 µm, was smaller compared to the treatment group which was 5.80 ± 0.20 µm ($P < 0.05$). It can be inferred that sembung leaf extract provides a protective effect against damage to Leydig cells due to hyperlipidaemia.

Keywords: Leydig cell, Hyperlipidaemia, High-Fat Diet, Sembung leaf extract, *Blumea balsamifera*, *in vitro* study.

INTRODUCTION

Infertility in married couples is a highly disruptive condition that can even threaten the integrity of the household (Hocaoglu, 2019). Infertility can occur due to disturbances of the reproductive system in women and decreased quality and quantity of sperm in men (Pizzorno, 2018). Based on world demographic data, as many as 12.5% of couples experience difficulties in having children, 50% of which is contributed by male infertility (Louis *et al.*, 2013; Kumar and Singh, 2015). Infertility tends to occur in big cities owing to the unbalanced lifestyle and diet for reproductive needs (Kulu, 2013; Panth *et al.*, 2018).

Lack of knowledge, information availability, and economic factors often act as the reasons for society's lifestyle, including the diet they adopt (de Ridder *et al.*, 2017). Unhealthy diet has been proven to greatly decrease reproductive function (Sharma *et al.*, 2013). Diets that tend to incorporate more fatty and high cholesterol foods have a risk of increasing blood lipid levels, known as "hyperlipidaemia" (Rhee *et al.*, 2019). The most common clinical features of this disease are increased levels of total cholesterol (Nelson, 2013), triglycerides (Chandalia and Abate, 2004), and LDL *(Low Density Lipoprotein)* (Hirano, 2018), and decreased HDL *(High Density Lipoprotein)* (Forrester, Makkar and Shah, 2005). Hyperlipidaemia has a positive correlation to the onset of various degenerated diseases such as coronary heart disease (Nelson, 2013), diabetes mellitus, dyslipidaemia, Alzheimer's (Carlsson, 2010), cancer (Huang *et al.*, 2017), obesity (Klop, Elte and Cabezas, 2013), stroke (Sekuri *et al.*, 2004), and decreased male reproductive function (infertility) (Saez and Drevet, 2019). Several effects of hyperlipidaemia on the male reproductive system include tubular atrophy, decreased spermatozoa motility, spermatozoa morphological abnormalities, inhibition of testosterone and LH *(Luteinizing Hormone)* secretion, Leydig cell degeneration, and impaired spermatogenesis.

Hyperlipidaemia plays a significant role in increased free radical production and lipid peroxide imbalance at the tissue level (Yang *et al.*, 2008). This condition can trigger oxidative stress which is an early pathophysiology of male infertility (Ribas-Maynou and Yeste, 2020). The research scale stated, hyperlipidaemia caused a significant decrease in plasma testosterone levels in rats. This decrease may result from disruption of the hypothalamic-pituitary-testicular axis, degeneration of Leydig cells, reduction of Leydig cell nucleus diameter, and decreased LH levels and testicular activity of 17β-hydroxysteroid dehydrogenase.

Substances that are able to ward off free radicals are called "antioxidants" (Haider *et al.*, 2020). Antioxidants can be enzymatic (primary antioxidants) or non-enzymatic (secondary antioxidants) (Mehta and Gowder, 2015). Secondary antioxidants can be easily obtained with high availability in nature, especially in plants that contain vitamins A, C, E (Baiano and Del Nobile, 2016), β-carotene, polyphenols, and flavonoid compounds (Xu *et al.*, 2017). One of the plants with a high antioxidant content is Sembung (*Blumea balsamifera*) (Pang *et al.*, 2014; Kusumawati and Yogeswara, 2016; Wang and Zhang, 2020). Sembung is known as a plant that can be used as traditional medicine (Islam *et al.*, 2013; Suweta, 2013). Balinese people in Indonesia generally use

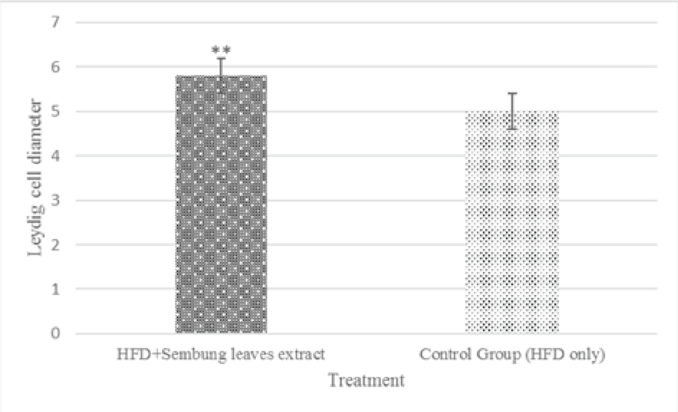

Figure 1. The mean ± SD number of Leydig cells in rats induced by High-Fat Diet (HFD) and oral sembung leaf extract. Note: ** indicates that there is a significant difference (P <0.05), n = 8 mice.

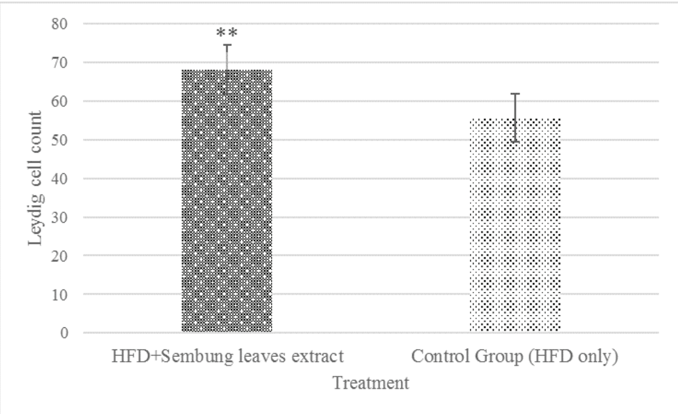

Figure 2. The mean ± SD diameter of Leydig cells in rats induced by High-Fat Diet (HFD) and oral sembung leaf extract. Note: ** indicates that there is a significant difference (P <0.05), n = 8 mice

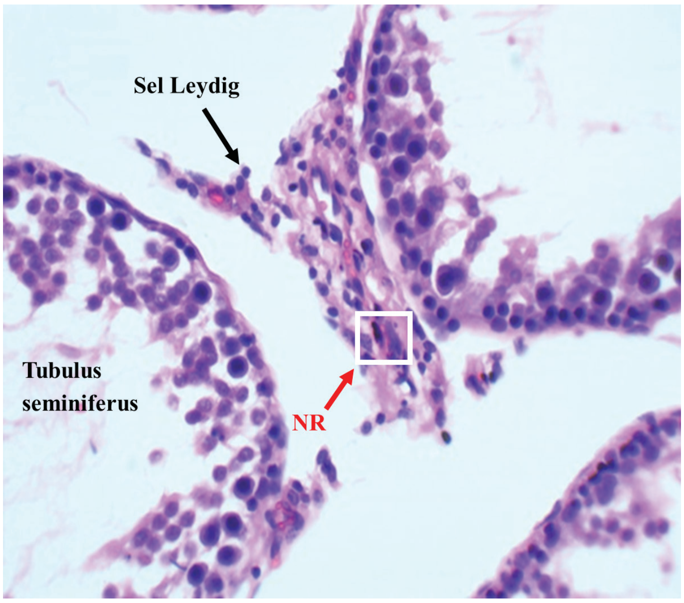

Figure 3. Group A Leydig cell histology (control); NR: Necrotic cell (200 x magnification)

Sembung leaves as an ingredient for a traditional drink called "Loloh" (Kusumawati and Yogeswara, 2016).

Sembung leaves have long been used in treating heartburn (Ali, Wong and Lim, 2005), diarrhea (Perawati, 2017), improving blood flow (vasodilation) (See, Arce

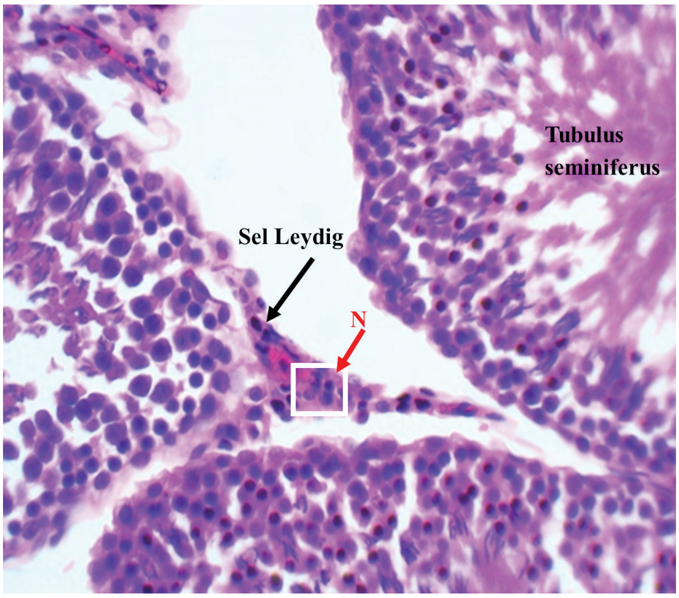

Figure 4. Leydig cell histology group B (treated with *B. balsamifera* extract 2 mg / mL). N: Normal Leydig cells. (200 x magnification)

and Deliman, 2016), and reducing blood sugar levels (Kusumawati *et al.*, 2019), and blood fats (Kusumawati *et al.*, 2018). Sembung plants contain active substances such as essential oils (Sakee *et al.*, 2011) (cineol, borneol, landerol, and camphor), flavonols (Saewan, Koysomboon and Chantrapromma, 2011), tannins (Boy *et al.*, 2018), and resins (Pang *et al.*, 2017). Flavonoid compounds contained in sembung leaves can act as immunomodulators and antioxidants so that it can help regenerate cells damaged by free radicals.

Based on the aforementioned facts, it is necessary to conduct research on the effectiveness of antioxidant compounds from Sembung leaf extracts on the morphology of Leydig cells induced by high-fat feed, based on variable cell abnormalities (degeneration / necrosis) so as to strengthen the potential regenerative effect of this plant extract.

MATERIALS AND METHODS

Ethical approval

Ethical approval for this study was obtained from the Animal Research Ethics Committee, Faculty of Medicine, Udayana University, Bali / Sanglah Central General Hospital Denpasar, Bali, Indonesia (approval no. 2020.02.2.0847).

Sembung *(Blumea balsamifera)* leaves extraction

Sembung leaves were obtained from Tista Village, Kerambitan District, Tabanan, Bali and then cleaned of foreign organic matter and dirt. The leaves were put into a juicer which led into the extraction process, carried out at the Biotechnology Laboratory, Universitas Dhyana Pura, Badung, Bali using ethanol extract. Maceration was done for 24 hours, the product of which were filtered several times and then the pellets/residue were taken. The residue obtained were then distilled to separate the alcohol from the sembung leaf extract. After being separated, the extract is evaporated to form a dry powder which is then stored for research purposes. The concentration of sembung leaf extract was calculated by comparing the leaf weight with the final volume obtained, therefore, the concentration was obtained in mg / mL units (Widhiantara *et al.*, 2018).

Research design

This research is an experimental study with a randomized post-test only control group design. The sample in this study was adult male Wistar rats *(Rattus novergicus)* aged 3-4 months with a body weight range of 150-200 grams. Because the sample, male Wistar rats that have met the requirements, is homogeneous, it was taken in a simple random manner. The selected sample was divided into 2 groups, namely:

A: hyperlipidaemia control group + sterile distilled water

B: group of sembung leaf extract per oral (p.o).

Each group contained 8 rats with an additional rat as a reserve (Widhiantara *et al.*, 2018; Haryanto *et al.*, 2019).

Induction of high-fat feed and sembung leaf extract

The high-fat diet was given to groups A and B *(pre-test)* for 30 days. Then, group A was only treated with sterile distilled water while group B was given 2 mg/mL of sembung leaf extract orally, each for 50 days *(post-test).* During the study period, rats were given standard feed (Chicken Feed, CP594, PT. Pokphand) with a composition of 13% water, 17.5-19.5% protein, 3% fat, 8% fibre, 7% ash, 0.9% calcium, and 0.9% phosphorus ad libitum. High-fat diets have a composition of 1% cholesterol, 5% egg yolk, 30% lard, 5% cooking oil, and standard foods up to 100% (Widhiantara *et al.*, 2018).

Tissue observation

Histopathological observations were carried out at Laboratorium Balai Besar Penyakit Veteriner, Denpasar, Bali. The organs observed were the testes which had been separated and then fixed in 10% formalin buffer solution for 3 hours, then dehydrated. Dehydration was done in stages, starting with 70% alcohol for ½ hour, then soaked in 95% alcohol for ½ hour, the first 100% alcohol (I) for ½ hour, the second (II) for 1 hour, the third (III) for 1 hour, and the fourth (IV) for 1 hour. It then proceeded with the clearing process which involved submerging the samples in xylol (I) for 1 hour, xylol (II) for 2 hours, followed by embedding in which the samples were inserted into paraffin (I) for 2 ½ hours and paraffin (II) for 4 hours. Paraffin blocks that had been cut using a 5 µm microtome were prepared and stained.

Staining was done with Hematoxylin-Eosin (HE) by deparaffinizing using xylol, hydrating with serial alcohol 100% (2x2 minutes), 95% (2 minutes), 90% (2 minutes), 80% (2 minutes), 70% (2 minutes) and then the staining itself with Hematoxylin for 1 minute, followed by washing with water. Afterwards, the staining process was continued by Eosin staining for 5 minutes, then washed 2 times with 75% alcohol, dehydrated with 95% alcohol, cleaned with xylene, and mounted using entellan (Widhiantara *et al.*, 2018).

Observations were made under an electric microscope (Olympus CX-21) which had been installed with a micrometre to calculate the number and diameter of Leydig cells as well as to observe the morphological quality which included necrosis and degenerated cells. Preparations were taken from the right and left testes with 3 incisions each. Every preparation was observed for its number and diameter of Leydig cells in 5 fields of view under an electric microscope with 400X magnification. The calculation results obtained were the mean number and diameter of Leydig cells for each treatment as well as a picture of the morphology of each individual's Leydig cells in 5 fields of view.

Data analysis

The results of the study on the number and diameter of Leydig cells were statistically analysed (Widhiantara, Arunngam and Milas Siswanto, 2018). The order of analysis was descriptive analysis, namely normality test and homogeneity test, followed by comparability test. To determine the effect of sembung leaf extract treatment, the mean post-test Leydig cell nucleus diameter of the control group was compared with the treated group, then the Independent t-test was used. Meanwhile, the Leydig cell morphology data were analysed descriptively and quantitatively by comparing the normal Leydig cell morphology in the control and treatment groups. The number of morphologically abnormal Leydig cells in percentage per field of view.

RESULTS AND DISCUSSION

Based on the results that have been obtained, a protective effect can be seen on the Leydig cells of rats that had been given high-fat food + sembung (*B. balsamifera*) leaf extract (Figure 1 and Figure 2). Meanwhile, feeding a single high-fat diet can directly or indirectly affect the growth, development, and function of Leydig cells in testosterone production. Directly, high-fat foods can increase ROS *(Reactive Oxygen Species)* so that there is an imbalance between the amount of free radicals and antioxidants in the body. This imbalance results in oxidative stress. Oxidative stress is able to trigger lipid peroxidation reactions in the cell membrane so that it has a negative effect on the morphology and function of Leydig cells. The indirect effect is able to react through the nervous system found in the hypothalamus, reducing the secretion of *Gonadotrophin Releasing Hormone* (GnRH) so that it affects the growth and development of Leydig cells in the testes.

The mean difference test using the independent t-test showed significantly different results in the two groups. These differences occur in the variables studied, namely the number and diameter of the Leydig cell nucleus. The cell morphological picture also showed an abnormal structure in the form of necrosis in Leydig cells of rats that were given a high-fat diet (treatment A) while rats that were given high-fat food + sembung leaf extract (treatment B) showed the histological dominance of normal Leydig cells (Figure 3,4).

Other studies have also stated that the post-prandial testosterone concentration has decreased significantly after eating fat-rich food, resulting in inhibition of testosterone production by chylomicrons (large triglyceride-rich lipoproteins produced in enterocytes from fatty foods such as fatty acids and cholesterol) (Volek *et al.*, 2001; Rahmany and Jialal, 2020). Sembung leaf extract daily administration to the treatment group as much as 2 mg/ml orally was proven to be able to prevent Leydig cell damage due to a high-fat diet. These results have corroborated previous studies which mentioned the improvement of spermatogenic cells after administration of sembung leaf extract to hyperlipidemic rats (Widhiantara *et al.*, 2018). The phytochemical content in sembung leaf extract in the form of flavonoids and tannins provides a strong reason for preventing lipid peroxidation reactions. Similar research results were also reported by (Singh, Parthasarathy and Mohan, 2014), that the alcohol and water extracts of *Blumea eriantha* DC were able to reduce plasma cholesterol, triglycerides, LDL and VLDL, increase plasma HDL levels, and show high antioxidant activity.

CONCLUSION

Hyperlipidaemia can cause structural abnormalities (necrosis) in Leydig cells, and this condition can be corrected by administering sembung leaf extract so that the number of cells and the diameter of the Leydig cell nucleus increase when compared to the group without such treatment. This result is the first report regarding the potential of sembung *(Blumea balsamifera)* leaf extract as a biomedical agent in improving the morphology of Leydig cells of rats induced by high-fat foods.

Further Research

Further research is still needed to complement the potential of sembung *(B. balsamifera)* leaf extract on the levels of TGA, MDA, and SOD so that the inhibitory pathways of the extract associated with oxidative stress can be identified. Further research is needed regarding the expression of reproductive-related genes that arise due to exposure to sembung leaf extract.

Authors' Contributions

I.G.W and A.A.A.P.P. supervised the experiment. P.A.W conducted, analysed, and prepared the manuscript. All authors read and approve the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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